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DIFFERENT ENVIRONMENTAL AND
PHYSIOLOGICAL CONDITIONS

by
Hardam Singh Azad

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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1968

Doctoral Committee:

Professor Jack A. Borchardt, Chairman
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A B S T R A C T
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Hardam Singh Azad

Chairman: Jack Borchardt

Continuous drainage of phosphorus from domestic, industrial, and agricultural sources has been shown to enrich receiving waters and thus enhance the rate of the natural process of eutrophication. Sufficient evidence has accumulated for engineers to feel that removal of this fertilizing element at its source could possibly result in better control of biological productivity in many ecosystems.

One of the major aims of this research therefore was to elucidate the cause-and-effect relationships that must exist among the factors that influence growth and the concentration of phosphorus in the aqueous environment.

The green algae *Chlorella* and *Scenedesmus* were grown as continuous uni-algal-cultures under rigorously controlled environmental conditions. A constant biomass density, during a given run, was maintained by employing a photo-electric-turbidostat apparatus.

Physical parameters including light intensity, algal cell density, diurnal cycle, temperature, and mixing have been studied. Investigation of chemical and physiological response patterns, generated by an orderly variation in nutrient

concentration, has also been reported.

Three distinct types of phosphorus uptakes by algae under steady state conditions have been demonstrated and defined. First, the zone of Metabolic Need or growth limitation. Second, the zone of Luxury Uptake, and third, the zone of excess phosphorus. A somewhat different regime pertains when the cells are pre-starved with respect to phosphorus. Visually such cells appear yellow to brown in color (instead of the normal green color). This type of response has been designated as Starvation Uptake and its total magnitude can be as large as 21% by dry weight of those green algae studied. The effect of the various physical-chemical parameters on the occurrence and magnitude of these phosphorus regimes has been presented. The data have been shown to be consistent with existing biochemical and physiological theories and are highly reproducible.

The phenomenon of P-residual at low algal cell densities and high temperatures, accurate prediction of the low cell density growth rate data by using an effective average light intensity method, the concept of optimum algal cell yield, and other factors having a potential for practical applications in algal ecology have been illustrated.

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CHAPTER I

INTRODUCTION

At least the industrialized nations now agree and emphatically demand that man-induced eutrophication of our natural waters be reduced and controlled. They are fully aware of the nuisance and destruction of the potential for multiple water use that accompanies such a phenomenon. Under these circumstances, it is unfortunate that water pollution control engineers and related scientists, faced with making an almost immediate decision on how to control and retard the pace of induced eutrophication, do not have the requisite knowledge to launch an effective and economical program on a large scale.

Temporary control measures such as killing aquatic plants by adding toxic chemicals (1)(2) or excluding light by spreading a thin film of carbon black (so dependent on winds and waves) do not remove the cause and thus, offer very little in terms of real control of eutrophication. Oglesby and Edmondson (3) suggested the "diversion" from or "dilution" in the receiving bodies of water, of the fertilizing elements as two possible methods of control. Obviously, neither of these two methods is generally applicable. "Diversion" usually means transferring the trouble from one place to another and the "dilution" method calls for an endless supply of nutrient-free water which even if available would hardly prevent biological concentration later on. Sealing lake bottoms (4) and/or supplying oxygen to the anaerobic hypolimnions may keep the oxidized microzones

intact and thus, interfere with nutrient cycles, but this would not keep new supplies of fertilizing elements from getting into the epilimnion. Direct harvesting of algae or indirect harvesting through cropping by herbivorous fish are both economically infeasible and basically ineffective methods for eutrophication control.

The removal of nutrients at their source appears to be one of the methods offering some promise in effective control of eutrophication. Most other methods have very limited applications and usually are effective only under special circumstances. Chemical methods for removing nutrients from waste waters such as precipitation and ion exchange need careful study and may be the answer to the problem in many cases. Even the very best operated secondary treatment plant, however, may leave behind enough organic matter in its effluent to foul up a delicate ion exchange system. The cost of chemicals themselves may make a chemical method economically infeasible. The process and its control may not be simple enough for generally reliable applications. All of these problems may dictate the use of biological techniques as being most practical for certain situations.

Unfortunately, biological methods have not received adequate study in depth and as a result, enjoy far less popularity than they might deserve. This should not discourage those, however, who know the history of the development of secondary sewage treatment processes and their current level of importance to the waste field.

This author feels that the potential of biological methods of nutrient extraction has often been underestimated and at the

present time, such judgements are made with inadequate evidence. Further understanding of the biometabolism of various nutrients is important not only from the standpoint of utilizing microorganisms most effectively to remove inorganic nutrients from wastewater but also from that of the aspect of their impact on limnological processes. Many applied scientists have carried out studies on nitrogen and phosphorus metabolism by autotrophs but such a simple question as, how many pounds of algal cells would be generated per pound of nitrogen or phosphorus added to a lake, remain essentially unanswered. Engineers and other eutrophication control scientists must elucidate the fundamentals of nutrient uptake by the microorganisms in order to develop information necessary for predicting a logical cost/benefit ratio for tertiary treatment.

Both algae and bacteria would strip significant quantities of fertilizing elements from their growth substrate under proper ecological conditions. Systematic investigations of different levels of nutrient uptake by microorganisms under different environmental conditions have not been carried out and consequently, this matter is in a state of confusion!

The terms luxury uptake, biological adsorption, occluded phosphate, and cation precipitation have been used in engineering literature rather indiscriminately. If these terms are valid, do they have particular significance, do we understand them sufficiently to reproduce them and can such knowledge help us to devise a technique which will be effective in the biological extraction of nutrients?

The major objectives of this study were to clarify mechanisms, quantitize the nutrient uptake potential of certain biota

under different environmental and physiological conditions, and to investigate additional factors influencing biological tertiary treatment. Algae were chosen as study organisms because the information gained would be of value in better understanding the extent of eutrophication to be expected from a given quantity of nutrient in addition to defining the different levels of nutrient uptake by cells under different growth conditions. The experiments were so designed as to explore the fundamentals involved in the bio-extraction process and thus, it is hoped that the results of this investigation would help in interpreting earlier nutrient uptake studies on algae and the related phenomena.

CHAPTER II

PHILOSOPHY OF LIMITING NUTRIENT

Practical considerations, relative to control of algal blooming, dictate that efforts be concentrated on removing only one or two crucial elements from nutrient rich waters rather than dissipating effort among a whole host of elements that "can be" growth limiting. The very mode of autotrophic growth and particularly, of algal growth would of course, concurrently deplete many nutrients from the growth medium. Their multiple nutrient extraction characteristic does, in fact, offer a relative advantage when employing biological methods of nutrient removal over chemical processes. In a slightly different way, this is in philosophical agreement with the conventional "safety factor" concept so vital in engineering design.

Although any of the micronutrients for algae such as Fe, Mo, Mn, Cl, V, Zn, Ca, B, Co, Cu, Si, Na, and perhaps others (Tables 1 and 2) and of the organic growth factors such as thiamine (vitamin B₁), biotin, cobalamin, (vitamin B₁₂) and certain amino acids (6)(7) alone can limit the growth of at least some algae, the chances of practically exerting control on their presence in the traces known to exist, are almost nil. Among the macronutrients, Na, K, Mg, S, O, and H are probably the least controllable due to their relative abundance in aqueous systems of concern. Carbon may be the next to be rejected from the control standpoint since CO₂ from air, CaCO₃ from geological formations, and the inorganic (plus some organic) carbon left in secondary effluents would

Table 1. Elements Required by Green Plants and Algae.

MACRONUTRIENTS (10^{-2} - 10^{-4} M)	MICRONUTRIENTS (10^{-5} M and Less)	
	Element	Possible Role (s)
Carbon	Iron:	Facilitates electron transfer during Photosynthesis, N-fixation, and enzymatics.
Hydrogen	Manganese:	Oxygen evolution and other metabolic function(s).
Oxygen	Chlorine:	Catalytic in oxygen evolution during Photosynthesis.
Nitrogen	Vanadium:	Accelerates Photosynthesis under high light intensity.
Phosphorus	Zinc:	Participates in photosynthesis at the CO_2 fixation level.
Sulphur	Copper:	Essential for respiration. Participation in photosynthesis? Other metabolic function(s)?
Potassium	Boron:	Required in N-fixation. Other metabolic function(s)?
Magnesium	Molybdenum:	Required in N-fixation. Other metabolic function(s)?
	Cobalt:	Required in N-fixation? Other metabolic function(s)?
Calcium (except algae)	Calcium:	Facilitates N-fixation. Other metabolic function(s)?
	Silicon:	Required by diatoms for cell wall formation
Sodium (blue-greens)	Sodium:	Metabolic function(s)?

Table 2.*Mineral Requirements for Chlorella pyrenoidosa
Based on Critical Concentrations Required in
the Culture Medium (5).

NUTRIENT	GROWTH TYPE	
	AUTOTROPHIC	HETEROTROPHIC
<u>Macronutrients:</u>		
NO ₃	2.5 x 10 ⁻² Moles	0.25 x 10 ⁻² Moles
Mg	0.2 x 10 ⁻²	2.0 x 10 ⁻²
K	4.3 x 10 ⁻⁴	4.3 x 10 ⁻³
P	1.8 x 10 ⁻⁴	1.8 x 10 ⁻⁴
S	2.0 x 10 ⁻⁴	2.0 x 10 ⁻⁴
<u>Micronutrients:</u>		
Fe	1.8 x 10 ⁻⁵	1.0 x 10 ⁻⁹
Zn	7.7 x 10 ⁻⁷	7.7 x 10 ⁻¹¹
Mn	1.0 x 10 ⁻⁷	1.0 x 10 ⁻⁹
Cl	3.4 x 10 ⁻²	3.0 x 10 ⁻⁶

*It should be noted from this table that the micronutrient (Fe,Zn,Mn,Cl) concentration required for photosynthesis is much higher than that required for other metabolic purposes.

normally furnish enough carbon for photosynthesis in most natural bodies of water.

Thus, nitrogen and phosphorus are the only two macronutrients on which it might be possible to exercise some regulatory control and thereby reduce the rate of eutrophication compared with the situation where no nutrient control is exercised. Nitrogen control alone, however, may not prove generally effective since many species of *Nostoc*, *Anabaena*, and *Cylindrospermum* (genera of the Cyanophyta, blue-green group of algae); photosynthetic bacteria (*Rhodospirillum rubrum*); heterotrophic bacteria, both aerobic (*Azotobacter vinelandii*) and anaerobic (*Clostridium pasteurianum*); and other forms maintain the capacity to fix atmospheric nitrogen. Furthermore, nitrogen is more abundant and more soluble under aerobic conditions than phosphorus.

If the bio-extraction process for phosphorus removal employs algal cultures, significant nitrogen is certain to be stripped from the nutrient rich waste water since healthy algae under normal growth conditions may be 70 per cent protein by dry weight (8). When the role of phosphorus in life is considered, it assumes even more importance as the desired control element. Three moles of ATP are required by each mole of CO_2 fixed and it is only through ATP that solar energy can be transferred to chemical energy by algae. Since phosphorus acts as fuel for the photosynthetic machinery, it should be possible to reduce the eutrophication problem promptly by cutting the supply of this ingredient at its source.

CHAPTER III

PERSPECTIVES OF PHOSPHORUS CONTROL

A. LIMNOLOGICAL VIEWPOINT

From the limnological standpoint, Hutchinson (9) reasoned, "of all the elements present in living organisms, phosphorus is likely to be the most important ecologically, because the ratio of phosphorus to other elements in organisms tends to be considerably greater than the ratio in the primary sources of the biological elements". He further stated on this basis that a deficiency of phosphorus was more likely to limit the productivity of any region of the earth's surface than would a deficiency of any other material except water.

Among the more recent publications, Patrick (10) pointed out that one of the causes for algae blooms could be the increase of phosphorus flowing through a system. Goldman and Carter (11) studied Lake Tahoe from this angle and found that in oligotrophic lakes nitrogen might be the most limiting factor during the major portion of the growing season. They also stressed, however, that in the past greater attention had been directed to phosphorus than to nitrogen because its general low supply in the lithosphere caused it to be a limiting factor for plant growth. These investigators concluded that the extreme sensitivity of Lake Tahoe waters to any change in nutrient regime clearly evidenced the potential of even secondary treatment plant effluent for speeding the eutrophication and destroying the clarity of that scenic and important natural resource.

Fitzgerald and Rohlich (12) testified that there was little doubt as to the need for more study of methods for reducing the amount of fertilizing elements (nitrogen and phosphorus) contributed by a sewage treatment plant to receiving waters. One interesting conclusion, based on their laboratory experiments, was that the rate of nutrient utilization and growth of *Chlorella* in primary or secondary effluent appeared to be independent of the organic matter present. They also suggested that a comparative survey of the relative importance of nitrogen or phosphorus removal in a particular sewage plant effluent might be in order to make the most efficient use of the environment. Olsen (13) introduced his study of phosphate equilibrium between reduced sediments and water by expressing general concern over the progressive enrichment of inland waters with phosphorus from continuously increasing discharge of phosphate-containing domestic and industrial wastes.

8. ENGINEERING THOUGHT AND EVIDENCE

One of the most publicized works done by engineers on nutrient and eutrophication relationships, which by now has become a classic, was on the pollution of the Madison Lakes in Wisconsin (14)(15). After 2 years of intensive study, this research group reached the following pertinent conclusions:

1. High concentrations of inorganic phosphorus and nitrogen are often the major cause of algal growths.
2. Sewage treatment effluent discharged into a

confined body of water may contribute a considerable portion of the total fertility.

3. Nitrogen can be made available to plankton by fixation from the atmosphere.

4. The removal of phosphorus from effluents should control one of the basic causes of blooms.

Thereafter, Sawyer (16) estimated in 1952 that the average phosphorus content of domestic sewage in the United States had probably doubled during the interval since World War II and that the phosphate consumption in the water-softening and detergent fields in 1950 was approximately 6 to 8 lb. per capita per year.

After a time lapse of twenty-three years, the chief investigator for Madison Lakes study stated, "It is conceded generally that the biological productivity of a lake, in terms of phytoplankton, is related directly to the nutrients available per unit volume of water in the trophic zone"(17). More specifically, he went on to say that phosphorus had been considered by many investigators as a key element in the control of biological activity. In his opinion, there was ample reason to believe that phosphorus would be used more and more as one of the parameters of eutrophication. Most recently, Buzzle and Sawyer (18) stated that it had been well established that control of living organisms could be accomplished by limiting the supply of a single essential food element. They argued against the effectiveness of nitrogen control but felt that this form of ecological control could be effected by controlling

phosphorus.

Many recent investigators have attributed the increased biological productiveness in almost all instances to excessive concentrations of phosphorus and nitrogen, particularly of phosphorus. Only a selected few from the extensive literature are cited here to represent the spectrum of current engineering thought and evidence.

Starting with the most recent, Bailey (19) stated that the rate at which a body of water would progress from the oligotrophic into the eutrophic stage depended almost entirely upon the quality and quantity of nutrient influx. Fruh and co-workers also observed (20) that other conditions being equal, the rate of increasing biomass for a given lake would depend on the kinds and amounts of nutrients present. They specified that nitrogen and phosphorus had assumed great importance in relating nutrients to productivity and indicated that nitrogen and phosphorus budgets for several watersheds had been compiled.

Pyper, Weber, and Borchardt (21) stated that nitrogen and phosphorus were of most general concern because they were usually present in adequate quantity and because they were so important for aquatic plant growth. Loehr and Stephenson (22) reasoned that even when abundant supplies of CO_2 and light energy were available to the algae in natural streams and lakes, nitrogen and phosphorus might be in short supply, thus limiting the amount of algae that could exist in these waters. The introduction of wastewater effluents into receiving waters would increase the quantity of available nitrogen and phosphorus, and the problems associated with increased algal growth would become

more acute.

Oglesby and Edmondson (3) predicted that decreases in eutrophication rates would be of a relatively permanent nature in cases where nutrient inflow was limited for prolonged periods. They reported that the increased algal population in Lake Washington evidently resulted from a large increase in the nutrients provided by wastewater. Limiting the nutrient supply by using diversion and dilution techniques gave early indications of success as a means for significantly decreasing eutrophication rates in this case.

Shapiro and Ribeiro (23) studied reasons for excessive algal growth in the freshwater Potomac estuary and reported, "It is clear that the growth of the algae is made possible by an adequate supply of nutrients, particularly phosphorus and nitrogen compounds".

Foster et al. (24) in their discussion of nutrient problems in Lake Tahoe cited a study made during 1963 by "outstanding sanitary engineers and limnologists" (McGauhey, Eliassen, Rohlich, Ludwig, and Pearson) which concluded that the nutrients in sewage effluents, although at that time being discharged to the ground, would ultimately reach the lake and produce increased populations of microorganisms. These experts felt that increased concentrations of phosphates and nitrogen in Lake Tahoe could trigger explosive growths of algae that would destroy its oligotrophy.

Oswald and Golueke (25) judged that eutrophication was "one of the day's most urgent problems in water pollution control" and observed that the presence of nitrogen and phosphorus

in surface waters was almost always accompanied by algae growth. The independent reasoning of Gates and Borchardt (26) and also of Pfeffer and McKinney (27) led them to state that control of either one or both the nitrogen or phosphorus content of the sewage effluent had to be attained to make the treatment of sewage complete.

In January 1965, AWWA Task Group 2610 P was appointed to consider the extent and severity of problems of water quality and treatment that are known to be, or are alleged to be, caused by the presence of nutrients in water. This study group gathered extensive information and reported (28) that the two most significant algal nutrients occurring in limiting concentrations were nitrogen and phosphorus. McCarty in reporting on the deliberations of the group (29), testified that domestic wastes were a large contributor of phosphorus so that removal of phosphorus from wastewaters should help significantly in reducing the rate of eutrophication downstream from points of discharge. This group also suggested that domestic wastes and perhaps some industrial wastes appeared to be the only sources in which the phosphate concentrations were sufficiently high to make removal feasible.

"Lake Erie dying but not dead" was the heading of a feature article edited by Morgan (30) and a solution suggested for halting the premature death was to reduce the input of phosphorus since it was considered the prime stimulation to the growth of aquatic plants and plankton.

Tenney & Stumm (31) felt certain that phosphorus was one of the major factors contributing to progressive stream

fertilization. Levin and Shapiro (32) agreed with the pre-dominant opinion that high concentration of phosphorus was the dominant factor in enhancing the natural pace of eutrophication. Bogan and co-workers (33) asserted that "eutrophication of receiving waters by nutrient rich wastes may be controlled by removal of phosphorus".

Similarly, Yee (34) Maloney(35), Borchardt and Azad (36), and many other workers have expressed the opinion that phosphorus is the most crucial and controllable element of all the nutrients required by algae. Missingham(37)recently published an extensive review on the occurrence of phosphates in surface waters and some related problems. He arrived at the following conclusions:

1. Phosphorus is in all probability the key element in lake fertilization.
2. Sewage plant effluent provides a potent source of phosphorus for surface enrichment.

C. BIOCHEMICAL AND PHYSIOLOGICAL ROLE

1. Phosphorus Compounds

Phosphorus is found in living cells as part of the phosphate group, in both the organic and inorganic forms. Phosphoric acid (H_3PO_4) is absorbed by plants as the mobile monovalent ion H_2PO_4 , but the phosphate ion (PO_4^{-3}), unlike the nitrate and sulfate ions, undergoes no reduction and plants absorb it as the orthophosphate ion (38).

The phosphorus atom is incorporated as the phosphoric acid group into phospholipids (Appendices I and II);

phosphorylated sugars and their intermediary metabolic breakdown products as found in the glycolytic and the alternate oxidative pathways (App. III) (40); nucleoproteins and nucleic acids (App. IV) (40); purine and pyridine nucleotides (App. V-D, E, & G); flavin nucleotides (App. V-F); coenzymes such as pyridoxal phosphate and thiamine pyrophosphate (App. V-B & C); adenosine mono-, di-, and triphosphates (App. V-A); phytin (App. V-H) (42); condensed phosphates (App. V-I) and into many other compounds.

2. Important Roles

Phosphate plays a key role in energy transfer, in respiration, in photosynthesis, and most probably in active transport. As a matter of fact, phosphorus participates in almost every reaction of cell metabolism and there is no substitute for phosphorus such as is the case when strontium may be substituted for calcium.

The fairly strong dissociation of phosphoric acid ($pK_a = 2.15$ at 25°C) usually increases when it is attached to an organic molecule. The phosphoric acid groups, along with the carboxyl group, play an important role in the formation and maintenance of the acidic properties of the amphoteric protoplasm, and electronegative surface potentials, etc. In addition, phosphate has the unique property of being able (by oxidative or photosynthetic phosphorylation) to change the steric linkage of O and P in such a way that the molecular energy is increased (38).

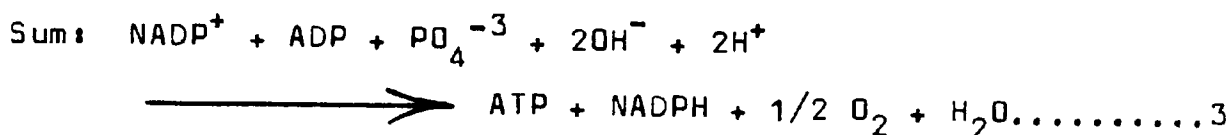
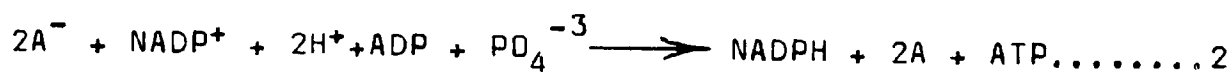
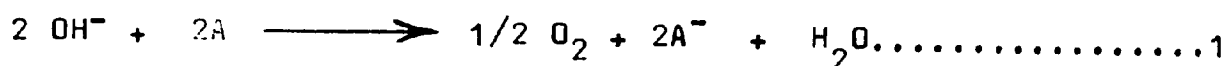
Among the phosphorus containing compounds listed earlier, phospholipids and condensed phosphates are much more important

than have been recognized generally in engineering literature. Hence, their crucial roles will be discussed below in some detail. A brief discussion of the role of phosphorus in energy transfer shall also be included here.

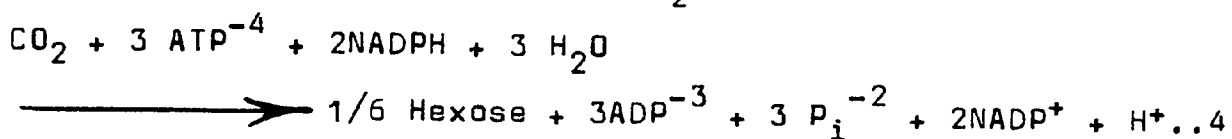
3. Energy Transfer

The distinction between photosynthetic (algae) and nonphotosynthetic (heterotrophic bacteria) cells lies in the manner in which ATP (App. V-A) and NADPH (App.V-E) - both being energy rich compounds, are formed (44). Nonphotosynthetic cells form these compounds at the expense of energy released from bio-oxidation of reduced substrates, whereas photosynthetic cells form them by absorption of light energy.

In equation form, the generation of this chemical energy by plants can be expressed as follows (44):



It should be noted that the only requirement for the continued assimilation of carbon dioxide by plants is a supply of ATP, together with reduced NADP (App.V-A & E). This may be clear from the following simplified equation for the overall photosynthetic assimilation of CO₂ (40):



Finally, it is only through these energy rich compounds that any anabolic (biosynthetic) reaction in any living cell can take place. Phosphorus is equally crucial in the catabolic (biodegradative) sequence of reactions in that the bond energy released could not be trapped in chemical form and would be wasted as heat without its intermediacy.

4. Condensed Phosphates

Condensed phosphates (App.V-I) represent a means available to the cell for internal storage of phosphorus and energy. It is important to realize that the phosphorus element as PO_4^{-3} could not be stored by the microorganisms in great quantity due to the high osmotic pressure that would be exerted within the cell in so doing. Hence, the condensed phosphates and the compounds like phytin (App.V-H) represent nature's method for storing phosphorus without cell rupture.

The presence of condensed phosphates has been reported in yeast (45)(46), in bacteria (47 to 49) (App.VI) and in algae (41)(50 to 52)(App.IV)(Table 3). Distribution of phosphorus within the growing algal cells was investigated by Miyachi and Tamiya (53) using the *Chlorella* which had been uniformly labeled with ^{32}P . They found that *Chlorella* cells contained three kinds of polyphosphate: poly-P "A" (acid soluble), poly-P "B" (acid insoluble but soluble in cold alkali at pH=8-10) and poly-P "C" (insoluble in cold acid or cold alkali at pH=8-10, but extractable with 2N KOH in 16-18 hours at 37°C). These authors stated that polyphosphate "A" and "B" appeared to correspond to the acid-soluble and

insoluble metaphosphates, respectively, obtained by Wiame from yeast (45). Polyphosphate "C" seemed to correspond to Langen and Liss' polyphosphate 4 found in brewer's yeast(53).

Hase et al.(41) isolated chloroplast and metachromatic granules from *Chlorella* cells and analyzed the phosphorus compounds contained in these organelles (App.IV). The volutin granule fraction was found to contain mainly the acid-soluble, nonnucleotidic polyphosphate (93.2 micro mole P/100 mg. volutin granule) designated as polyphosphate "A" in App. IV. RNA was also present in these granules. The cytological observations on the volutin granules (41) and some other evidence (50) strongly suggest that polyphosphate "A" participates in the metabolism concerned with cell division although its actual biochemical role is yet obscure.

Furthermore, the synthesis of polyphosphate "C" fraction (App.IV) occurs only in light (51), whereas that of polyphosphate "B" takes place independent of light which suggests that polyphosphate "C" may be localized in the chloroplast. Kanai et al. (51) also concluded that:

1. In the light, endogenous phosphates supply phosphorus for the synthesis of DNA and phosphoprotein, whereas exogenous PO_4^{-3} is the source for RNA synthesis.
2. In the dark, polyphosphate "B" serves as a phosphorus source, at least partly, for the synthesis of RNA.

Table 3. Algae in Which Polyphosphate have been Demonstrated (52).

CYANOPHYTA

Anabaena variabilis	Oscillatoria amoena
Cylindrospermum licheniforme	O. limosa
Gloeothece sp.	Phormidium ambiguum
Lyngbya aerugineo-coerulea	P. frigidum
L. amplivaginata	P. uncinatum
Oscillatoria sp.	

CHLOROPHYTA

Acetabularia mediterranea	Hydrodictyon reticulatum
Chlorella sp.	Mougeotia sp.
Chlorella ellipsoidea	Oedogonium sp.
Chlorella pyrenoidosa	Rhopalocystis oleifera
Chlorella vulgaris	Scenedesmus sp.
Cladophora sp.	Spirogyra sp.
Cosmarium sp.	Ulothrix sp.
Enteromorpha sp.	Zygnema sp.

BACILLARIOPHYTA: Fragilaria sp. Navicula sp.

CRYPTOPHYTA: Chilomonas sp.

RHODOPHYTA: Ceramium sp.

XANTHOPHYTA: Vaucheria sp.

EUGLENOPHYTA: Euglena gracilis

CHAROPHYTA: Chara sp.

The almost universal presence of condensed phosphates in yeast, bacteria (App.VI), and algae (Table 3); and the other evidence reported heretofore would strongly suggest that the volutin granules play a vital role in cell division and that "only the chromosomes coated with volutin are capable of division"-a conclusion long drawn from observations on yeast metabolism (46).

5. Phospholipids

The phospholipids are basically involved in the structural framework of the protoplasm, although some droplets may be found dispersed in the cytoplasm. They occur as part of the chloroplast structure, the protoplasmic membranes, and the limiting membrane of the chloroplasts. Obviously, phospholipids do not function primarily as reserve food material (like the fats proper) and such compounds must have other roles to play.

It should be clear from their structure (App.II) that they combine the fatty hydrophobic properties of the true fats with the hydrophilic nature of the phosphate group and for this reason, are fundamentally dipolar. This quality would permit them to project fatty acid residues into fat and phosphate residues into water at a fat-water interface. Hence, their ability to stabilize certain interfaces within the cell could be one of their most important roles.

Kirk and Tilney-Sassett (39) arrived at a figure of 21% for the total lipid content of spinach chloroplasts

which had not lost their soluble protein. Only 9.1% by weight of the total lipids were phospholipids (App.I). This could be interpreted to mean that the later represented 1.91% by weight of the spinach chloroplasts. Assuming the phosphatidyl glycerol (App.II-A) molecular formula ($C_{40}H_{70}O_{10}P$) as an approximate formula for all the phospholipids, the phosphorus content for the phospholipid part of spinach chloroplasts would appear to be $.0191 \times 31/741$ or about 0.08%. This percentage turns out to be about one-tenth of the one calculated from Appendix IV (41) for *Chlorella* chloroplasts (completely free of metachromatic granules).

Kamen (54) believed that lipid components could account for as much as 40% of the total dry weight of chloroplasts and were essential both as structural and as metabolic factors. Even assuming this figure for the lipid content of spinach chloroplasts of Appendix I, among other possible interpretations, one might conclude that *Chlorella* chloroplasts could contain 5 times as much phosphorus per unit weight as do the spinach chloroplasts in their phospholipids. Unless the phosphorus present in all other forms than phospholipids can account for this difference, it would appear that the *Chlorella* chloroplasts contained more phosphorus than the spinach chloroplasts did.

This conclusion inevitably leads to the assumption that phosphorus plays an even more crucial role in the chloroplasts of a unicellular plant than in those of higher plants. Since the phosphate ion is mobile within multi-cellular plants, it can be assumed that each cell within the whole could share and therefore would not store as much phosphorus as the unicellular

cell. Also, the single cell carrying out all life processes within its limits, may require phosphorus for more purposes at any one time, than would any one cell of the aggregate in a higher plant.

6. Active Transport

It has been concluded that active transport, like other endergonic (energy requiring) processes, depends on energy derived more or less directly from "energy-rich" organic phosphates such as ATP. The evidence that inhibitors of phosphorylation such as dinitrophenol also inhibit salt absorption, supports this (55). Estimates are that in certain circumstances, at least three ion pairs may be transported across the cell membrane for each high energy phosphate bond utilized.

Complete knowledge relative to the exact mechanism by which the phosphate bond energy is utilized in active transport must await further studies but several hypotheses have been proposed. Two of these hypotheses are illustrated in figure 1. Sutcliffe (56) observed that the accumulation of Na^+ and Cl^- ions by washed red beet tissue at 25°C was prevented by chloramphenicol, a specific inhibitor of protein synthesis, at concentrations which do not affect oxygen absorption. On the basis of this evidence, the author concluded that active transport was more closely linked to protein synthesis than to some other aspects of metabolism and favored the hypotheses illustrated in figure 1-A.

Whatever the mechanisms may be, it is clear that

phosphorus plays an exceedingly important role in the active transport as it does in nearly every other phase of algal metabolism.

The evidence for the importance of phosphates, discussed in this chapter should not leave the impression that the other nutrients listed in Tables 1 and 2 are not as essential for the growth of algae. In fact each is critical, and the cell cannot live without them.

Figure 1. Carrier Mechanisms in Which ATP is Implicated (55).

A. Mechanism Linking Salt Uptake to Protein Synthesis.

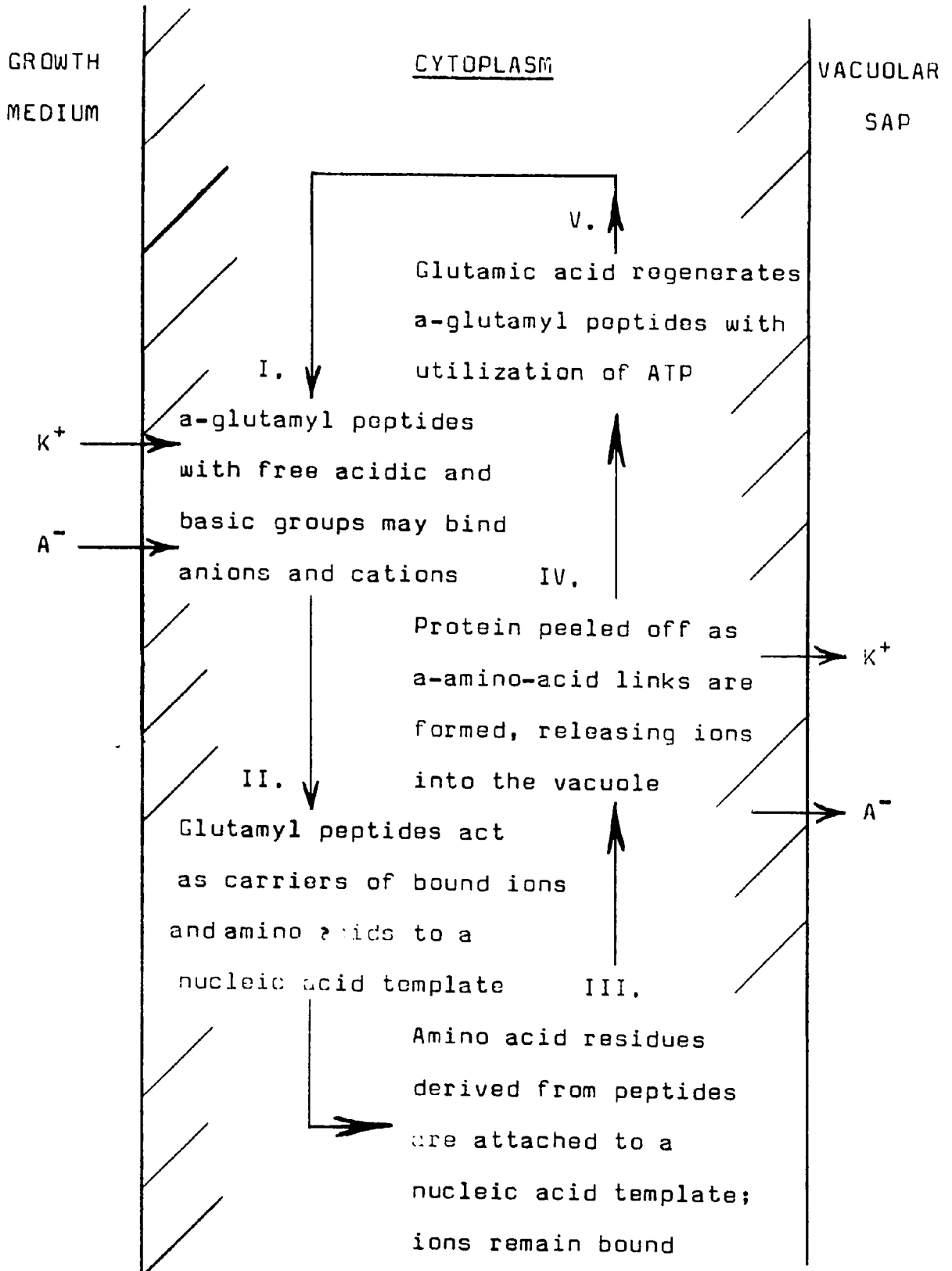
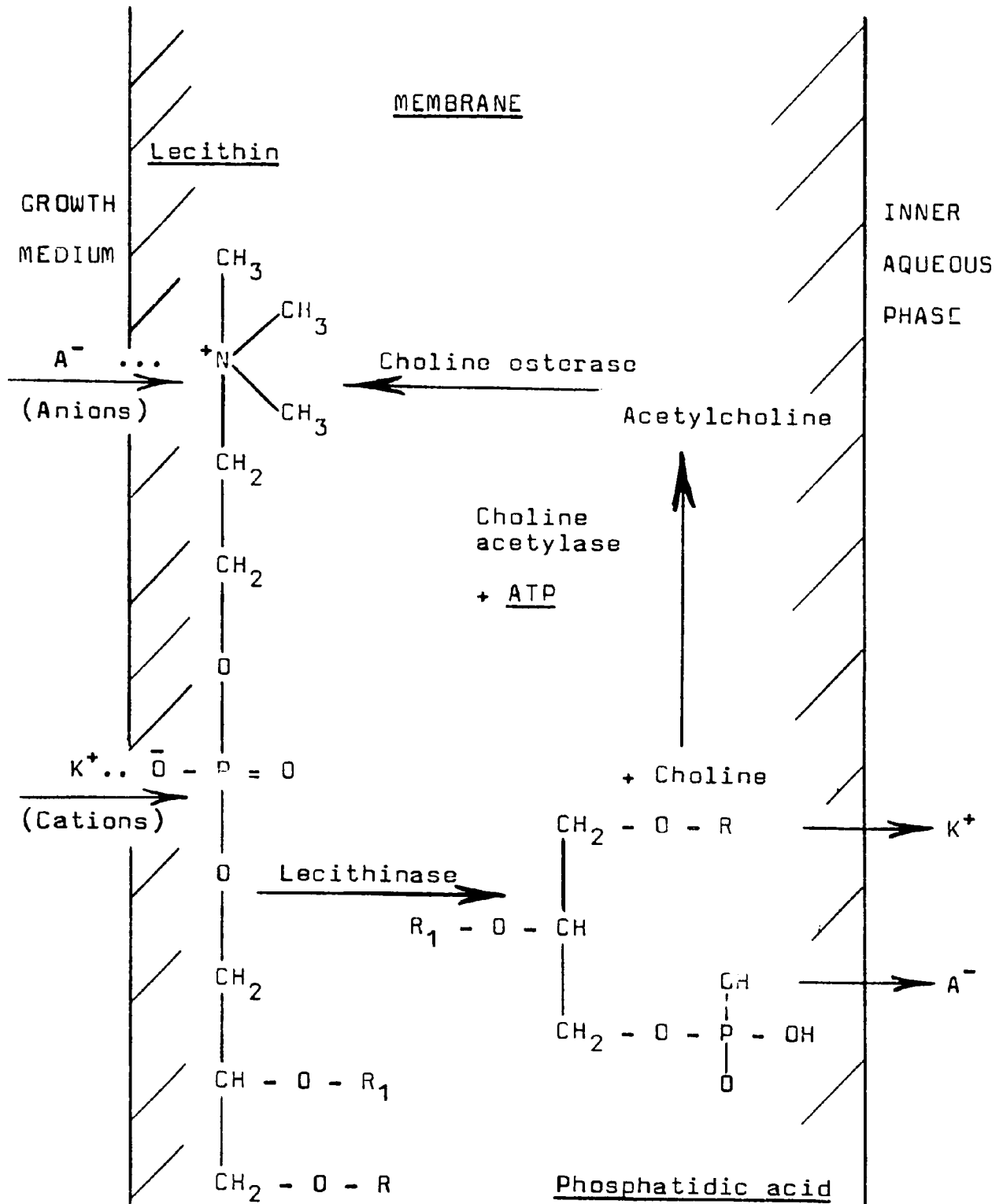


Figure 1. (Contd.)

B. Cyclic Mechanism of Active Transport Involving Lecithin as an Amphoteric Carrier.



CHAPTER IV

NUTRIENTS ESSENTIAL FOR ALGAL GROWTH

In addition to phosphorus, many other elements are essential for the growth of algae. The term "essential" as applied to the elements concerned in nutrition is not relative to quantity in its significance. The fact that several thousand times as much potassium as manganese is utilized by a plant does not make potassium "more essential" than manganese. In this case, while both the elements are vitally necessary, potassium is necessary in greater amounts than manganese. Nutrients essential for algal growth are usually grouped on a "quantity" basis as Macro-and Micro-(also minor-, rare-, or trace-)nutrients. The elements required by green plants and algae are listed in Table 1 under these categories. Some species of algae are not capable of synthesizing certain essential enzymes or amino acids, and as a consequence, require the so called "organic growth factors" or organic micronutrients such as thiamine, biotin, cobalamine, and some amino acids in their growth substrate in addition to the macro-and micro-nutrients, when grown in pure cultures.

The various essential elements perform many important functions in cell metabolism. Some save the microbe from osmotic shocks, some are fundamental constituents of metabolic products; and others may catalyze certain chemical processes within the cell. Among a number of attempts (57)(58) made to relate the essential elements and their chemical properties

to function and mechanism of action, one of Thatcher's(58) might be interesting here. He stated that nearly all the elements known or proposed to have a function in plant metabolism occur in the first 4 periods of the periodic table. Thatcher further classified (a) H and O as energy-exchange elements; (b) C,N,S, and P as energy storers; (c) Na,K,Ca,and Mg as translocation regulators; and (d) Mn,Fe,Cu,Zn,and Co as oxidation-reduction regulators. However, the functions for the remaining essential elements were listed as unknown.

A. MACRONUTRIENTS

The roles of the macronutrients carbon, oxygen, hydrogen, phosphorus, and nitrogen in life are so widespread and generally known that these will not be discussed here. Calcium may be a macronutrient for higher plants and N-fixing blue-green algae but only a micronutrient for other algae, as will be discussed later. Only N-fixing blue-green algae have been reported to require sodium as a macronutrient while this element serves the other algae as a micronutrient. Sulfur,potassium, and magnesium are the three macronutrients for all plants and will be discussed here.

1. Sulfur

Sulfur being a constituent of the amino acids cysteine, cystine, and methionine serves as an important component of proteins. It plays a direct role in metabolism by way of the sulfur-bearing vitamins, such as thiamine, biotin, and coenzyme A. Glutathione is a sulfur containing tripeptide.

The characteristics of glutathione reductase (59) have demonstrated that sulfur is also directly involved in electron transport. Cyclic S-S-compounds play an important role as regulators of the redox state of many enzymes (60).

The metabolism of sulfur is similar to that of nitrogen, in that the sulfur atom as such enters directly into organic linkages (38). Furthermore, nitrogen and sulfur utilization by the microorganisms go hand in hand. Sulfate ion is the main source of sulfur for plants and Gilbert (61) indicated that the amount of sulfur varies considerably in different genera and families of plants, usually ranging between 0.1 and 1.0% on a dry weight basis. The intracellular inorganic sulfate acts as a sulfur reserve drawn upon in the synthesis of organic substances. Robbins and Lipmann (67) identified 3^1 -phospho-adenosine- 5^1 phosphosulfate (PAPS) as being the active sulfate donor from which SO_4 can be transferred to various acceptors by sulfurylase and specific sulfokinases to form sulfate esters or sulfamino compounds (63).

"Sulfur-deficient plants are chlorotic and have an impaired photosynthesis" (42). Pirson (60) attributed this to an indirect effect of sulfur deficiency on the protein level and the chlorophyll content of the chloroplasts. Cell division may also become abnormal in sulfur-deficient plants (64).

2. Potassium

Although the specific role of potassium in plant metabolism is as yet obscure, it is absolutely essential to plants and cannot be completely replaced by other elements

such as chemically similar sodium, rubidium, and lithium(42). Sodium, lithium, and rubidium (but not cesium) may substitute in part, but not completely, for the potassium requirement in certain plants.

Because of its high ionic mobility, potassium operates almost exclusively as a free ion and plays a dominant role in regulating the ionic balance, including the pH (38). It also influences the physical properties of the protoplasm. From enzymology aspects, potassium can activate a number of enzyme systems such as fructokinase, pyruvic acid kinase(App. III-21), and transacetylase. Webster (65) reported an absolute requirement for K^+ in the activation of enzymes that synthesize certain peptide bonds and that K^+ enhances the incorporation of amino acids into protein.

Potassium deficient algae may show a decrease in photosynthetic activity, insufficient chlorophyll development in some species, disturbed carbohydrate metabolism, increased respiration, accumulation of certain other elements, a lower protein content, and a higher percentage of soluble organic nitrogen compounds. Unquestionably, potassium is essential for maintaining cell organization, permeability, and hydration (42).

3. Magnesium

Magnesium is the only metal contained in chlorophyll and comprises 2.7% of the molecule by weight (42). A chemical exchange of magnesium with other metal atoms is possible only at the loss of the photosynthetic properties of the chlorophyll

molecule.

Studies of isolated enzyme systems from plant and animal tissues have demonstrated that Mg^{++} plays a predominant role in the activity of the various enzymes concerned with carbohydrate metabolism (Table 4:4,5,13-15,18,20-22). Other divalent ions such as Mn^{++} , Co^{++} , Zn^{++} , and Cr^{++} may substitute for Mg^{++} in certain reactions, but only at the loss of some activity. Magnesium also activates the catalysis of those enzymes involving nucleotide substrates (Table 4:1-3, 6-8,11,12,16 & 26). The pattern has emerged that Mg^{++} (and to a certain extent Mn^{++}) is required as the intermediate carrier primarily for those reactions involving group transfer, namely those in which phosphate participates (Table 4). Magnesium has also been shown to be the metal component of inorganic pyrophosphatase (Table 5:8).

Magnesium deficiency causes a loss in the yellow pigments xanthophyll and carotene(66) in addition to the expected chlorosis (yellowing).

8. MICRONUTRIENTS

Calcium (a macronutrient for higher plants and N-fixing blue-green algae), sodium (a macronutrient only for N-fixing blue-green algae), chlorine (believed to be a macronutrient for higher plants by some researchers), iron, manganese, vanadium, zinc, copper, boron, molybdenum, and cobalt are the generally accepted micronutrients for most algae. Silicon is known to be essential only for diatoms.

Selenium has been referred to as an "essential" element for the plants that grow on seleniferous soils but, generally speaking, this element is not a nutrient but an ionic antagonist. There is some evidence that selenium can partially replace sulfur in the synthesis of proteins in Chlorella vulgaris (67) but only as an antimetabolite. Similarly, arsenic can antagonize phosphorus and chlorate can serve as an antimetabolite of nitrate. Certain other elements, electro-negative or positive, can also exchange with chemically related nutrient elements and cause trouble in the metabolism of microorganisms but the discussion of this general phenomenon is outside the scope of this manuscript.

Only a brief review of the essentiality and role of the true inorganic micronutrients will be presented here. The organic micronutrients, (vitamins and certain amino acids), not being a general requirement by algae, will not be considered.

1. Calcium

Higher plants and N-fixing blue-green algae may require calcium as a macronutrient but green algae, at best, are believed to require only microquantities of this element. Walker (68) provided experimental evidence of *Chlorella* becoming calcium-deficient when EDTA is present in the substrate but also reported that strontium could correct the deficiency better than calcium.

Hewitt (69) and Pirson (60) indicated that calcium, to some extent, acts in a complementary fashion to potassium in

maintaining cell organization, hydration, and permeability, and thus, indirectly influences many enzyme systems. McElroy and Nason (70) reported that Ca^{++} acts as an activator for such isolated enzymes as arginine kinase (Table 4:7), adenosine triphosphatase, and adenyl kinase. Calcium has been shown to be an integral component of amylase from various microorganisms and animal tissues (42). The enzyme lecithinase c, which splits choline from phosphatidyl choline (App. II-D, Fig. 1-B & Table 4-24) is known to be activated by calcium (71)(72). This element has been implicated as an essential metal for the synthesis of polymetaphosphate during N-fixation in Azotobacter vinelandii (73).

Ca^{++} , like K^{+} , also plays an important role in the general acid-base balance of the cell and this element is believed to be important for buffering protoplasm (38).

2. Sodium

Blue-green algae definitely require sodium for their growth and development (5). Allen and Arnon (74) showed that 5 ppm Na^{+} was sufficient for optimum growth of Anabaena cylindrica and potassium, lithium, rubidium and cesium could not substitute for sodium. Kratz and Myers (75) found that logarithmic growth of Anabaena variabilis, Anacystis nidulans, and Nostoc muscorum could not be maintained without sodium. Nostoc muscorum has been reported to require from 3 to 5 ppm sodium regardless of whether the nitrogen source is NO_3^{-} , NO_2^{-} , or NH_4^{+} (5). Higher concentrations of sodium are needed when the medium contains higher levels of K^{+} . The chlorophyll

Table 4. Enzymes Activated By Various Ions (42)

Enzyme	Reaction	Metal(s)
1. Glucokinase	Glucose + ATP $\longrightarrow$ Glucose-6-P+ADP	Mg ⁺⁺ , Mn ⁺⁺
2. Hexokinase	Fructose, Glucose, or Mannose + ATP $\longrightarrow$ Hexose-6-P+ADP	-do-
3. Phosphoglucokinase	Glucose-1-P+ATP $\longrightarrow$ Glucose-1,6-di-P+ADP	-do-
4. Phosphoglucomutase	Glucose -1- P $\longrightarrow$ Glucose - 6-P	Mg ⁺⁺ , Mn ⁺⁺ , Co ⁺⁺ , Cr ⁺⁺
5. Phosphoglycerate Kinase	3-P-glycerate $\longrightarrow$ 1,3-enol-P-pyruvate+H ₂ O	Mg ⁺⁺ , Mn ⁺⁺ , Zn ⁺⁺
6. Adenosine Kinase	Adenosine + ATP $\longrightarrow$ ADP + AMP	Mg ⁺⁺ , Mn ⁺⁺
7. Arginine Kinase	Arginine + ATP $\longrightarrow$ Arginine - P + ADP	Ca ⁺⁺ , Mn ⁺⁺ , Mg ⁺⁺
8. NAD ⁺ Kinase	NAD ⁺ + ATP $\longrightarrow$ NADP ⁺ + ADP	Mg ⁺⁺ , Mn ⁺⁺
9. Yeast apyrase	ATP + H ₂ O $\longrightarrow$ AMP + PO ₄	Mn ⁺⁺
10. Glutamyl transferase	Glutamine + NH ₂ OH + ATP $\longrightarrow$ Glutamohydroxamate + NH ₃	Mn ⁺⁺
11. Glutamine synthetase	Glutamic Acid + NH ₃ + ATP $\longrightarrow$ Glutamine + ATP	Mg ⁺⁺ , Mn ⁺⁺
12. Dephospho-CoA Kinase	Dephospho-CoA + ATP $\longrightarrow$ CoA + ADP	-do-
13. Pyruvic carboxylase	Pyruvate $\longrightarrow$ Acetaldehyde + CO ₂	-do-

Table 4. (Contd.)

Enzyme	Reaction	Metal(s)
14. Pyruvic oxidase	$\text{Pyruvate} + \text{PO}_4 \longrightarrow \text{Acetyl-P} + \text{CO}_2$	-do-
15. Oxalacetic decarboxylase	$\text{Oxalacetic Acid} \longrightarrow \text{Pyruvic Acid} + \text{CO}_2$	$\text{Mg}^{++}, \text{Co}^{++}, \text{Zn}^{++}, \text{Mn}^{++}$
16. Isocitric dehydrogenase	$\text{Isocitrate} + \text{NADP}^+ \longrightarrow \text{Oxalosuccinate} + \text{NADPH}$	$\text{Mg}^{++}, \text{Mn}^{++}$
17. Oxalosuccinic decarboxylase	$\text{Oxalosuccinate} \longrightarrow \text{Ketoglutarate} + \text{CO}_2 + \text{O}_2$	Mn^{++}
18. a-Ketoglutarate oxidase	$\text{Ketoglutaric Acid} \longrightarrow \text{Succinic acid} + \text{CO}_2$	$\text{Mg}^{++}, \text{Mn}^{++}$
19. Malic enzyme	$\text{Malate} + \text{NADP}^+ \longrightarrow \text{Pyruvate} + \text{CO}_2 + \text{NADPH}$	$\text{Mn}^{++}, \text{Co}^{++}$
20. Condensing enzyme	$\text{Oxalacetate} + \text{Acetyl CoA} \longrightarrow \text{Citric acid}$	$\text{Mg}^{++}, \text{Mn}^{++}$
21. Yeast phosphatase	$\text{Glycerophosphate} \longrightarrow \text{Glycerol} + \text{PO}_4$	$\text{Mg}^{++}, \text{Mn}^{++}, \text{Co}^{++}, \text{Fe}^{++}, \text{Ni}^{++}$
22. Phosphatases (Acid & Alk.)	Numerous phosphorylated substrates	$\text{Mg}^{++}, \text{Mn}^{++}, \text{Co}^{++}, \text{Ni}^{++}, \text{Fe}^{++}$
23. Arginase	$\text{Arginine} + \text{H}_2\text{O} \longrightarrow \text{Ornithine} + \text{Urea}$	$\text{Mn}^{++}, \text{Co}^{++}, \text{Ni}^{++}, \text{Fe}^{++}$
24. Lecithinase	$\text{Lecithin} \longrightarrow \text{P-choline} + \text{diglyceride}$	$\text{Ca}^{++}, \text{Mg}^{++}, \text{Co}^{++}, \text{Zn}^{++}, \text{Mn}^{++}$
25. Cysteine desulphydrase	$\text{Cysteine} \longrightarrow \text{H}_2\text{S} + \text{NH}_3 + \text{pyruvate}$	$\text{Zn}^{++}, \text{Mg}^{++}, \text{Mn}^{++}$
26. DNase	Depolymerization of DNA	$\text{Mg}^{++}, \text{Mn}^{++}, \text{Co}^{++}, \text{Fe}^{++}$
27. Nitrite reductase	$\text{NO}_2^- + \text{NADH} \longrightarrow \text{NH}_3 + \text{NAD}^+$	Mn^{++}
28. Hydroxylamine reductase	$\text{NH}_2\text{OH} + \text{NADH} \longrightarrow \text{NH}_3 + \text{NAD}^+$	Mn^{++}

Table 5. Specific Metalloenzymes (70).

Enzyme	Reaction	Metal
1. Carbonic Anhydrase	$\text{CO}_2 + \text{H}_2\text{O} \longrightarrow \text{H}_2\text{CO}_3$	Zn
2. Dehydropeptidase	Glycyldehydrophenylalanine $\longrightarrow \text{NH}_3 + \text{Phenylpyruvate}$	Zn
3. Glycylglycine dipeptidase	Glycylglycine $\longrightarrow$ Glycine	Zn
4. Carboxypeptidase	Carbobenzoxycarbonyl-L-phenylalanine $\longrightarrow$ Phenylalanine	Zn
5. Alcohol dehydrogenase	Ethanol + NAD^+ $\longrightarrow$ Acetaldehyde + NADH	Zn
6. Glu-dehydrogenase	Glutamate + NAD^+ $\longrightarrow$ Ketoglutarate + NADH + NH_3	Zn
7. Lactic dehydrogenase	Lactate + NAD^+ $\longrightarrow$ Pyruvate + NADH	Zn
8. Inorganic Pyrophosphatase	Pyrophosphate + $\text{H}_2\text{O} \longrightarrow \text{PO}_4^-$	Mg
9. Succinic dehydrogenase	Succinic acid - 2 H $\longrightarrow$ Fumaric acid + 2 H	Fe
10. Catalase	$2 \text{H}_2\text{O}_2 \longrightarrow 2 \text{H}_2\text{O} + \text{O}_2$	Fe
11. Peroxidase	H_2O_2 oxidation of aromatic amines and other compounds	Fe
12. Cytochromes	Electron transport	Fe
13. NADH - Cytochrome reductase	$\text{NADH} + \text{cytochrome c (Fe}^{+3}) \longrightarrow \text{NAD}^+ + \text{cytochrome c (Fe}^{+2})$	Fe
14. Uricase	Uric acid + O_2 $\longrightarrow$ Allantoin + $\text{H}_2\text{O}_2 + \text{CO}_2$	Cu
15. Tyrosinase	Tyrosine + $1/2 \text{O}_2$ $\longrightarrow$ Dihydroxyphenylalanine	Cu
16. Laccase	Phenols $\longrightarrow$ O-, and P-quinones	Cu
17. Ascorbic acid oxidase	Ascorbic acid $\longrightarrow$ Dehydroascorbic acid	Cu
18. Prolidase	Glycylproline $\longrightarrow$ Proline	Mn
19. Nitrate reductase	$\text{NO}_3^- + \text{NADPH} + \text{H}^+ \longrightarrow \text{NO}_2^- + \text{NADP}^+ + \text{H}_2\text{O}$	Mo
20. Xanthine oxidase	Xanthine + O_2 $\longrightarrow$ H_2O_2 + Uric acid	Mo
21. Aldehyde oxidase	Acetaldehyde + cytochrome c (Fe^{+3}) $\longrightarrow$ Acetate + Cytochrome c (Fe^{+2})	Mo
22. Hydrogenase	H_2 + cytochrome c (Fe^{+3}) $\longrightarrow$ cytochrome c (Fe^{+2})	Mo

content of sodium-deficient cells is not affected but the cells definitely contain less phycocyanin (5).

Na^+ has been considered to be capable of substituting for potassium to some extent. There is also some evidence that sodium acts antagonistically towards K^+ . The considerably stronger hydration of Na^+ compared with that of K^+ could, when there is an exchange, cause great alterations in the water balance (state of swelling) of the protoplasm (38). Among other things, this can certainly influence the synthesis of proteins.

As would be obvious from the discussion above, the status of Na^+ as a micronutrient for green algae is not established and its exact role in algal metabolism, if any, is far from being clear at the present time.

3. Chlorine

Broyer et al (76) showed Cl^- to be an essential nutrient and it has been argued (42) that this element should be considered a macronutrient for higher plants. Other than that bromine can partially substitute for this element, its mode of action in plant tissue is unknown.

Chlorine is an essential micronutrient for algae and is known to act as a catalyst in the oxygen evolution reaction in algal photosynthesis (Equation 1). Arnon (77) delineated the necessity of chloride ion for noncyclic photophosphorylation and for the riboflavin phosphate pathway of cyclic photophosphorylation. Both these pathways are known to be functional in chloroplasts. Since chloroplasts can also follow

the vitamin K pathway of cyclic photophosphorylation (which does not require Cl^-) however, deficiency of Cl^- can only reduce and not stop the growth of algae.

Eyster (5) obtained double the amount of growth of *Chlorella* by adding only 3.4×10^{-6} moles of NaCl. He found the critical concentration of NaCl yielding optimum effect for autotrophic growth to be about 3.4×10^{-2} moles, whereas only 3.0×10^{-6} moles of Cl^- were required for the heterotrophic growth of the *Chlorella* (Table 2). Since the heterotrophically grown *Chlorella* too requires some Cl^- , this element must have additional function(s) in the algal metabolism of at least some species.

4. Iron

The biochemical importance of iron lies mainly in its ability to change its valency. Iron is an indispensable constituent of peroxidase; catalase; cytochromes: c, b_6 , f, and oxidase; photosynthetic pyridine nucleotide reductase (Table 5: 9-13); and certain other porphyrins. An iron nonheme enzyme has been shown to be necessary for N-fixation by certain microorganisms (5). This element has also been found essential in the biosynthetic incorporation of magnesium into the protophorphyrin (precursor of chlorophyll) molecule (78)(79).

The most obvious symptoms of iron deficiency in algae are chlorosis and growth retardation. Wiessner (80) reported that deficiency of this micronutrient reduces the synthesis of proteins within the chloroplasts, and the decreased content of chlorophyll reduces the photosynthesis. He also

pointed out that larger quantities of iron must be supplied when EDTA is present in the medium.

According to Myers (81), adequate amount of Fe for *Chlorella* in the culture medium varies from 1.8×10^{-7} to 2.6×10^{-8} M. More iron is needed for autotrophic growth (1.8×10^{-5} M) than for heterotrophic growth (1.0×10^{-9} M) of Chlorella pyrenoidosa (Table 2). Eyster (5) showed that *Chlorella* had to have a minimum of 1.8×10^{-5} M Fe for maximum growth and did not grow when the iron concentration in the medium was below 1.8×10^{-7} M. He further indicated that, under higher illumination intensities and denser culture conditions, the iron requirement may increase ten fold.

Walker (82) found that normally grown Chlorella pyrenoidosa contains about 30 micro gram Fe per one gram dry weight i.e. .003% by weight. Iron, being the 4th most abundant element on the earth's surface (4.2%), it would be expected to be present in sufficient quantities in nearly all soils for supporting photosynthesis. It could however, become "unavailable" under certain circumstances by forming insoluble compounds and thereby limit photosynthesis.

5. Zinc

Zinc has been established as being an absolute necessity for the growth of algae. This metal is an important constituent of carbonic anhydrase, dehydropeptidase, glycylglycine dipeptidase, carboxypeptidase, alcohol dehydrogenase, glutamic dehydrogenase, and lactic dehydrogenase (Table 5:1-7). Vallee and co-workers (83) obtained some evidence that

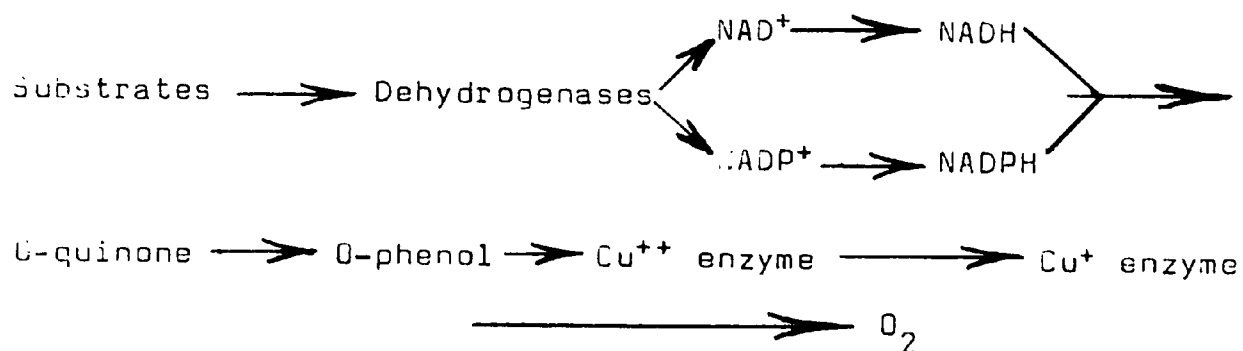
glyceraldehyde-3-phosphate; α -glycerophosphate; malic, and glucose-6-phosphate dehydrogenases are also pyridine nucleotide-dependent zinc-metalloproteins. The general occurrence of carbonic anhydrase in Scenedesmus quadricauda, Chlorella pyrenoidosa, and in Nostoc muscorum has been established (84) (85). Since zinc is a constituent of carbonic anhydrase, it may be participating in photosynthesis at the level of CO_2 fixation. Stegmann (86) showed that a decrease in chlorophyll formation and in photosynthetic activity accompanied zinc deficiency in Chlorella. There is also a significant amount of evidence available to indicate that zinc is intimately concerned with the activity of the Hill Reaction in photosynthesis (5).

Provasoli and Pintner (87) reported that several algae required about 10^{-6}M zinc in media containing EDTA (ethylenediaminetetraacetic acid). Walker (82) investigated the symptoms of zinc deficiency in Chlorella pyrenoidosa and found a significant relative decrease in dry weight at zinc concentrations below 10^{-7}M . According to Eyster (5) about 4.5 microgram of zinc is required for the growth of Chlorella in NO_3^- medium and 6.5 microgram in urea medium for each gram dry weight of cells. Furthermore, the autotrophic growth of algae requires more zinc than does heterotrophic growth (Table 2).

6. Copper

Copper is known to be essential for the respiration of higher plants. Tyrosinase (polyphenolase), laccase, and

ascorbic acid oxidase (Table 5:15-17) are plant enzymes which contain copper as a metal constituent. The enzymes ascorbic acid oxidase is believed to act as a terminal oxidase (corresponding to the cytochrome system in animal tissues and in yeast) bringing about electron transfer from reduced ascorbic acid to atmospheric oxygen (Table 5:17). Tyrosinase, on the other hand, probably catalyzes the oxidation of NADH and NADPH (5) according to the following scheme:



There is some indirect evidence and speculation that copper also participates in the photosynthesis of higher plants but this remains to be ascertained.

Walker (63) reported the requirement for copper by green algae, *Chlorella* grown in a glucose-nitrate-EDTA-salt medium. He showed that Be, Ag, Ba, Co, Fe, Zn, Mn, Mo, Bi, Tl, Ga, Al, Cd, V, Se, Ti, Zr, As, Bi, Sn, Cr, Hg, Na, K, B, Ag, Pb, or Au ions, added singly, could not replace copper. He indicated that copper concentrations below 1.0×10^{-7} M showed deficiency symptoms. Provasoli and Pintner (87) demonstrated that certain algal flagellates also require this element. More modern research would be necessary to establish the copper requirement by algae in general.

7. Molybdenum

Molybdenum is essential for NO_3^- utilization (Table 5-19) and for N_2 fixation by algae. No molybdenum is required by Chlorella pyrenoidosa, Nostoc muscorum, and Scenedesmus obliquus when NH_4^+ or urea furnishes the nitrogen (5)(89). The critical concentrations of Mo for N_2 fixation and for NO_3^- utilization by Nostoc muscorum were found to be $1 \times 10^{-7}\text{M}$ and $1 \times 10^{-10}\text{M}$, respectively. Chlorella pyrenoidosa also required $1 \times 10^{-10}\text{M}$ molybdenum for nitrate utilization. Similarly, molybdenum-deficient Scenedesmus obliquus cells failed to assimilate $\text{NO}_3^- - \text{N}$ (88)(89).

Below 10^{-9}M Mo, cell division is inhibited, chlorosis appears, and much starch tends to accumulate in the cell. Furthermore, molybdenum addition overcomes the deficiency symptoms (89). Molybdenum also appears to control in some way the supply of hydrogen donors or energy carriers necessary for protein synthesis (80 & Table 5:20-22). This would lead to reduction in protein synthesis in Mo-deficient cells.

8. Manganese

A wide variety of enzymatic reactions (Table 4:1-28) are activated by manganese including the oxidation-reduction, decarboxylation, hydrolysis, and group transfer reactions. Manganese would appear to be the predominant metal ion in the citric acid cycle (42). Manganese has also been shown to be a component of prolidase (Table 5:18).

According to Pirson (60), manganese deficiency in green algae lowers the rate of photosynthesis without appreciably

affecting the respiration, nitrate reduction, and the oxidative assimilation of glucose in the dark. Furthermore, the deficiency of this metal does not necessarily lower photosynthesis by reducing the chlorophyll content of the cell since the decrease or restoration of photosynthesis takes place without appreciable lag upon removal or addition of manganese (60).

This element is required for the evolution of oxygen regardless of the Hill oxidant used (5). The size of the photosynthetic unit (about 2500 chlorophyll molecules) is regulated by the role of manganese in photosynthesis since *Chlorella* grown with a minimum amount of manganese for maximum growth yields a chl: Mn ratio that tends to indicate such a relationship (5). The role of manganese in photosynthesis is also clear from the evidence (Table 2) that much more of this metal is required for autotrophic growth (1×10^{-7}) than for heterotrophic.

Manganese-deficient cells of autotrophically grown *Chlorella* are characterized by being abnormally clumped, about twice the volume of normal cells, and eventually, by a reduction in chlorophyll (90).

Antagonism between manganese and K^+ , Na^+ , Ca^{++} , or Fe^{++} has been reported (80) and must be considered in any precise work on the role of manganese in algal metabolism. Relations of this metal with nitrogen and phosphorus metabolism may also be interesting and important.

9. Boron

Although its exact role remains unclear, boron has been shown to be essential for higher plants (91). The nitrogen fixing blue-green algae also require this element (5).

Boron is believed to act as a coenzyme for inositol synthesis; to stimulate N-fixation by *Azotobacter*; to stimulate tyrosinase activity; to increase oxidase activity; to cause liberation of inorganic phosphates, glucose accumulation, and lack of ATP; and to accumulate certain soluble nitrogen compounds. It probably plays a part in the transportation and utilization of carbohydrates and possibly, has a function in protein metabolism (5). Calcium accentuates boron deficiency i.e. has an antagonistic effect.

Eyster (5) demonstrated the essentiality of boron for the growth of *Nostoc muscorum*. He reported the optimum concentration for nitrogen fixation to be about 0.1 ppm ($0.9 \times 10^{-5}M$) and hypothesized that if boron were required by algae only for some other function(s), the amount would be far less than 0.1 ppm.

Although, McIlrath and Skok (92) demonstrated an increased growth of *Chlorella* (increase in cell number and dry weight) with 0.5 ppm B, further research is necessary for determining the essentiality of boron for green algae in general.

10. Vanadium

Vanadium is the 20th most abundant element in the earth's crust and its average concentration in the lithosphere is

100 ppm, as compared to 10 ppm for molybdenum (42). Due to the chemical similarities between V and Mo, V could partially replace Mo as a catalyst in nitrogen fixation by *Azotobacter* and *Clostridium* as reported by certain workers. On the other hand, Allen (93) reported that Mo requirement of the blue-green alga Anabaena cylindrica for N-fixation and NO_3^- - reduction could not be replaced by V. The controversial nature of the evidence available would seem to indicate that the role for V in N-fixation has as yet not been established (42).

Experiments of Arnon (94) indicated that the chlorophyll content per Scenedesmus obliquus cell is little affected by V deficiency but the dry weight is markedly reduced and symptoms of chlorosis are produced. Photosynthesis, based on a unit-chlorophyll basis at 1850 foot candles, was about twice as high in the cells with V as in the cells deprived of vanadium. He detected no significant difference however, at 186 foot candles. Further research is necessary for establishing general essentiality of vanadium for algae.

11. Cobalt

Cobalt has been shown to be able to activate various enzymatic reactions (Table 4:4,15,19,21,23,24,& 26). Buddhari(95) reported a distinct cobalt requirement for Anabaena cylindrica and Nostoc sp. based on dry weight, chlorophyll, and phycocyanin determinations of cultures grown with and without added cobalt in the form of $\text{Co}(\text{NO}_3)_2$. He observed no growth in the absence of cobalt but good growth in its presence. The critical and toxic concentrations reported by Buddhari

were 0.5 microgram Co/l and 10.0 milligram Co/l, respectively.

Since cobalt can be replaced by relatively minute amounts of vitamin B₁₂ and it is known to be a constituent of this vitamin, it would appear that cobalt should be required by all algae otherwise capable of synthesizing vitamin B₁₂. Krauss (96) reported that the growth of Scenedesmus obliquus was stimulated by cobalt. This element has also been found essential for soybean, alfalfa, and rubber plants (5). In spite of this evidence, the essentiality of cobalt for plants in general is not established and its role, if any, in algal metabolism is far from being clear.

12. Silicon

Diatoms are known to have silicon in their cell walls, Lewin (97)(98) grew Navicula pelliculosa in synthetic medium containing 1.0 ppm Si and observed that silica uptake was an aerobic process. The uptake of silica was completely inhibited in the absence of oxygen in the medium.

Very little is known about the role of silicon in the metabolism of cell protection of other algae and it is generally assumed not to be a micronutrient for other types than diatoms.

It may be added here that the experimentally observed essentiality or non-essentiality of a given nutrient is a function of many chemical characteristics of the medium and previous physiological history of the microorganism. Almost all the evidence reported here was obtained using limited volume (batch) cultures where the chemical and physiological states

continually change as the microbes grow and thus, the results may be overshadowed by many secondary factors. As will be discussed later, in the opinion of this author, continuous culture studies would have yielded much more reliable information on the essentiality of these nutrients and their specific roles in plant metabolism.

CHAPTER V

EQUIPMENT AND ANALYTICAL METHODOLOGY

A. SELECTION OF THE CULTURING TECHNIQUE

The physico-chemical environment of algal cultures in open water being essentially uncontrollable, it is almost mandatory that rigorous phosphorus uptake studies be carried out using laboratory cultures. These cultures, on the other hand, must be so designed as to provide information that can be projected to the natural systems with some assurance. To date, most of the physiological, biochemical and nutritional studies on algae have been conducted using batch cultures.

These limited-volume cultures have furnished some useful information but they are decidedly unsatisfactory for many experimental goals. Environmental conditions such as light, nutrient and physiology of the 'average' cell continually change and consequently, growth is rarely balanced, i.e. properties of the cell, such as mean size and composition, do not remain constant (8). Any sample taken from a batch culture would include cells in all stages of their growth cycle and consequently, the measurements would be mean values giving no clues to the fluctuations that may be occurring during the growth and reproduction of the individual cells (8). Finally, the results obtained by different investigators using batch cultures of the same organisms, presumably under the same physico-chemical environment, rarely agree and one is forced to believe that batch culture results are highly system specific and extremely

difficult, if not impossible to interpret.

The automatic continuous culture techniques stabilize the physico-chemical environment, hold a culture at a given point on its growth curve by regulating the input of fresh medium, and thereby enable a precise and continuous control of population density. "Chemostat" and "Turbidostat" are the two basic types of continuous culture devices founded on these principles. In the chemostat technique, fresh medium is added at a constant rate whereas its rate is regulated by the culture growth in a turbidostat. Constant culture volume is maintained by an overflow device in both. The chemostat, used most extensively for bacterial cultures, has its pitfalls and has failed to gain popularity among the investigators of algal photosynthesis. "In this type of culture, a steady state is attained only if growth is limited by low light intensity or by the rate of supply of a nutrient so that the relative growth rate must always be less than the maximum possible" (8).

The turbidostat culture method introduced by Myers and Clark in 1944 (99), has since been used with success for culturing *Chlorella* (100); *Monodus* (101); *Scenedesmus*, *Euglena*, *Anabaena*, and *Anacystis* (102). Soon after steady state conditions are established in a turbidostat, all the conditions affecting growth remain constant and the growth is limited only by factors truly intrinsic to the alga. The cell populations produced in this type of apparatus have rather constant characteristics.

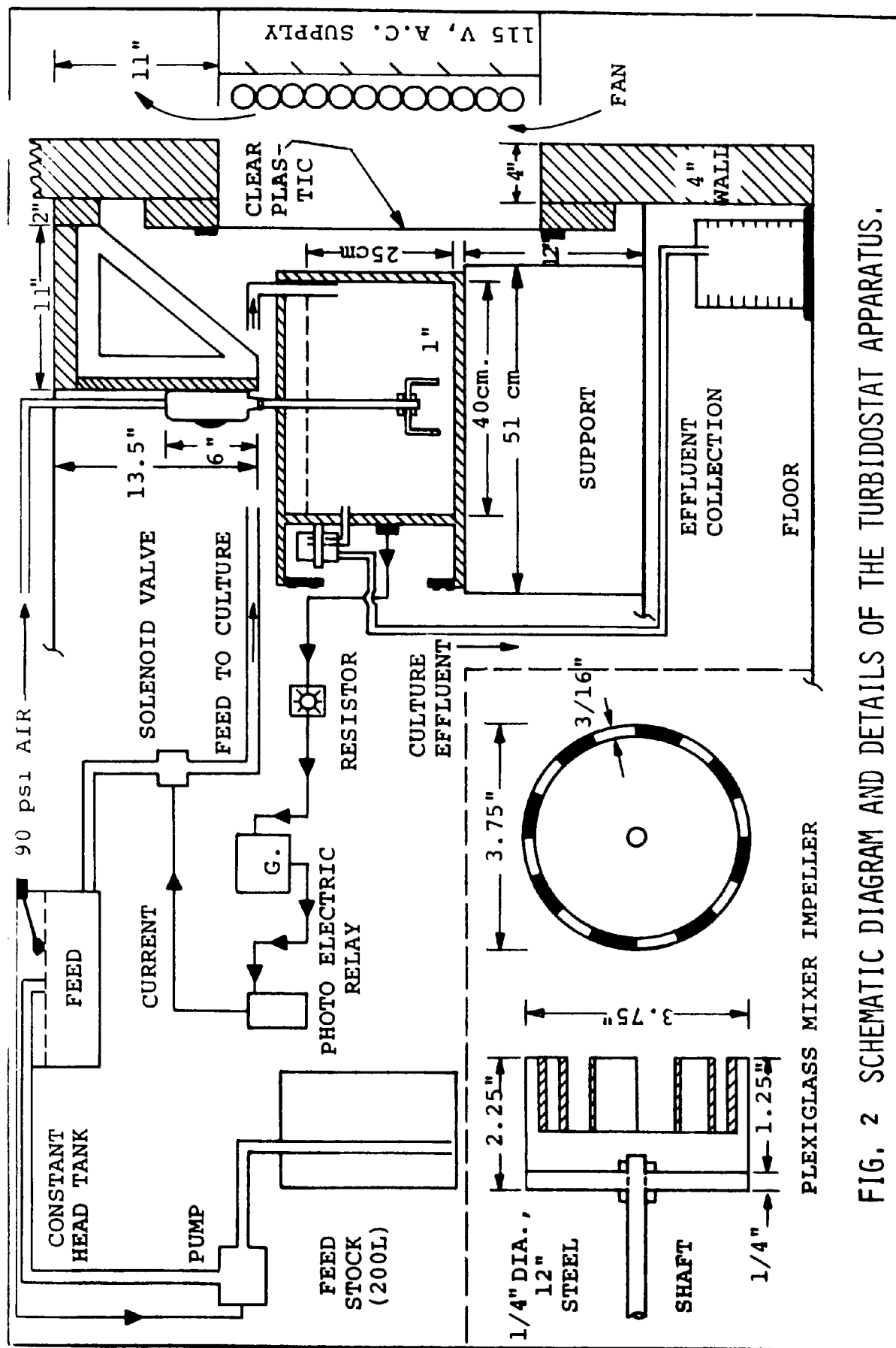
This method of algal culturing was successfully adapted

to various engineering studies at the sanitary engineering laboratories of the University of Michigan (26) (104-107) and elsewhere (21). Encouraged by the aforementioned evidence and logic, this investigator used the turbidostat equipment of this laboratory in most of his studies. Some batch studies of short duration were conducted using an apparatus to be described below.

8. THE TURBIDOSTAT APPARATUS

Two photoelectric cells connected in parallel, an electric current resistance regulator, a spotlight galvanometer, a sensitive phototube, an amplifier and power relay, and a solenoid valve were the essential features of the turbidostat equipment employed in this investigation to hold the culture density constant during any experiment. A schematic diagram of this apparatus, of the reactor (culture tank), and the sketches of some pertinent components are shown in figure 2.

The internal dimensions of the culture tank used throughout this investigation were 25 x 30 x 40 centimeters. Leaving 5 centimeters at the top for the freeboard, an actual culture cross-section of 25 x 25 cm. was exposed to light. Since there were eight grooves (initially designed for varying the culture depth at will) in the reactor, the actual culture volume was 26.25 liters instead of the calculable ($25 \times 25 \times 40 =$) 25 liters. It should be noted that the culture depth was kept constant at 40 cm. throughout this study and the schematic diagram shown in figure 2 holds true for all the experiments



reported.

The shape, size, and location of the mixer impeller with respect to the culture were fixed as shown in figure 2 for the entire duration of this study. Once these parameters, including the length of the shaft and the characteristics of the driving machinery (air driven hand drill in this case) were fixed, the revolutions per minute of the impeller shaft, measured by a stroboscope, represented the degree of mixing provided during any experiment.

The source of light shown in figure 2 consisted of 12,110-watt "Gro-Lux" fluorescent lamps, spaced 2 inches center to center. There were 6 switches on the light source each controlling the operation of two lamps.

This type of constant density culture apparatus was designed by Witt (104) and further described, along with minor modifications, by Gates (106) and Echelberger (107). A complete scheme of the electrical circuits for density control was shown by Gates in his figure 5. He also presented in his figure 6 a schematic diagram of the electrical circuits used for pH control in the initial phases of this study. For most of this investigation however, no such pH control was necessary since the bicarbonate ion added to the medium, in addition to whatever carbon dioxide diffused into the culture at the turbulent culture-air interface, provided adequate sources of carbon and appeared to buffer the system at about pH 8.3.

Further details of the design of the culture tank used in this study can be found in figures 1 and 2 of Gate's thesis (106), and also in figure 1 of Echelberger's thesis (107).

Gates also described in some detail the operation of the air turbine used in this study for mixing culture.¹ The "Gro-Lux" fluorescent lamps, developed particularly for plant growth objective by the Sylvania Corporation, were described by Echelberger and are different from the light sources used by Witt, Gates, and this author for batch study.

Temperature, Light intensity, mixing, and culture density were the main physical variables in this study. For a ready reference, the mechanisms used for the desired control of these parameters are described in Table 6.

Table 6. Physical Variables and Their Control.

VARIABLE	CONTROL MECHANISM
<u>TEMPERATURE</u>	The entire experimental apparatus was housed in a thermostatically controlled walk-in-incubator. The light bank was installed outside to cut down the heat and two fans were installed inside the room for more efficient cooling of the culture. Any desired culture temperature from 15 to 45°C could be obtained by thermostatic regulation of the room temperature.
<u>LIGHT INTENSITY</u>	Any one or all of the six switches could be turned off at will and thereby, obtain the desired illumination at the face of the culture ranging from 0 to 2400 foot candles. Minor adjustments in incident light could also be made by moving the culture tank away from

Table 6 (Contd.)

VARIABLE	CONTROL MECHANISM
<u>LIGHT</u> <u>INTENSITY</u>	or closer to the light source.
<u>MIXING</u>	After the size, shape, and location of the mixer impeller and the shaft were fixed, the quantity of compressed air supplied to the air turbine at a constant pressure regulated the revolutions per minute of the mixing implement. This was easily controlled by putting a valve in the air line.
<u>CULTURE</u> <u>DENSITY</u>	The photoelectric circuit could be brought into balance at any desired culture density and conveniently, from 8 to 100 mg/l dry weight of algae. When the cell density increased due to growth, this circuit became unbalanced and the photoelectric relay opened the solenoid valve which introduced fresh substrate to the culture. As soon as the culture was diluted to the desired density, the solenoid valve was automatically closed by the photoelectric circuitry.

C. THE BATCH CULTURE EQUIPMENT

Some pure culture studies of relatively short duration were conducted using the apparatus shown in figure 3. The light source consisted of 13,40-watt cool white fluorescent

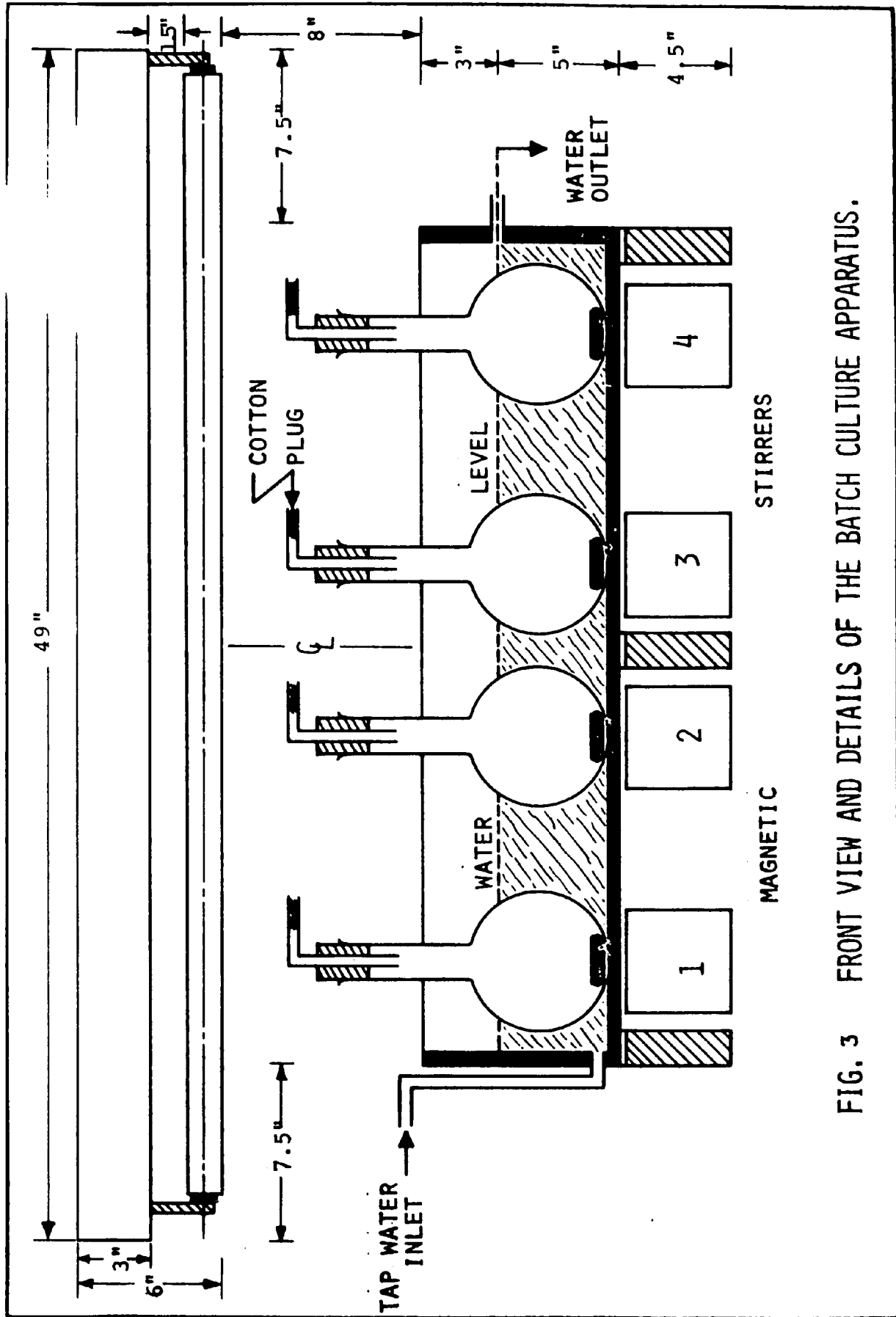


FIG. 3 FRONT VIEW AND DETAILS OF THE BATCH CULTURE APPARATUS.

bulbs spaced 1.75 inch center to center. Further description of the quality of this light and the design of the fixture can be found elsewhere (104)(106). The average illumination produced at the water level throughout the water bath was about 1500 foot candles.

Four, 2 liter capacity, flat bottom, tooled mouth, Florence flasks were employed as culturing containers. Proper precautions were taken for avoiding contamination of the pure cultures, including the use of sterile pipets, magnets, rubber stoppers, and cotton plugs, etc. A constantly running tap water through the 34 x 8 x 9 inch plexiglass water bath shown in figure 3 provided a relatively constant culture temperature of 25⁰C. An approximately uniform rate of mixing was provided to the 2 liter cultures by the fixed size magnets and the magnetic stirrers. No measurements of the actual degree of mixing were made other than appropriately setting the regulators of the different 'magmixes' so that the turbulence in all four cultures visually appeared the same.

D. ANALYTICAL PROCEDURES

Several physical, chemical and biological tests were run routinely to determine the steady state conditions in the turbidostat. Only after the culture had attained equilibrium with the desired variables, the pertinent determinations were recorded to show the cause and effect relationships. The routinely measured physical parameters included mixer speed,

incident light intensity, algal density, temperature, a visual observation of the color of algae, and the growth rate of the algae. Among the chemical determinations, phosphorus and pH were the most important and routine observations, although some analyses were carried out for inorganic carbon (alkalinity), nitrogen (mainly ammonia), and the chloride ion. Routine microscopic observations of algae and photographs taken by a photomicroscope were the main biological analyses, in addition to the algal cell counts included in the physical tests.

1. Physical Tests

i. Mixer Speed:

The revolutions per minute (RPM) of the impeller shaft were measured two to five times daily with a stroboscope, capable of measuring 250 to 18,000 RPM. At times, line air pressure (and therefore, RPM) did fluctuate somewhat from the normal operation level of 90 psi pressure. With about one third the air valve open, a speed of about 1200 RPM was maintained. At this level, no particular mixer speed adjustments were found necessary due to pressure variations.

ii. Incident Light Intensity:

Light intensity measurements were made at about the center of the front face of the culture tank at least thrice daily with a Weston Model 756 illumination meter and a Weston illumination target equipped with a viscor filter. This target is sensitive only to the light from 420 to 710 millimicron wavelength and thus, any energy provided to the culture by

other wavelengths than these was not measured. About 90 per cent of the incident light actually passed through the plexi-glass and reached the liquid face.

Line voltage fluctuations were minimized by placing the light bank on a relatively independent circuit within the building but nevertheless, there were occasions when slight changes existed. Any significant decrease in light intensity due to the aging of bulbs were corrected by replacing the old bulbs with new ones.

Echelberger showed the typical light intensity distribution over the front face of the reactor in his thesis (107). The spot chosen to measure the foot candles during this study was such that the reading represented the average illumination falling on the entire front face. All the light intensity measurements made during a specific phase of this study were averaged to report the foot candles for that phase.

iii. Algal Density:

Three different techniques were used at least twice daily for routine check on the cell density in the turbidostat: (1) Absorbance (2) Hemacytometer count and (3) Algae dry weight. After measuring absorbance of many monochromatic beams, within the 'visible' range, by algal suspensions with a Coleman Model 14 spectrophotometer, using various light paths over a period of several months, it was found that the wavelength 690 $m\mu$ always gave the highest absorbance. Also, these results were more consistent with the other two accompanying measurements than the results obtained by using other monochromatic

lights. Since the principal chlorophylls of green algae are known to absorb maximally from 680 to 700 $m\mu$ (40), the absorbance measurements at 690 $m\mu$ were thought to have a sound basis and were used for the rest of the study.

Per cent transmittance (and not absorbance) versus dry weight of algae gave a straight line plot. Using a 20 millimeter light path and 690 $m\mu$ monochromatic light, the straight line was found to hold from zero to at least 60 mg/l dry weight of 'well fed' algae. This relationship checked when a straight line plot also resulted from zero to 30 mg/l of the same algae using a 40 mm. light path. This plot proved highly useful for a frequent check on the cell density in the reactor and several times, for manual feeding when the turbidostat was out of order either due to breakdown or due to self-imposed darkness in the culture. Further details of the per cent transmittance reading procedures and of its significance can be found elsewhere (106)(107).

Routine microscopic counts of algal cells were made as described by Gates (106) and Echelberger (107). Other than being a relative and qualitative measurement, the algal cell count per se did not prove very useful in this investigation and was the least reliable of the three techniques used for measuring the algal cell density.

The 24 hour dry weight of algae was also determined exactly as described by Gates and Echelberger. Dry weight evaluations were highly reliable when a large sample taken from culture effluent (500 to 1000 ml) was passed through a high grade, 1.2 micron pore size, millipore filter and when proper

precautions were taken in the gravimetric procedures. Since the mg/l dry weight of algae in the synthetic medium most nearly represented the actual weight of the algal protoplasm grown on the supplied nutrients, this determination was given the most attention throughout this study.

The dry weight density was found to correlate very well with the per cent transmittance as long as the culture was not starved with respect to phosphates and every other nutrient was in excess. Upon the significant induction of phosphorus starvation in the algae however, these two determinations did not follow exactly the same pattern. As the algae color deviated from its healthy brilliant green to yellowish to brownish due to the increased phosphate starvation, the per cent transmittance and algae dry weight plot became less and less rigorously applicable. The algae dry weight steadily increased during the phosphate starvation period without a corresponding equivalent decrease in the per cent transmittance.

With the exception of the brownish algae (highly starved) however, the two parameters still remained reliably correlatable. Nonetheless, appropriate corrections in the experimental results had to be made since this phenomenon also manifested itself in the operation of the turbidostat apparatus during the prolonged P-starvation experiments.

iv. Temperature:

A laboratory thermometer was installed in the culture through a hole in the top of the reactor and the temperature

in degrees centigrade was recorded at least 4 to 6 times a day.

v. Visual Observation:

Visual observations of the apparent color of the culture were frequently recorded, particularly during the phosphate starvation experiments. The change in the color of the algae was easily discernible after the phosphorus deficiency had ensued. As illustrated later in this manuscript, a chronological record of visual observations can provide very useful information on the level of phosphate starvation once the color pattern has been studied before.

vi. Growth Rate:

The constant density culture effluent was collected in a graduated container and the accumulated volume was recorded as frequently as desired. When the cumulative overflow in liters was plotted against time in hours, the slope of the resulting straight line yielded the growth rate in liters per hour. Since the algal cell density in the culture effluent was always the same as in the reactor and thus, was known, the growth rate of the algae could be expressed in other forms including the form of $\log_e$ units per day as shown by Gates and Echelberger in their theses.

It should be noted however, that the growth rate expressed in other forms than liters per hour does not become more valid or theoretically sound. At the present stage of knowledge, no growth rates of the same alga expressed in various forms by different investigators can be assigned a universal

meaning and they all tend to be system specific to some extent. The virtue of using liters per hour, lies in the fact that it does not give such an illusion!

2. Chemical Tests

i. pH Determinations:

A line voltage operated pH meter, standardized against pH 7 and pH 10 buffer solutions daily, was used for all pH determinations on the synthetic feed and the algal culture. Since no free carbon dioxide was fed to the algae for most of these studies, a careful pH control on the synthetic feed was as essential as actually checking the pH of the culture. The hydrogen ion activity being less time consuming, was carried out at least 4 to 5 times daily. It proved useful in checking the algal growth activity as well as the chemical precipitations in the feed tank. Appropriate pH adjustments, when needed, in the feed and the culture were easily made using a dilute acid solution or occasionally, a dilute alkaline solution.

ii. Phosphate Analysis:

During the initial phases of this research, the aminonaphtholsulfonic acid method for orthophosphate was used as described in the 12th edition of Standard Methods (108). For most of this investigation however, only total phosphate determinations were made using the method for quantitative determination of total phosphorus in water described by Gales, Julian, and Kroner (109). Routine total phosphate analyses of

the synthetic feed, the reactor, and of the effluent filtrate (the culture fluid which passed through the 1.2 micron pore size high grade membrane filter) were conducted using the procedure outlined in Appendix VII.

Many phosphate solutions of different concentrations in distilled water and the synthetic feed at the culture pH were passed through the same filtering apparatus to see if any loss of phosphates in the filtrate occurred but no such evidence was obtained. On this basis, the phosphate in algae was calculated by subtracting the phosphate in the effluent filtrate from that determined for the reactor contents. Since all these values were total phosphates, a mass balance was always checked under steady state conditions as following:

$$\begin{aligned} \text{PO}_4 \text{ in Feed} &= \text{PO}_4 \text{ in Reactor} = \text{PO}_4 \text{ in Algae} \\ &+ \text{PO}_4 \text{ in Effluent Filtrate} \end{aligned}$$

When the feed did not contain significant quantities of phosphorus and this element was directly added as a shock to the reactor, the above balance could not be struck. The knowledge of the quantity of phosphates added and of the hydraulic washout characteristics of the reactor however, significantly helped in checking on the accuracy of the phosphate analyses of the reactor contents.

The author found this method of total phosphorus determination to be reliable as long as the iron concentration in the diluted sample did not exceed 1.0 mg/l Fe and the dry weight algal density was kept below 25 mg/l. The results were expressed as milligrams of PO_4 per liter of sample and found to be highly reproducible from run to run, as well as within

replications of three to five. With few exceptions, the critical phosphate tests were always run in duplicates or triplicates and many times, different dilution factors were used between these replications.

A reliable and complete phosphate analysis for as many as 80 samples was carried out in a normal 8 hour working day by a single assistant having no previous laboratory experience, after having been trained to run these tests. Standard curves of the type shown in Appendix VIII were used. One or more knowns were always run with each batch of samples to assure desired accuracy and precision.

iii. Alkalinity Measurements:

In several phases of this investigation, routine total alkalinity determinations were made on the synthetic feed and the effluent filtrate using techniques as described in the 12th edition of Standard Methods (108). The alkalinity results were reported as mg/l inorganic C for a direct comparison with the phosphorus results. It should be noted here that these values of inorganic carbon did not include the carbon dioxide present in the reactor and therefore, could not be used for a quantitative analysis of the nutrition of algae with respect to carbon.

iv. Nitrogen Analysis:

Only a limited amount of organic, nitrite, and nitrate nitrogen testing was done in accordance with the Standard Methods (108). These results have not been reported in this thesis due to an insufficient establishment of the

nitrogen-nutrition pattern of algae. Ammonia-N was the only significant nitrogen source in the substrate (traces of NO_2^- and NO_3^- from the other reagents added to the growth medium, and the N_2 gas from air were considered to be insignificant sources for any substantial nutrition of the green algae). As a result, the $\text{NH}_3\text{-N}$ was tested for much more frequently than the other nitrogen fractions. This was done mainly to assure an adequate supply of NH_3 to the algae and in batch studies, to observe its extraction from the medium in relation to the phosphorus uptake. The direct nesslerization method of ammonia-N determination described in Standard Methods (108) was used and the results were expressed as mg/l N.

v. Chloride Determinations:

Sodium chloride was used as a tracer to check the hydraulic characteristics of the continuous culture tank. Mercuric nitrate method for chlorides in water, outlined in Standard Methods (108), was adopted and the results were reported as mg/l Cl.

3. Biological Tests

Routine microscopic observations, particularly under oil immersion using a fixed slide, were found very helpful to determine the relative purity of the culture, changes in the morphology of the cells, and in understanding the intracellular structure of the algal cells. This author, not being an algal taxonomist, developed proper respect for the alga cell as an

individualist and learned to control the culture environment to get the type of the cells desired through the use of this technique. Some photomicroscopic work was also done to provide evidence for the cell types used in this investigation. A few photomicrographs are presented in the next chapter. The microscopic cell counts have been mentioned under the physical tests.

CHAPTER VI

CULTURE TYPE AND SUBSTRATE

A. CULTURE ORGANISMS

The proper choice of a well defined growth medium depends upon the microorganism to be cultured. For an optimum and generally reliable nutrient extraction process, selection of the organism must take into account the physico-chemical environment and the variability that would naturally exist in a large scale tertiary treatment plant. Species which are hypersensitive to any antagonistic effects would normally be excluded, and more persistent ones selected. *Chlorella* and *Scenedesmus* genera of the order Chlorococcales of the class Chlorophyceae of algae (green) have been studied most extensively and found to grow under a great range of variations in their ecological environment. These organisms have been employed successfully for several other engineering studies (26)(104-107), and appeared entirely suitable for the current investigations.

B. THE OPTIMUM SUBSTRATE

Having chosen these green algae as the culture organisms, certain micronutrients (Table 1 and Chapter 4) could be excluded and the quantities of others reduced in the growth medium. The exclusion of silica would, for example, discourage diatoms while keeping the sodium, calcium, boron, molybdenum, and cobalt levels low should provide an unfavorable diet for the

blue-greens. When a careful balance of nutrients was struck in this manner, the chances for the growth of culture contaminants were greatly reduced and the desired type of cells could be grown with success as a uni-algal-culture, particularly at their optimum temperature and pH. Furthermore, a rigorous control of the physico-chemical environment of the desired culture discouraged the appearance of new organisms not accustomed to these conditions, while encouraging the growth of the adapted species.

In the selection process for the growth medium, another factor that should be included, is the chemical composition of wastewater treatment plant effluent. Witt (104), Gates (106), and Echelberger (107) grew the above noted green algae in treated sewage and reported success in their culturing. The information available on the chemical analysis of this type effluent (106)(107) was considered in arriving at the constituents of the synthetic growth medium for the present investigation. An elementary analysis of domestic sewage from four different cities in California as reported by Allen (110) is presented in Appendix IX for further comparisons.

A full description of the exact composition of the growth substrate and comparisons of the quantities of individual nutrients supplied to the ones present in sewage or experimentally determined to be 'critical' for the growth of the green algae are given in Appendices X, XI, and XII. The critical concentrations in Appendices XI and XII were reported for batch cultures under relatively ill-defined environmental and physiological conditions and therefore, cannot be given too

rigorous an interpretation.

C. WHY NOT PURE CULTURE

This investigator did not feel that a pure culture of a given strain of a particular species was absolutely necessary in studies of this nature. Fogg (8) stated, "It must be largely true that the total biomass of phytoplankton is determined by the physical and chemical factors in the environment and is not dependent on the species which are represented in it". Danforth (111) reported that the nutritional behavior, of the algal species that he studied, showed slight correlation with taxonomic relationships. Collyer and Fogg (112) documented the observation that different species of algae have a tendency to resemble each other in the relative amounts of crude protein, fats and hydrolyzable polysaccharide which they contain when grown under approximately similar conditions.

Parsons, Stephens, and Strickland (113) demonstrated that the gross composition of the cell material of an alga is dependent more on environmental circumstances than on class or species. Some of their results are summarized in Table 7. The ash content of different species varied greatly (particularly high in diatoms) but when allowance was made for this, by expressing the amounts of major fractions in terms of the total organic carbon in the cells, the composition of the cells were generally similar. The Dinophyceae have thick cellulose walls and if this were taken into account, their cell composition (low protein/C ratio) would conform more closely to that of

others than is shown in Table 7. The maximum variation in protein content of these five classes of algae is only about two fold.

Spoehr and Milner (114) on the other hand, showed that the variation in protein content of Chlorella pyrenoidosa may be more than ten fold depending upon the conditions under which it is grown!

These considerations however, should not be taken to imply that kinetics of growth for various algae are always the same under similar environmental conditions. In general, the larger the size of the cell, the slower the growth rate to be expected. Also, a change in a given environmental factor would not necessarily change the growth kinetics of different species in the same direction and to the same extent.

The foregoing evidence would support the statement however, that the nutrition pattern of all cells grown in a carefully controlled uni-algal-culture should be very similar, if not identical. Furthermore, the proportion of the culture contaminants with respect to the total number of cells in a uni-algal-culture being insignificant (less than 5% by weight under the worst conditions), their effect on the results attributed to a given algal culture would, under no circumstances be more than the limits of the accuracy of the various analyses employed in any rigorous pure culture study.

This investigator used uni-algal-cultures of *Scenedesmus* during most of these studies. Since the environmental conditions maintained in these cultures favored their growth, no significant difficulty was encountered in preserving their relative

Table 7. Comparison of the Cell Composition of Species of Marine Phytoplankton, Grown under Similar Chemical and Physical Conditions, in Terms of the Ratio of Components to (Oxidizable) Carbon (113).

ALGAL GROUP	PROTEIN/C	CARBOHYD./C	FAT/C
Chlorophyceae:			
<u>Tetraselmis maculata</u>	1.42	0.41	0.07
<u>Dunaliella salina</u>	1.43	0.80	0.15
Chrysophyceae:			
<u>Monochrysis lutheri</u>	0.94	0.59	0.22
<u>Cricosphaera carterae</u>	1.41	0.45	0.12
Bacillariophyceae:			
<u>Chaetoceros sp.</u>	1.12	0.22	0.21
<u>Skeletonema costatum</u>	1.38	0.79	0.17
<u>Coscinodiscus sp.</u>	1.08	0.27	0.11
<u>Phaeodactylum tricornutum</u>	0.88	0.64	0.17
Dinophyceae:			
<u>Amphidinium carteri</u>	0.69	0.75	0.44
<u>Exuviella sp.</u>	0.70	0.84	0.34
Myxophyceae:			
<u>Agmenellum quadruplicatum</u>	0.86	0.75	0.31

purity. The routine microscopic observations were most helpful in determining the level of contamination in the *Scenedesmus* culture (generally very low). Many photomicrographs were taken during the course of this investigation so that the changes in the cell morphology and in the species within the culture genus could be recorded. What this observer designated as a uni-algal-*Scenedesmus* culture, based upon many photomicroscopic observations, is presented in Figure 4. Also included in this figure are the photomicrographs showing what was similarly designated as a uni-algal-*Chlorella* culture. The effect of environmental and physiological conditions on the morphology of algal cells is apparent too from this figure.

When the level of contamination of *Chlorella* in *Scenedesmus*, or vice-versa, was found to be more than 5%, the culture was called 'predominantly *Scenedesmus*', or 'predominantly *Chlorella*'. The bacterial contamination, significant only during the river water runs, was recorded as such on the appropriate figures to be found later in this manuscript. The effect of this contamination on the phosphorus nutrition of algae could hardly be detected. During the pure batch *Chlorella* culture studies, no significant contamination was ever noted under the laboratory microscope.

Figure 4. Photomicrographs of the Green Algae *Scenedesmus*
and *Chlorella*.

A. Photomicrographs in Natural Color.

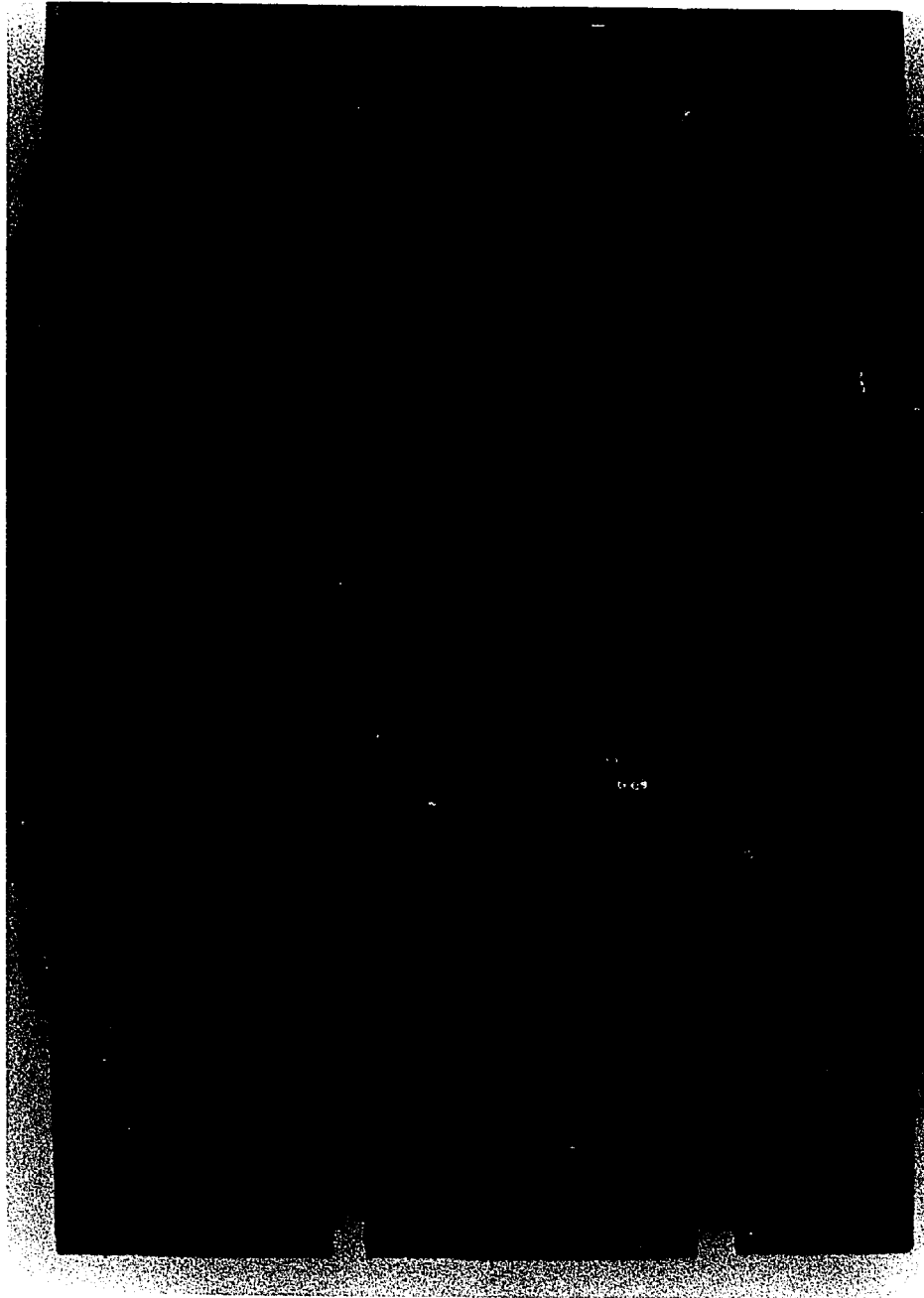


Figure 4 (Contd.)

B. Information Concerning the Photomicrographs.

1. Uni-Algal-Scenedesmus (150X).

Continuous, constant density culture. Contained 3.6% PO_4 by dry weight of algae. Had been grown at 25°C, pH of 8.3, 1600 F.C., and 1500 RPM.

2. Uni-Algal-Scenedesmus (645X).

Same as number 1.

3. Predominantly Scenedesmus (645X).

Continuous, constant density culture. Phosphorus content from 5 to 9% PO_4 by dry weight of algae. Growth conditions: 20-25°C, pH from 8.2-8.4, 1500-2000 F.C., and 1200-1500 RPM.

4. Uni-Algal-Scenedesmus (645X).

Batch culture. Contained 3.4% PO_4 by weight at the time of this photomicrograph. Approximate growth conditions: 25°C, 1500 F.C., 1200 RPM, and pH 7.7.

5. Scenedesmus and Chlorella (645X).

Culture conditions similar to the ones noted for number 3.

6. Pure Chlorella (645X).

Batch culture. About 3 weeks old. High cell density and P-content of the cells. Growth conditions approximated the ones indicated for number 4.

7. Uni-Algal-Chlorella (645X).

Continuous, constant density culture. Phosphorus content = 14% by weight of cells. Growth conditions: 25°C, 1550 F.C., 750 RPM, and pH 8.4.

8. and 9. Pure Chlorella (645X).

Batch culture. Conditions similar to the ones indicated in number 6.

CHAPTER VII

PRELIMINARY STUDIES

Before using the turbidostat apparatus for evaluating the effect of various physical, chemical, and physiological parameters on the phosphorus uptake process of green algae, a check for complete mixing in the reactor was felt to be necessary. In addition, the reliability and sensitivity of the photoelectric circuit used for maintaining a given algal density in the reactor had to be ascertained. The response of a steady state culture to a sudden breakdown in one or more environmental controls also had to be evaluated. Since a wide range of phosphorus concentrations were to be fed to a constant density culture, some idea of the effect of this variable on the uptake of carbon by the algae was considered useful.

Despite all the problems outlined in chapter V, the limited volume (batch) culture being relatively simple and requiring little effort to maintain, was considered for use wherever possible. The ease with which a pure culture could be maintained in the batch apparatus was the main reason for substituting it for the turbidostat in short term studies concerning NH_4^+ and PO_4^{3-} ion adsorption by Algae. Since the steady state situation in a batch, comparable to that in a constant density culture, is never reached, a direct correlation of the results obtained using the two techniques is not possible. It was not the goal of this study to try to

find ways to translate batch culture results into comparable continuous culture values. Determining the often quoted cation-anion parallel in ion absorption with respect to NH_4^+ and PO_4^{3-} ions was considered useful and this could easily be done employing the batch culture.

All these preliminary considerations as well as the factors concerning the use of the synthetic feed are discussed below item by item.

A. A CHECK FOR COMPLETE MIXING IN THE REACTOR

To assure complete mixing in the reactor was among the most important considerations in checking the reliability of function of the turbidostat apparatus. In order to evaluate the efficiency of culture-substrate mixing in the reactor, the possibility of short-circuiting of the influent feed, and to assess the exact rate of decrease in concentration of the phosphate in the culture when added as a shock, tracer studies were conducted.

The technique employed was essentially to feed Ann Arbor tap water at a specific rate into the reactor after it had been filled initially with a known solution of sodium chloride in tap water. During these tracer experiments, the rate of mixing was kept low (500 RPM) so that the lower RPM limit for complete mixing could be fixed. The chloride concentration in the effluent was measured and compared with the theoretical chloride concentration which can be predicted by the following equation:

$$C_t = C_o \cdot e^{-t/D} \dots\dots\dots 5$$

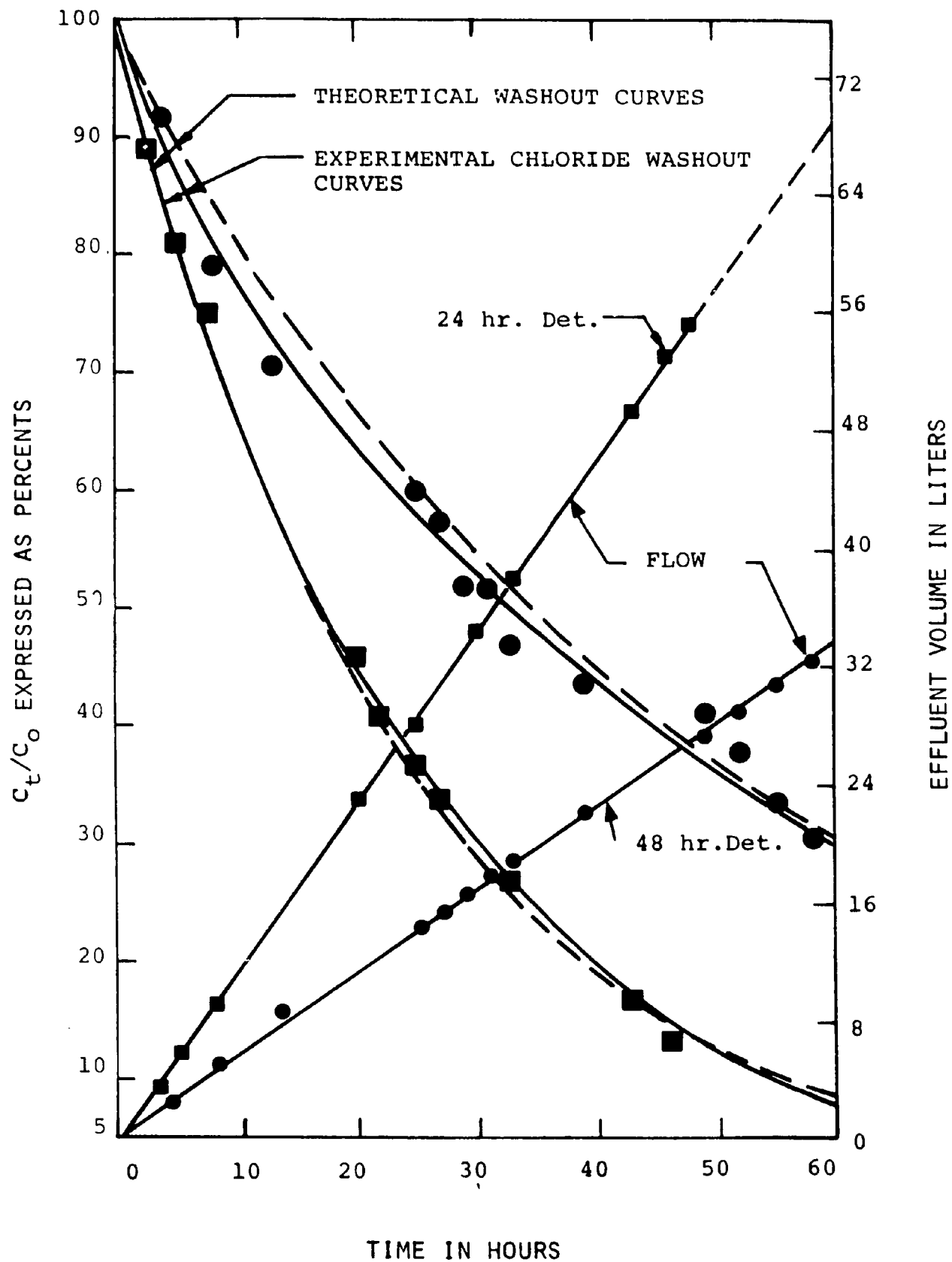


Figure 5. Theoretical and Experimental Washout Curves for the Completely Mixed Algal Culture Tank

In this equation, C_t is the concentration in the reactor (or in the effluent) at time t in hours, C_0 is the initial concentration, and D is the theoretical detention time calculated from the hydraulic flow rate. The curve produced by this equation is called a "washout" curve.

Figure 5 shows a comparison of the theoretical and experimental washout curves for 24 and 48 hour detention times. These results demonstrated that the reactor provided complete mixing of the influent substrate with the culture.

A complete mixing with respect to the algal cells in suspension was determined by comparing the algal density in the reactor under actual operation with that in the culture effluent. Under conditions of complete mixing, both these densities must be equal. Both of these techniques showed that the reactor used throughout this investigation was truly a 'completely mixed' system.

B. SUITABILITY CHECK ON SYNTHETIC SUBSTRATE

Since the synthetic feed though carefully prepared, still could lack some important growth factors that might be present in natural waters, the growth rate and the phosphate uptake behavior of algae in a supplemented river water feed were determined prior to use of the synthetic substrate in the turbidostat apparatus. The rate of growth of the algae and the phosphorus uptake pattern in the river water feed were found to be very similar to the values obtained by employing the synthetic feed prepared using Ann Arbor tap water. These results gave considerable support to future generalizations

which might be made concerning the data and encouraged extensive use of the synthetic media in these studies. Experiments concerning this factor are reported on in the next chapter.

C. FACTORS AFFECTING STEADY STATE GROWTH

When all the conditions affecting growth are held constant, the growth rate of microorganisms which have been adapted to the prevailing conditions attains a constant value. This growth rate is designated as 'steady state growth rate' and is highly reproducible. As long as the growth conditions remain unchanged, the growth of a constant density culture also remains unchanged. However, since the growth of microorganisms itself changes the growth conditions in a batch culture, the parameters in 'steady state growth rate' are not immediately translatable when observing results in limited volume cultures.

The steady state growth characteristics of the turbidostat culture can be seen in Figure 6. In addition, the remarkable constancy in phosphorus and carbon uptake by the algae is a feature of this figure worth noting. It is also clear from this figure that a sudden change in one of the critical growth parameters, produces a corresponding change in the steady state growth rate.

When the culture was suddenly exposed to darkness, after having been grown at 1200 foot candles for 2 days, the steady state growth rate immediately declined. The lights were turned off only for 3 hours but it took the mixed culture about

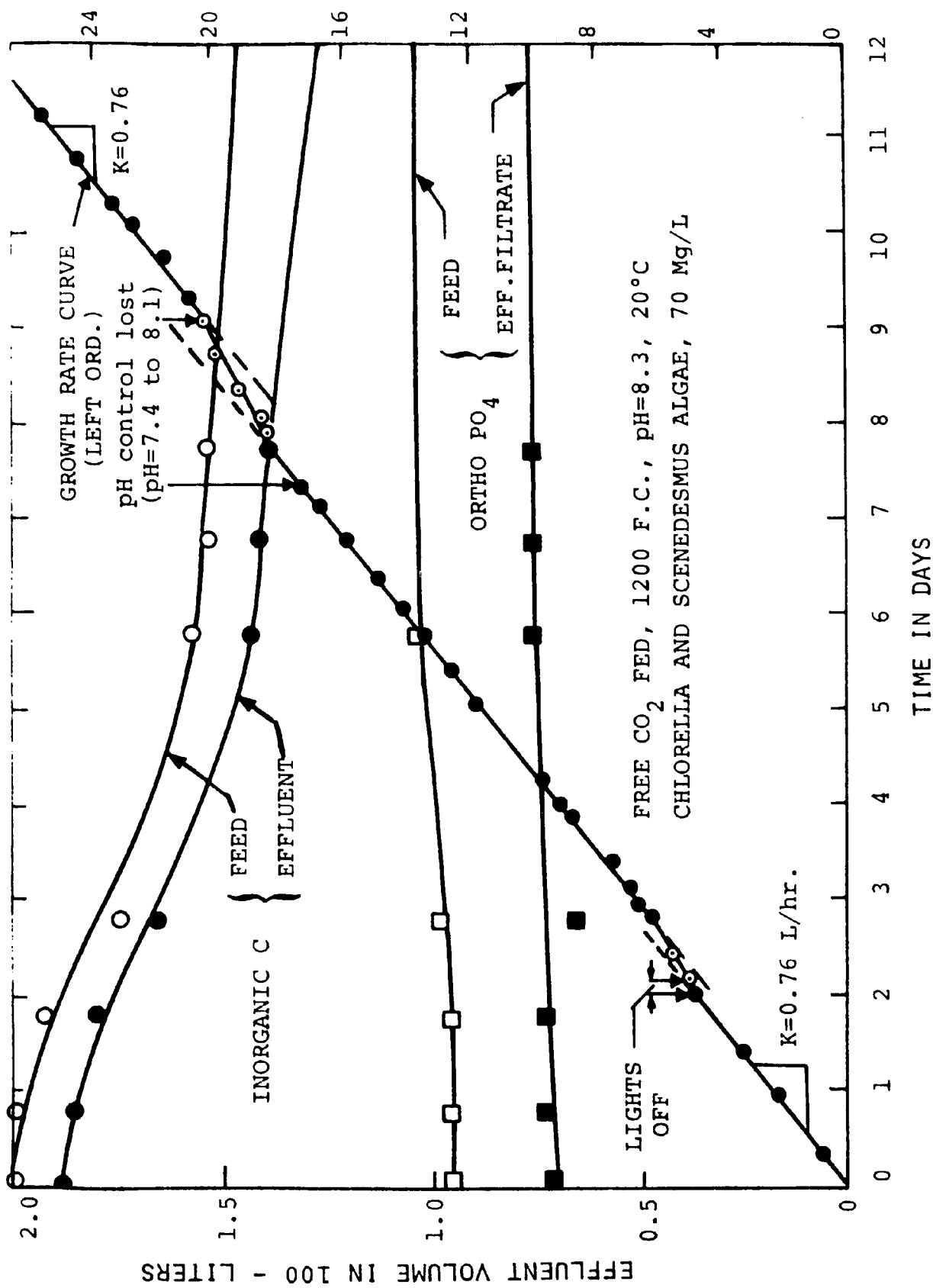


Figure 6. Inorganic Carbon and Phosphorus Uptake Patterns of Steady State Continuous Algal Cultures

16 hours to resume its previous steady state growth rate, after the original level of illumination was restored. It would appear that either the light absorbing pigment content of the algal cells declined or got 'inactivated' to some degree during the dark period since there was a considerable amount of 'lag' before the culture resumed its maximum growth under the experimental conditions. Another possible interpretation might be that some crucial chemical compounds such as nucleic acids and certain enzymes directly involved in cell division and cell synthesis were used up but not replenished during the darkness. If this is true, it is possible to visualize this lag without postulating any significant change in the light absorbing pigments since some time would be required to synthesize enough of these compounds before experiencing the maximum steady state growth.

Similarly, the value for the steady state growth rate declined when the pH control was lost and it increased (see figure 7) with an increase in temperature. Since the pH gradually decreased from its steady state value of 8.3 to 7.4 due to an unwanted addition of CO_2 , the decrease in the steady state growth rate was also gradual. The culture immediately assumed its maximum steady state growth however, when the original steady state pH was restored. This pattern was also true for temperature except that the growth rate gradually increased instead of decreasing. All this is in contrast to the response of culture to the restoration of light and a very important feature of algal growth characteristics.

Many determinations of algal cell density in the reactor

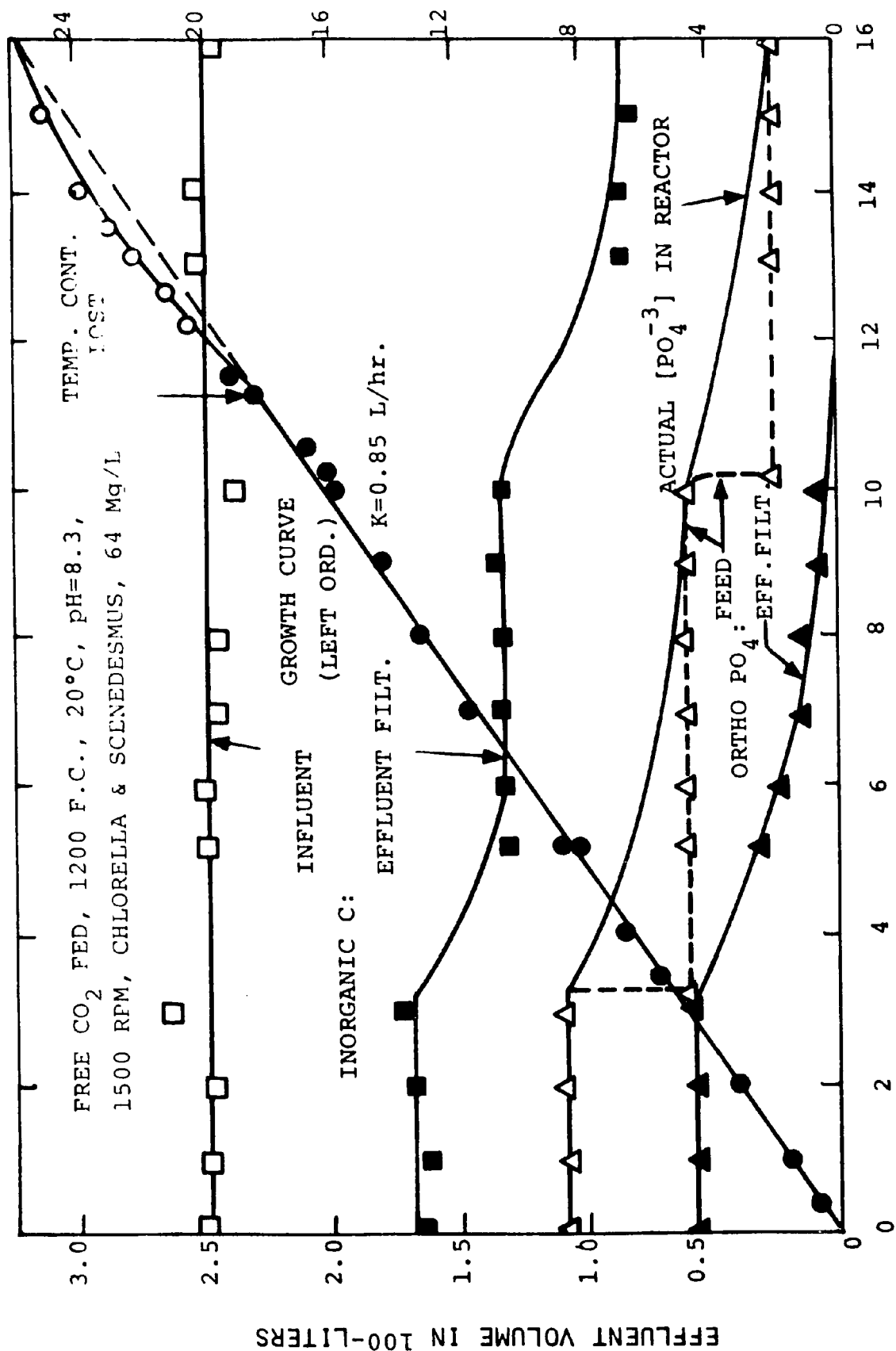


Figure 7. Effect of Stepwise Reduction of PO₄ on the Uptake of Carbon by Algae at pH 8.3

and in the effluent were carried out during the 12 day period shown on figure 6 to check the reliability and sensitivity of the photoelectric circuit used to maintain 70 mg/l algae in the reactor, and also to evaluate if the culture was a completely mixed system with respect to the algal cells in suspension. The results left no doubts that the reactor was in fact a completely mixed system and the turbidostat apparatus functioned very well.

D. EFFECT OF PHOSPHORUS SUPPLY ON CARBON UPTAKE

The remarkable constancy in phosphorus and carbon uptake by a steady state culture was pointed out in the last section when discussing figure 6. The concentrations of phosphorus and carbon in the substrate were kept constant throughout that experiment as one of the conditions for a steady state culture.

However, when the concentration of phosphorus in the reactor was varied systematically and the supply of carbon and other parameters was held constant, an increased uptake of carbon with a decreased supply of phosphorus to the culture was observed as shown in figure 7. Since free CO_2 was fed to the culture to maintain a constant pH of 8.3 and it was not included in the inorganic-C measurements, it may be argued that the algae started picking up more HCO_3^- and less free CO_2 than before upon a reduction of phosphorus in the medium, and that the total carbon uptake by algae remained constant. Another argument of pure culture proponents might be that this phenomenon was manifested by a relative increase in

Scenedesmus and decrease in Chlorella cells during the course of this experiment since it was a mixed culture. Both of these arguments are quite valid and were considered further.

Many microscopic observations made during the experiment did not reveal any significant change in the relative cell populations of Scenedesmus and Chlorella. Also, for the sake of a scientific argument, one may ask the question: Why would the Scenedesmus cells take over when the growth conditions and the supply of phosphorus were still adequate for the growth of Chlorella and the relative growth kinetics certainly did not favor Scenedesmus? The fact that the steady state growth rate remained unchanged may also count against the possibility of a major relative change in the algal species during the experiment.

The second argument was checked out by growing algae in the absence of free CO_2 feed and holding the pH constant at a different level (pH of 7.8) by properly adjusting the pH of the nutrient influent. These results are shown in figure 8. It is obvious from these data that a decrease in phosphorus supply in the medium does in fact induce the algae to pick up more carbon.

Information available on the effects of phosphorus supply on the composition of higher plants has been reviewed by Hewitt (115). While citing the work of Eaton (116)(117)(118), he stated that phosphorus deficiency in Helianthus annuus (sunflower), Glycine max (soybean), Brassica nigra (black mustard) caused increased concentrations of total sugars, reducing sugars, sucrose, and starch in young plants; of all

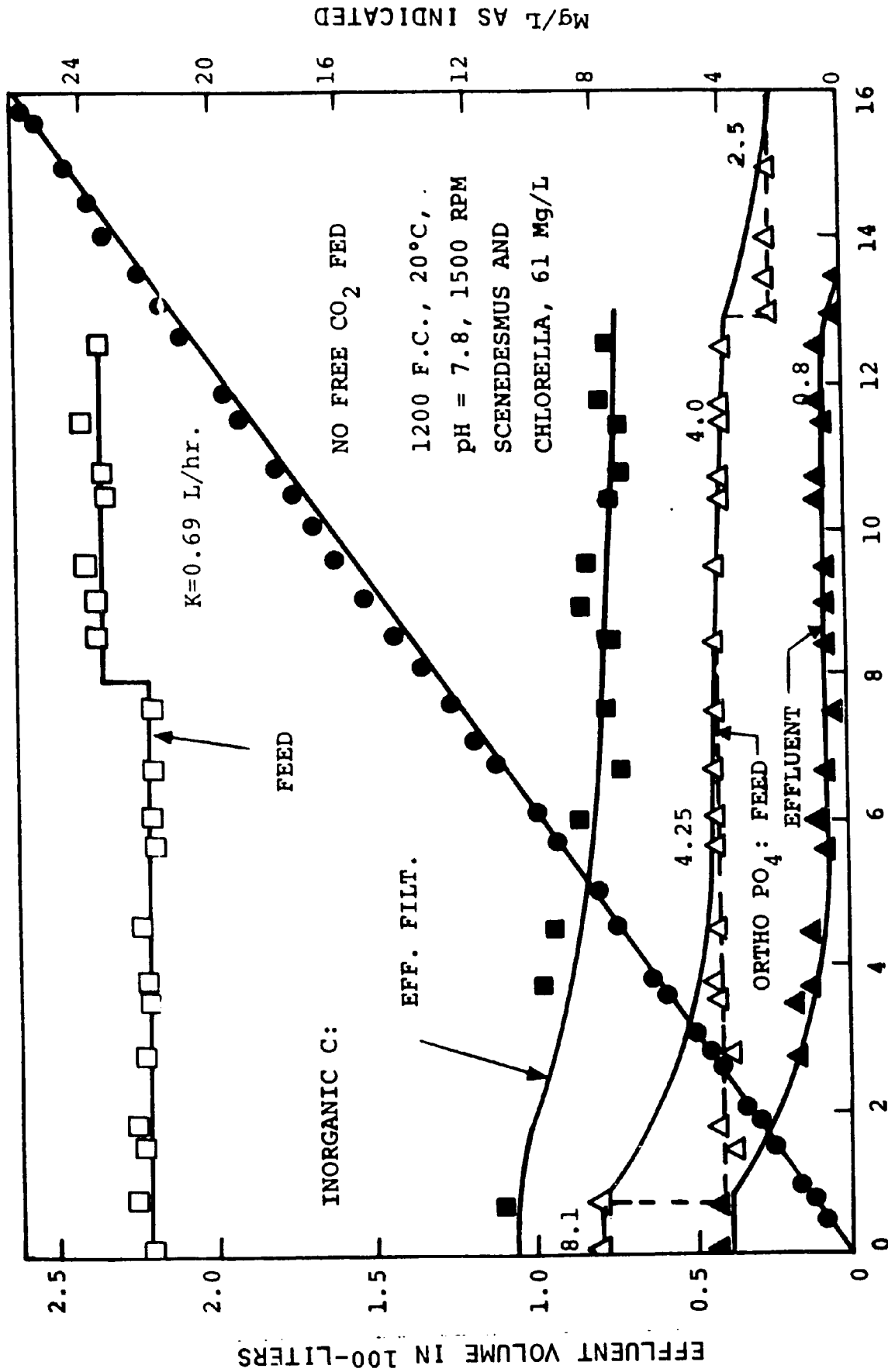


Figure 8. Effect of Stepwise Reduction of PO₄ on the Uptake of Carbon by Green Algae at pH 7.8 and under NO CO₂ Feed Conditions

the carbohydrate fractions; and of total and reducing sugars and sucrose respectively, in the three plants. Although phosphorus 'deficiency' is not the same thing as 'decrease' in supply without affecting the growth rate, the situation is very analogous. This evidence provides some support for the interpretation of the phenomenon observed above in the case of algae. Hewitt (115) also concluded that decreased phosphorus supply to plants often results in their increased nitrogen uptake. The real reasons for a decrease in PO_4 causing an increase in carbon uptake by algae are obscure at this time.

E. INTERNAL STORAGE OF PHOSPHORUS BY ALGAE

One very important observation to be made from figures 7 and 8 is, that irrespective of the actual amounts of carbon and phosphorus extraction by algae, the steady state growth rate remains constant. When 8.1 mg/L ortho- PO_4 was fed to the culture, the algae continuously extracted about 4.1 mg/L of ortho- PO_4 from the medium, leaving the remainder in solution, and it extracted all phosphate when the ortho- PO_4 in the feed was 4.1 mg/L or below (figure 7). This means that under these experimental conditions, algae could pick up continuously any amount of phosphate from 2.5 to 4.1 mg/L PO_4 without showing any effect on the steady state growth rate.

It is clear from this evidence that algae are capable of internally storing the phosphate and that the stored

phosphorus does not enhance their growth rate. Since the amount of storage that is apparent from figure 8 is different from the value stated above, it is also obvious that the extent of the internal storage of phosphorus depends upon the environmental conditions and more carefully controlled experiments would be necessary to find the proper correlations. This observation would suggest too that there are definite limits within which the supply of phosphorus to a given weight of algae will be consumed without having any effect on the steady state growth rate and that below the lower limit the phosphorus supply would control the amount of algal growth when the other conditions still remain favorable and fixed.

F. RELATION BETWEEN NITROGEN AND PHOSPHORUS UPTAKE

Although the main objective of this study was to elucidate the phosphorus uptake pattern of algae under different growth conditions, relating the uptake of phosphorus with that of nitrogen to a limited extent was considered desirable. This was accomplished using the batch culture apparatus and pure *Chlorella* in experiments of relatively short duration. The results obtained during two such experiments are presented in figures 9 and 10.

Pure cultures of *Chlorella* were grown in the synthetic feed described earlier and under the environmental conditions indicated on these figures. At the beginning of the experiment reported on in figure 9, the dry weight density of algae and the total phosphorus concentration in these cells were 500

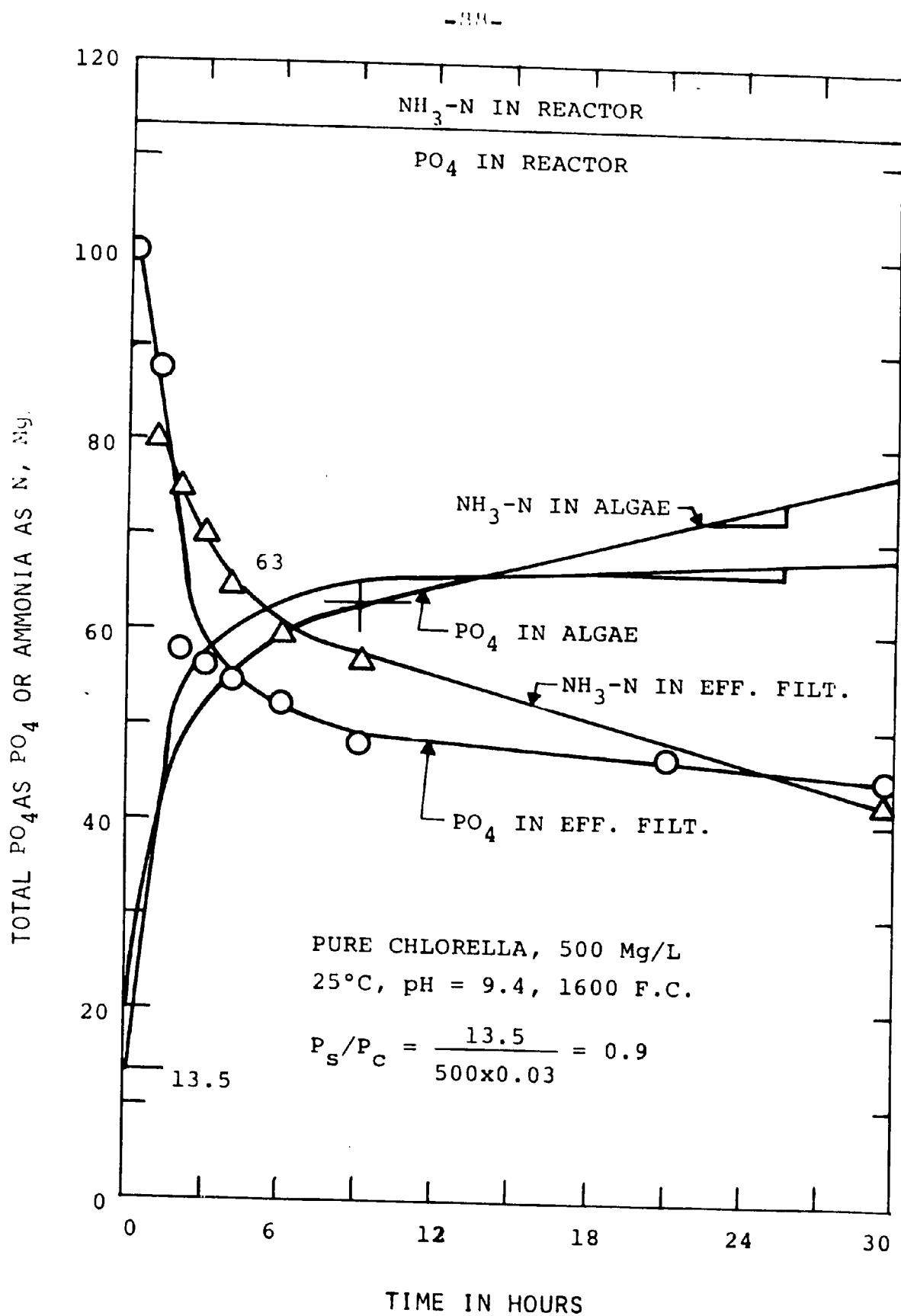


Figure 9. Simultaneous Uptake of Nitrogen and Phosphorus by Chlorella in a Batch Experiment

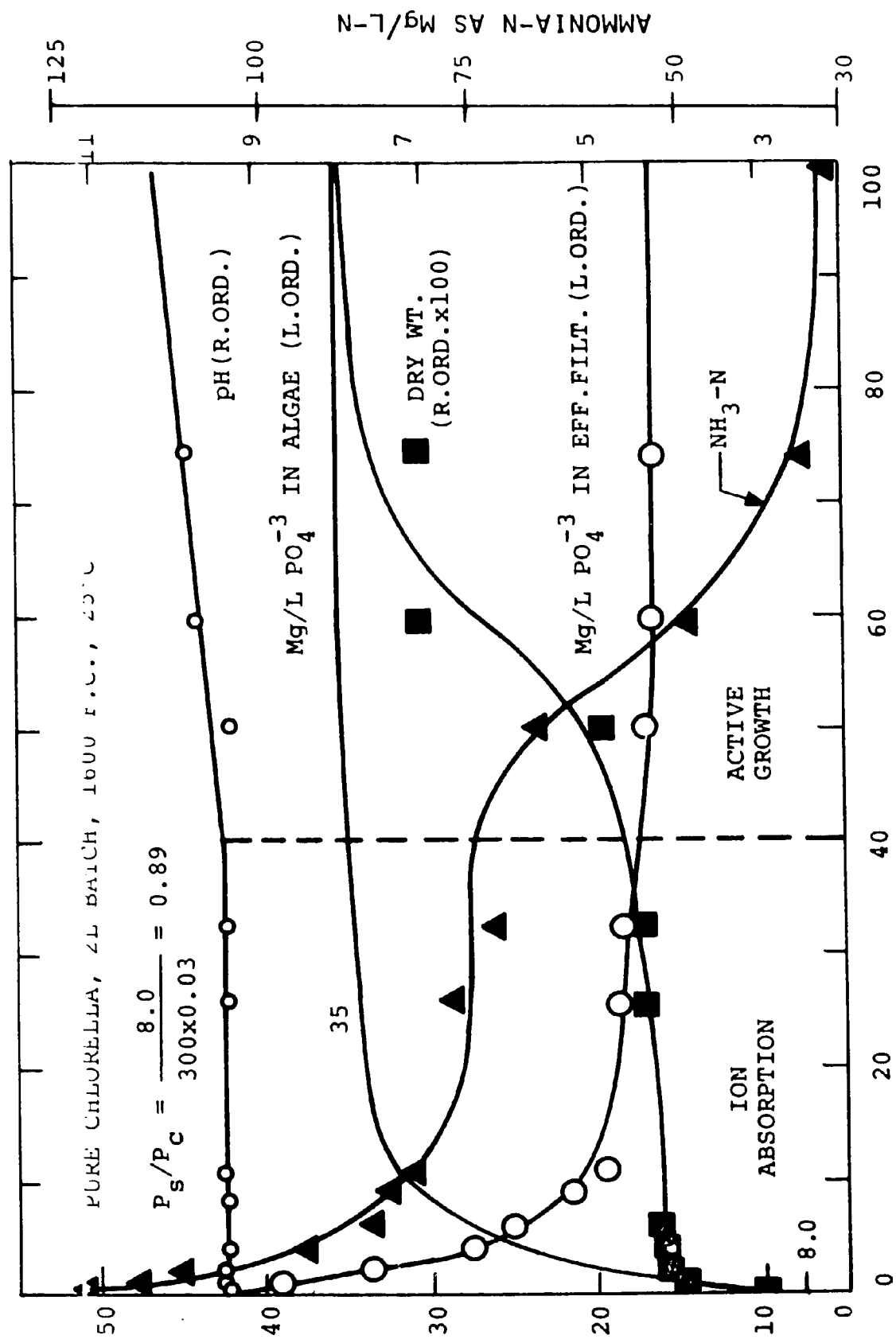


Figure 10. Simultaneous Uptake of Nitrogen and Phosphorus During Ion Absorption but not During Active Growth of Chlorella

mg/L and 13.5 mg/L, respectively. The culture was shocked with $\text{NH}_3\text{-N}$ (added as NH_4Cl solution) and ortho-phosphate (added as K_2HPO_4 solution) such that the respective concentrations in the reactor were brought to the level shown in figure 9 at time zero. It is clear from this figure that nitrogen and phosphorus were picked up simultaneously from the medium and their initially high rates of extraction were very similar. After a period of 9 hours, the relative rate of nitrogen uptake by *Chlorella* increased.

At 9 hours, the 500 mg/L algae had already accumulated 63 mg/L PO_4 which represents more than 12% PO_4 by weight of the cells! It was difficult to believe that this amount could be the normal metabolic capacity of *Chlorella* and therefore, these results were questioned and reserved for further clarification. However, this phenomenon could not be taken lightly since the percentage of phosphorus picked up by the cells during the experiment illustrated on figure 10 was again very similar. For the time being, the P_s/P_c ratios noted on figures 9 and 10 may be ignored. The significance of this ratio and the characteristic phosphorus uptakes associated with it are discussed in Chapter IX of this thesis along with the above noted phenomenon.

One very important feature of figure 10 is the evidence that after the culture had absorbed ammonia and phosphate simultaneously, in a similar fashion to the one illustrated in figure 9, no additional phosphorus was taken up by the algae during its active growth phase. At the same time however, nitrogen was being picked up from the medium in quantities

directly proportional to the growth of the algae.

Since no net amount of phosphorus from the medium was utilized by algae during growth (figure 10), the internal storage of phosphorus previously accumulated by the cells must be transferable to their progeny. This observation would help explain why after a spring overturn in an eutrophic lake, the algal blooms continue for months but no major phosphorus concentrations are found in their growth medium.

The phosphorus that comes in the epilimnion with the hypolimnetic waters during the period of lake mixing could be stored by the phytoplankton that already exists in low numbers in the epilimnion or grows at a fast rate during this period of nutrient abundance. Since the stored phosphorus could be passed from cell to cell and also could be liberated from the dying cells, one could see a self-perpetuation of algal blooms for quite some time without observing too much soluble phosphorus in the lake waters. A comparison between the percentages of phosphorus in the algae of figures 8 and 10 reveals a difference of about 4 fold and that, by no means, could be considered as the maximum possible range!

These preliminary studies proved very useful and this investigator, after studying the material available in the literature, decided to undertake a systematic study of the various observations reported above and of several others. It should be apparent that some of the evidence, already presented, needed further verification through carefully controlled experiments and by following different approaches. Since the phosphorus deficient cells appeared to behave differently

from those grown in an excess of phosphorus, the next chapter will deal only with the latter while a report on the investigation of the former type of cells is deferred to Chapter IX of this manuscript.

CHAPTER VIII

ENVIRONMENTAL CONDITIONS AND P-UPTAKE BY ALGAE

Metabolism and thus, man's eating habits are influenced greatly by the environment in which he lives. Since the microbial world cannot influence the extreme fluctuations in heat, cold, humidity, solar radiation, food supply, and the many other factors inherent in the environment as man does, the microorganisms must be able to exercise an extraordinary amount of control on their internal metabolism if they are to survive. This investigator was interested in evaluating some of these characteristics in the photosynthetic microorganisms such as, the ability to extract phosphorus from the growth medium when exposed to various phosphate concentrations, light intensities, temperatures, rates of mixing, and diurnal effects.

Philosophically, it was predicted at the very outset that algae would store phosphates above and beyond their usual metabolic needs to protect themselves against a possible future shortage of this nutrient in their growth environment. In addition, it seemed logical to predict that there must be some minimum steady state phosphorus concentration required in the substrate for the maximum growth of algae under each set of environmental conditions and that below this concentration, the steady state growth rate of algae must decline. Furthermore, this CRITICAL CONCENTRATION OF PHOSPHORUS (CCP) was expected to vary with the environmental conditions for

growth.

The phenomenon of the LUXURY UPTAKE OF PHOSPHORUS (LUP) by algae, i.e. storage above and beyond their usual metabolic need, was encountered early in the preliminary studies. As discussed in the last chapter and illustrated in figures 7 and 8, the LUP did not affect the steady state growth rate of the algae. However, it was apparent that environmental factors would influence the magnitude of LUP.

A systematic study of the effect of light intensity, algal cell density, diurnal cycling, temperature, and of mixing on LUP and CCP is reported in this chapter. The turbidostat apparatus, the synthetic feed (unless otherwise reported) and environmental conditions as indicated on each figure, were employed during these studies.

It should be noted that in many respects, the light intensity and the algal cell density effects must be interrelated since decreasing algal cell density means increasing illumination available to each algal cell for photosynthesis even when the source of light is emitting a fixed number of foot candles. Similarly, the diurnal cycle represents an extreme case for the availability of energy (zero during darkness and the full intensity during the lighted period). Temperature and mixing both play their parts in the transport of nutrient elements from the bulk phase to the inner cell boundary and from this standpoint, should have some correlation. It might be assumed, that for the phosphate molecule and the algal cell to come close enough to each other for any reaction, the concentrations of both of them would be

important and therefore, should be correlatable. Many other interrelations of this sort could be found, if carefully studied.

However, the main objective of this study was not to correlate these numerous phenomena. Instead, the aim was to observe the culture response by varying one parameter at a time, while holding all other values constant; and thus, characterizing the cause-and-effect relationships concerning the 'controlled' variable. Even then, several significant interrelationships between the culture response to the different environmental parameters, should become apparent by comparing the summary graphs presented in this report. Such an interrelation between the light intensity and the algal cell density is illustrated below in detail.

The rate of growth of an algal mass culture in a natural environment depends on many physical, chemical, and physiological factors some of which may be easily controlled while the others may be essentially uncontrollable. In general, physical factors are the least controllable. Solar radiation, being the principal driving force for all photosynthetic reactions in the universe, is the most important physical factor and man's ability to control this parameter is minimal. Since temperature depends to a large extent on light intensity in nature, any variations in the luminous energy would also affect the growth of algae by affecting the temperature.

Due to these considerations, more emphasis on the elucidation of light intensity effects was desired in this study

than on the other factors. Although the light intensity, algal cell density, and the diurnal cycle effects are discussed below under separate headings for the sake of clarity of presentation, the reader should not forget that they all represent manifestation of luminous energy applied to the culture in one form or another. In this controlled algal culture study, temperature was not a function of light intensity. Therefore, this factor should be considered independent of the effects of light intensity. The effect of mixing on the growth and P-uptake behavior of algae has certain aspects in common with both the light intensity and the temperature effects. For this reason, a discussion of the mixing effects does not appear until the end of this chapter.

A. LIGHT INTENSITY EFFECTS

Photosynthesis has been defined to be a series of processes in which electromagnetic energy is converted to chemical free energy usable in biosynthesis (54). It should be apparent then, that the total number of 'effective' photons (quanta of electromagnetic radiation) available to the culture would determine, within limits, the photosynthetic activity as measured by the growth rates.

1. EFFECTIVE PHOTONS

The quality of light too is very important and was

fixed during these studies since only Gro-Lux bulbs were used. According to the manufacturer, the spectral energy distribution of these bulbs falls between the wavelengths of 400 and 700 millimicrons, with peaks at 450 and 660. This would approximately correspond to the light absorption characteristics of the major chlorophyll fractions.

The energy content of a photon of any wavelength of light is given by the relation:

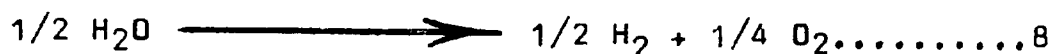
$$E = hc/\lambda \dots\dots\dots 6$$

where h is Plank's constant ($= 6.6 \times 10^{-27}$ erg-sec), c is the velocity of the electromagnetic radiation wave packet ($= 3 \times 10^{10}$ cm/sec, the velocity of light), and λ is the wavelength in centimeters. One may also easily express the energy content in electron-volts (54) as following:

$$E_{ev} = 12,350/\lambda \dots\dots\dots 7$$

where the wavelength λ is in angstrom units and the number 12,350 has resulted from proper substitutions in equation 6.

The systematics of photosynthesis has been discussed quite ably by Kamen (54) and accordingly, the primary reaction in photosynthesis is the photolysis of water. Energetically, the absorbed photon to be 'effective' in photosynthesis must supply sufficient energy, after transformation and conversion to electronic excitation energy, to equal at least 1.2 volts for driving the following reaction to the right:



Also, it may be noted from this equation that $4 \times 1.2 = 4.8$ volt-equivalents, or $4.8 \times 23,000 = 112$ K cal of free energy are needed per mole of O_2 produced or CO_2 reduced by

photosynthesis.

It should now become apparent what was meant above by 'effective' photons. The energy content of photons decreases as the wavelength of light increases. If one substitutes a value of $6900 \text{ \AA}$ for the wavelength in Equation 7 (the major long wavelength absorption band for green algae used in this study), the energy content for the absorbed photon is 1.79 electron-volts. This energy is more than adequate for supplying the driving force for the reaction of Equation 8 and therefore, is 'effective' in photosynthesis. It would appear from Equation 7 that the photons of light up to a level of 1029 millimicron wavelength could be 'effective'. However, since some energy is lost in transformation and conversion to excitation energy, the infrared wavelengths (above 700 millimicron) are not considered effective in green plant photosynthesis.

Photons of lower wavelength than approximately 470 millimicron would certainly contain more than an 'effective' amount of energy but there are no known pigments in photosynthetic organisms that can absorb these quanta. Chlorophyll b and the carotenoids (mainly β -carotene) of multicellular green plants and of green algae are known to have their major absorption bands around 480 millimicrons. Chlorophyll c of the diatoms and of the flagellates, maximally absorb the 470 millimicron wavelength light (40). Hence, the light of much lower wavelengths than about 450 millimicron should also not be considered effective in photosynthesis.

Clearly then, only the light of wavelengths between 450

and 700 millimicron is critical in algal photosynthesis. The foot candle meter used during these studies actually measured for the most part the visible light intensity (from 420 to 710 millimicron wavelength) and was therefore, appropriate.

2. MEASUREMENTS

The cell density of the culture and other parameters were held constant during these experiments and the light intensity at the front face of the culture chamber was varied from 400 to 2250 foot candles (1 Lux = 0.0929 F.C.). Simultaneous light measurements were made at front and back face of the reactor, except when no light could be detected during the 400 foot candle run, at the back face of the culture tank. In this way, the actual number of foot candles or the proportion of incident light absorbed by a given algal density during any experiment could be determined with ease. These types of measurements proved very useful, as discussed below.

3. EXPERIMENTAL RESULTS

The results obtained during four different experiments, particularly designed to evaluate the effect of light intensity on the critical concentration of phosphorus (CCP) required for the maximum growth of the uni-algal-Scenedesmus culture, are illustrated in figures 11,12,13,14, and 15. Average values of the significant environmental factors, measured routinely during these runs, are noted on the figures as are

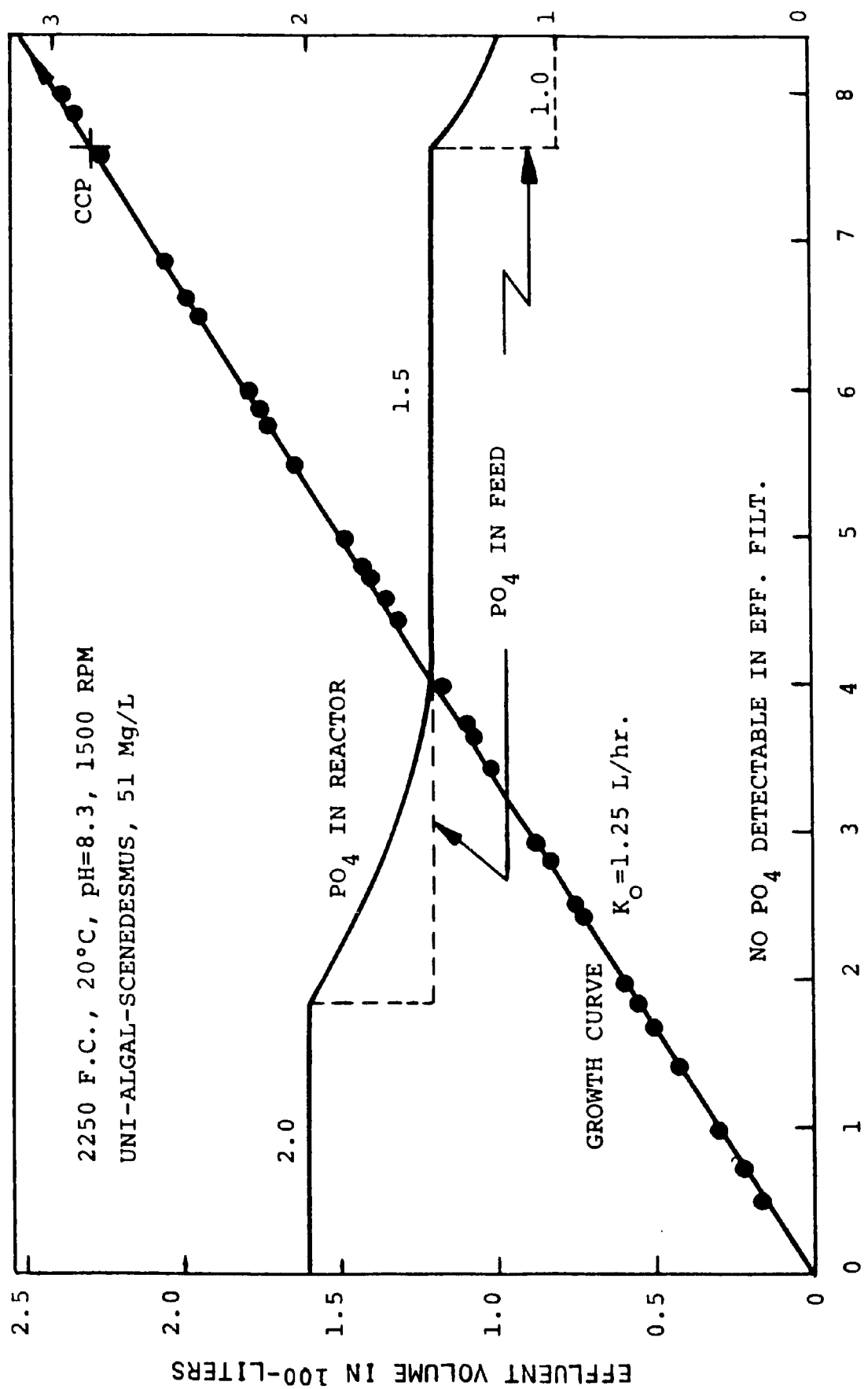


Figure 11. Effect of Stepwise Reduction of PO₄ on the Growth Rate of Green Algae at 2250 F.C.

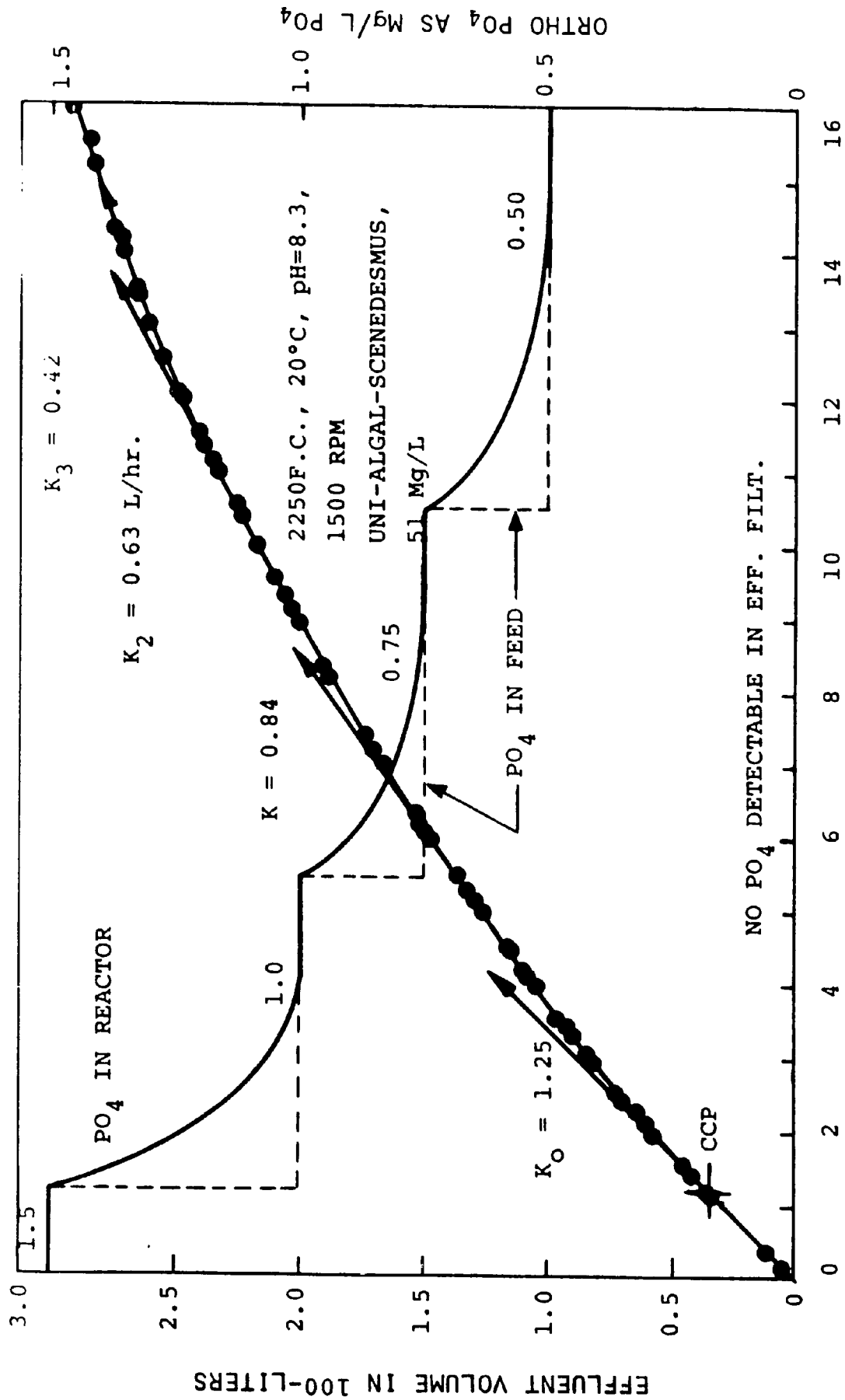


Figure 12. Effect of Stepwise Reduction of PO₄ on the Growth Rate of Green Algae at 2250 F.C.

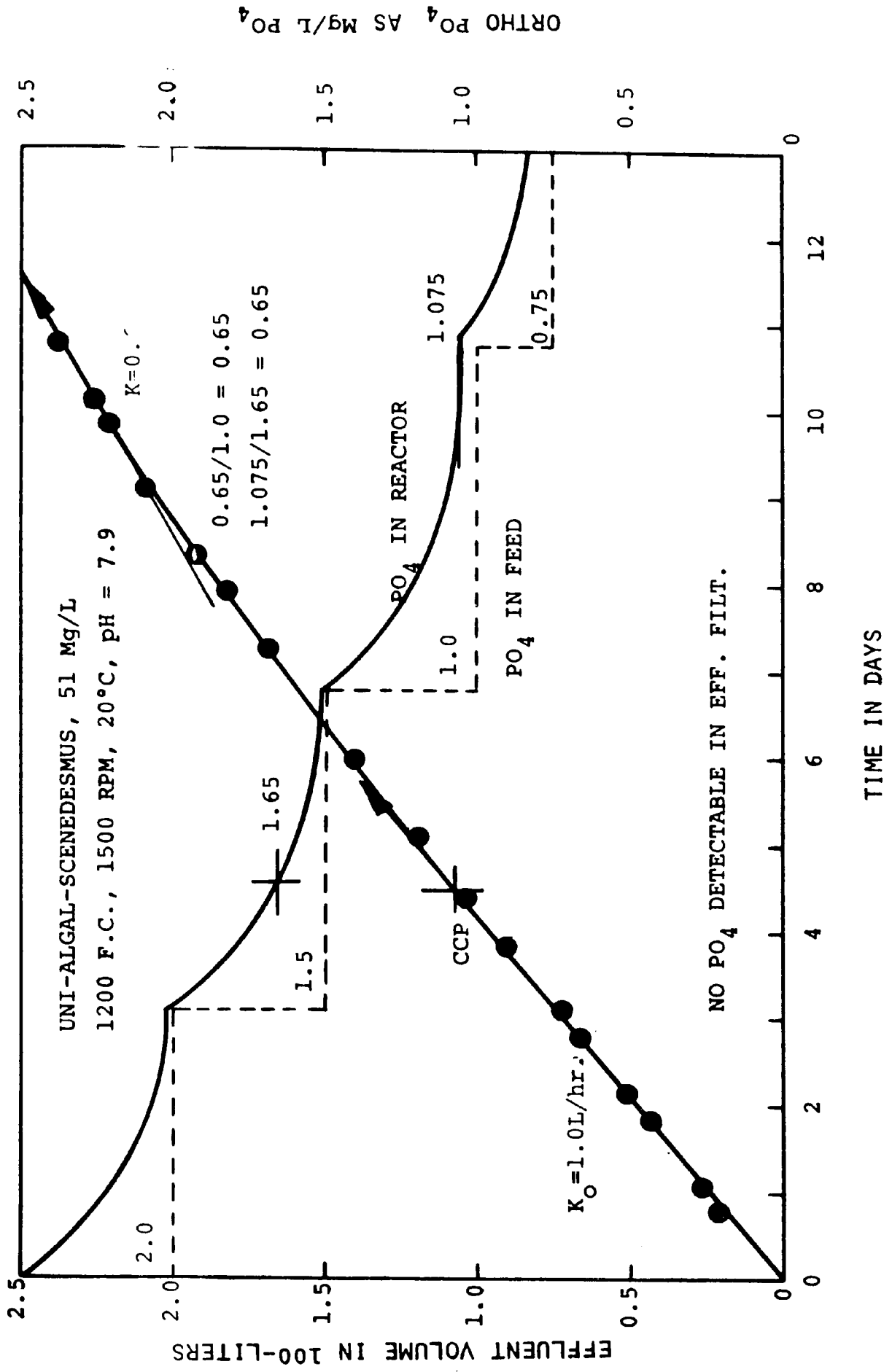


Figure 13. Effect of Stepwise Reduction of PO₄ in Feed on the Growth Rate of Green Algae at 1200 F.C.

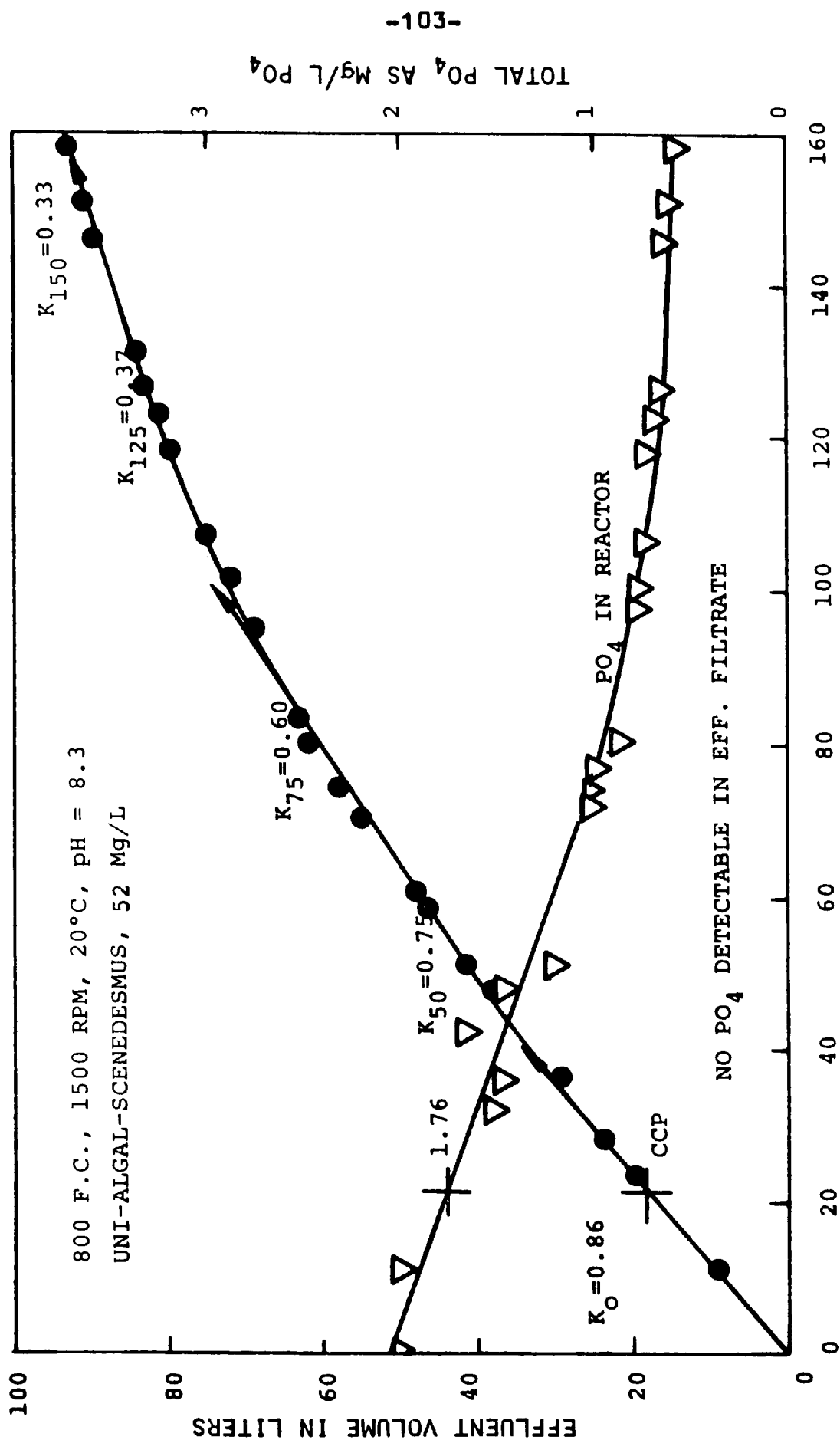


Figure 14. Effect of a Continuous Decrease in PO₄ on the Growth Rate of Green Algae at 800 F.C.

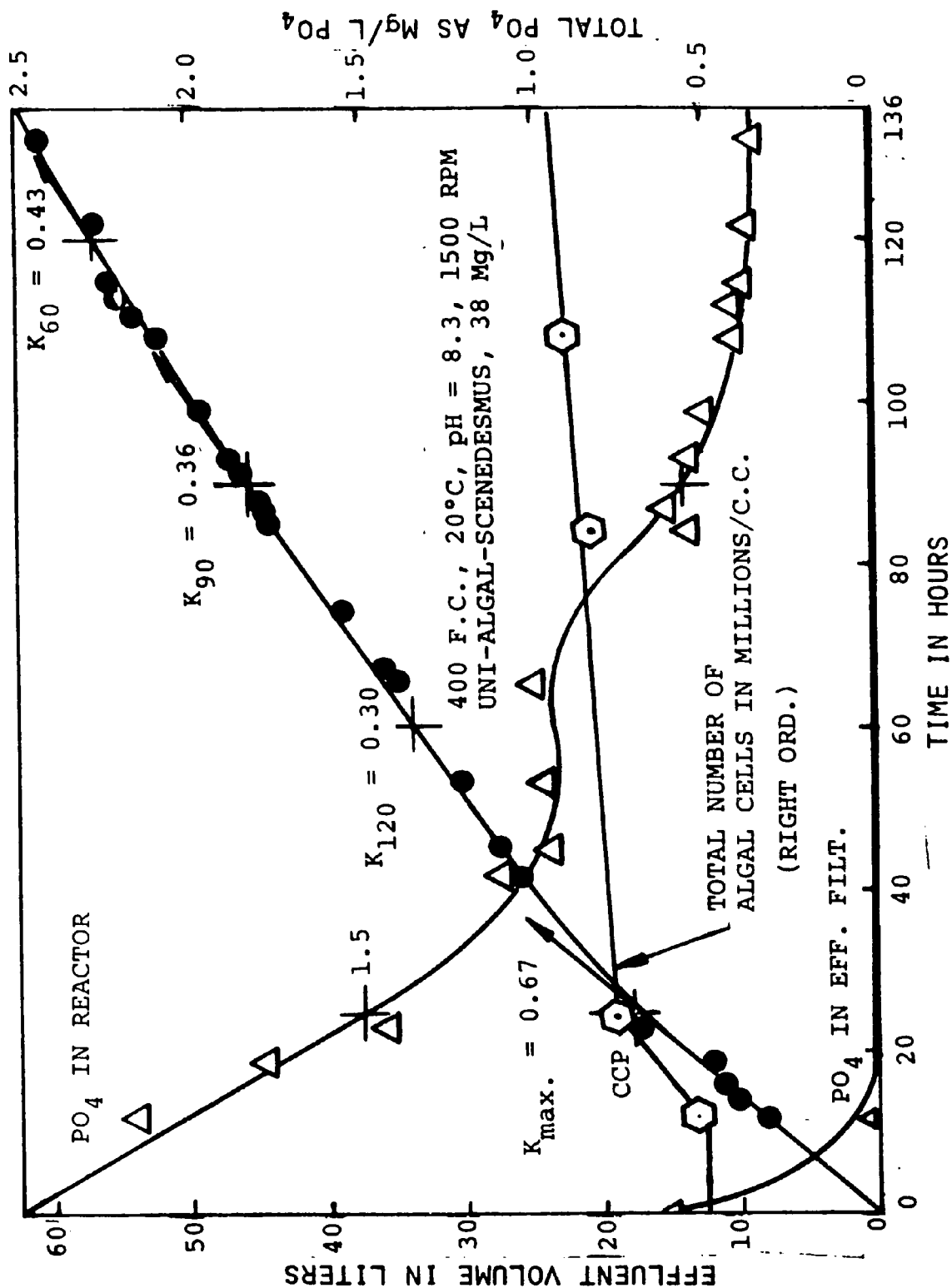


Figure 15. Effect of a Continuous Decrease in PO_4 on the Growth Rate and Cell Number of Algae at 400 F.C.

the various concentrations of phosphorus. Deviations from the mean values reported were small as evidenced by the consistency of the data.

The first experiment, reported in figures 11 and 12, was continued for more than two weeks. During this prolonged test and the one reported in figure 13, the method of phosphorus supply consisted of adding a known quantity to the synthetic feed as shown by the dotted lines. The actual concentration of phosphate in the reactor is indicated by the continuous curve which takes into account the hydraulic washout phenomenon caused by a steady overflow of the constant density culture due to the algal growth. Obviously, the latter curve represents the concentration of phosphate in the culture chamber that was actually available to algae for growth and not the concentration added in the feed.

Since the reactor was a completely mixed system, and the temperature and turbidostat conditions were favorable for nutrient absorption, the algal cells quickly attained equilibrium with the phosphate supply available in the culture media. Hence, there was no lag in the algal growth response (as measured by growth rate in liters per hour to the concentration of PO_4 in reactor, once the culture had been adapted to the experimental conditions and the steady state situation had been established.

This observation was used during the experiments reported in figures 14 and 15 and the mode of phosphorus supply was changed. After the culture had grown under an excess of phosphate and under the experimental conditions for about 2 days,

substrate containing no phosphate was fed and the concentration of phosphate in the reactor continuously decreased as shown in these figures. It should be noted that this method of phosphorus supply to the culture resulted in a considerable saving in the experimental time without sacrificing the reliability or accuracy of the results.

i. Cell Number Increase in Low Light

The effect of low light intensity on the relative number of algal cells in the turbidostat culture is shown in figure 15. Since dry weight density and per cent transmittance of the culture were held constant, the steady increase in cell numbers must mean a steady decrease in organism size, and thus result in holding the culture mass constant. Microscopic observations on the size of the cells confirmed this reasoning to be true.

ii. Discussion of CCP and Growth Rate Results

Summary graphs of the effect of light intensity on the maximum steady state growth rate and on CCP are shown in figure 16. Other than normalizing the maximum steady state growth rate and the CCP values where needed (using a technique described in the next section), the appropriate data points of this figure are taken directly from figures 11 to 15. Three regions of light intensity effects on the growth rate of *Scenedesmus* are indicated with proper notations on this figure. The shape of this growth rate curve is very similar to the ones

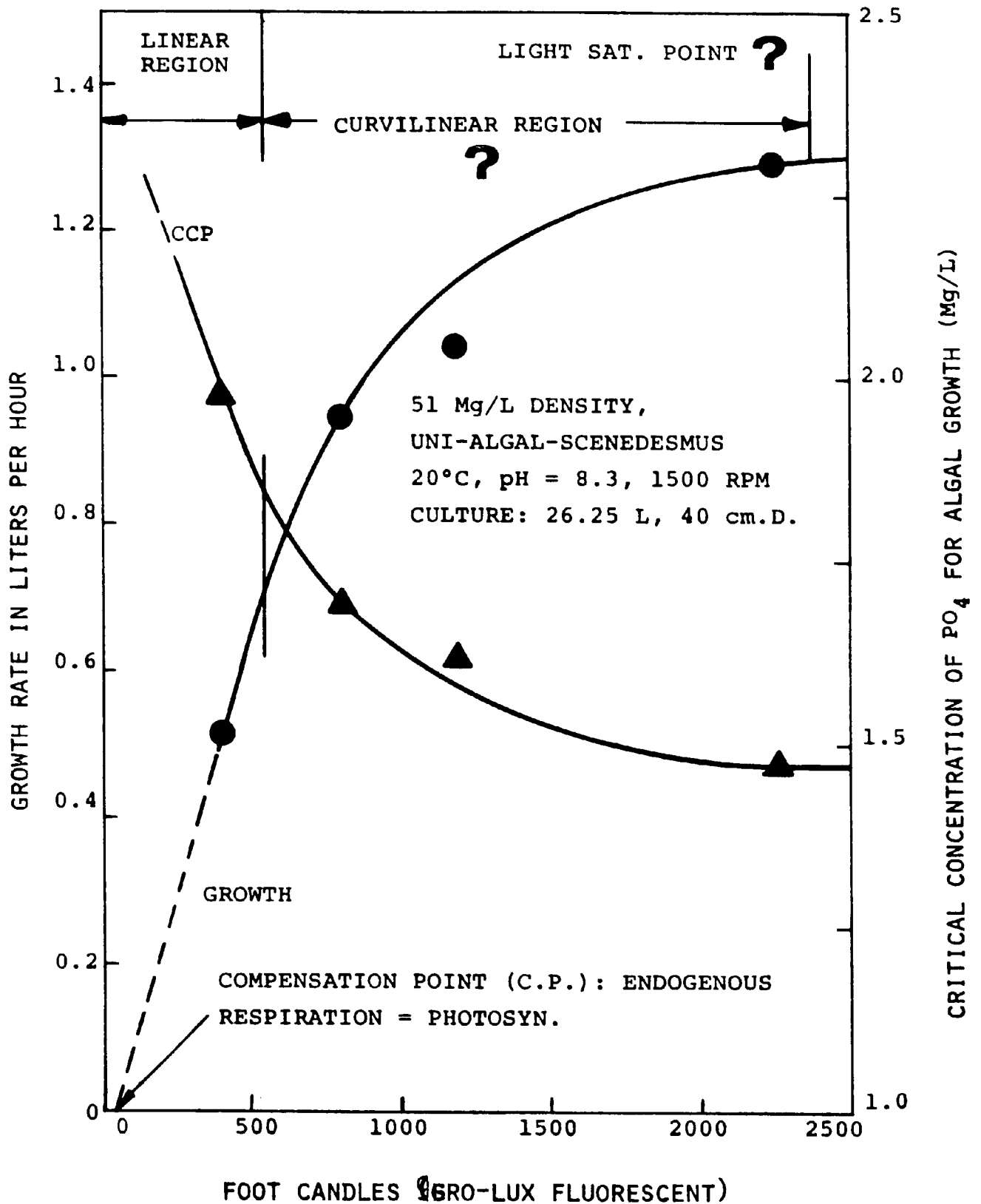


Figure 16. Growth Rate of and CCP for Green Algae as Functions of Light Intensity Measured at the Face of Culture Tank

reported by Fogg (8), Myers (119), Luebbers and Parikh (120), and by Witt and Borchardt (105).

Theoretically, the lower values on the growth rate curve must extrapolate to some finite value and not to zero light intensity since some light is required for the cellular 'over-head' without any increase (119). This light intensity is called the compensation point (C.P.) in figure 16. The compensation intensity has been observed experimentally to be about 20 foot candles for a mixed algal culture from a waste stabilization lagoon using the Warburg respirometer and the criterion of no net oxygen production (120). According to Oswald and Gotaas (121), a mixed culture of *Scenedesmus* and *Chlorella* showed no net growth below 30 foot candles. Since the precise lighting conditions and the quality of light that each alga is exposed to, would determine the value of this light intensity as well as of the saturation point, the great difficulty in comparing determinations of this sort made by different investigators or by the same investigator using different apparatus, should be obvious.

In mass culturing of algae, the 'classical' shape of the algal growth curve as shown in figure 16 was found misleading by this author. That is why the curvilinear region and the saturation point of this curve are questioned. This type of growth curve has some meaning only when each cell receives well defined, equal and continuous radiation from all directions. In a mass culture, on the other hand, unilateral and intermittent (due to high cell densities) are the rules rather than exceptions! Hence, there is every reason to

doubt the validity of the characteristics apparent from such a curve.

As shown in figures 11 to 15, the steady state growth rate of algae finally begins to decline, below a certain concentration of PO_4 in the reactor, when the cells are exposed to lower and lower levels of phosphate. This concentration has been designated since as the 'Critical Concentration of Phosphate (CCP)'. The CCP is indicated on these figures as the point where the growth rate of algae begins to decline from the maximum steady state growth rate. This point was in response to the concentrations of PO_4 at that particular time noted on the ' PO_4 in reactor' curves.

The effect of light intensity on CCP has been summarized in figures 16 and 19. As discussed on the preceding page, the light intensity definitely affects the value of CCP. The higher the available light intensity, the lower the concentration of PO_4 that is needed to support the maximum steady state growth rate of algae.

Below the critical level of phosphate, a steady change in the color of the algae, from brilliant green to yellowish green to yellow, was noted. As reported earlier, the algal density determinations and the operation of the turbidostat became less reliable at the advanced stages of phosphate starvation of the culture. The growth rates also declined to very low values and the experiments were terminated at times shown in the figures 12 to 15. After each experiment was finished, the algae was supplied adequate quantities of phosphates to regain their brilliant green color. The physiological

state of the algal cells and their nutritional behavior was brought to normal, and steady state conditions were restored before beginning the next experiment.

Concentrations of phosphorus lower than the CCP yielded proportionately lower growth rates but concentrations higher than the CCP (the 'luxury uptake' region) did not affect the specific steady state growth rate. In other words, if the phosphorus concentration in the reactor was half the value of CCP, the growth rate was also half the maximum steady state growth rate of the algae; but the growth rate was constant and independent of the phosphate concentration above the critical level. This is illustrated very well in figures 11 to 15.

4. LIGHT ABSORPTION CHARACTERISTICS OF ALGAE

Oswald and Gotaas (121) developed their general equation for the design of waste stabilization ponds based on the assumption that all algal suspensions absorb light in agreement with the Beer-Lambert Law. It is obvious from figure 17, prepared from the experimental observations made throughout this study, that this law does not generally hold. If this relation were generally applicable, the plot of the light absorption factor (A) would be a horizontal straight line (constant) and definitely not a parabolic type of smooth curve!

This figure would also explain why MacKenthum and McNabb (122) could not plot their light penetration data, obtained from several stabilization ponds in Wisconsin when there was

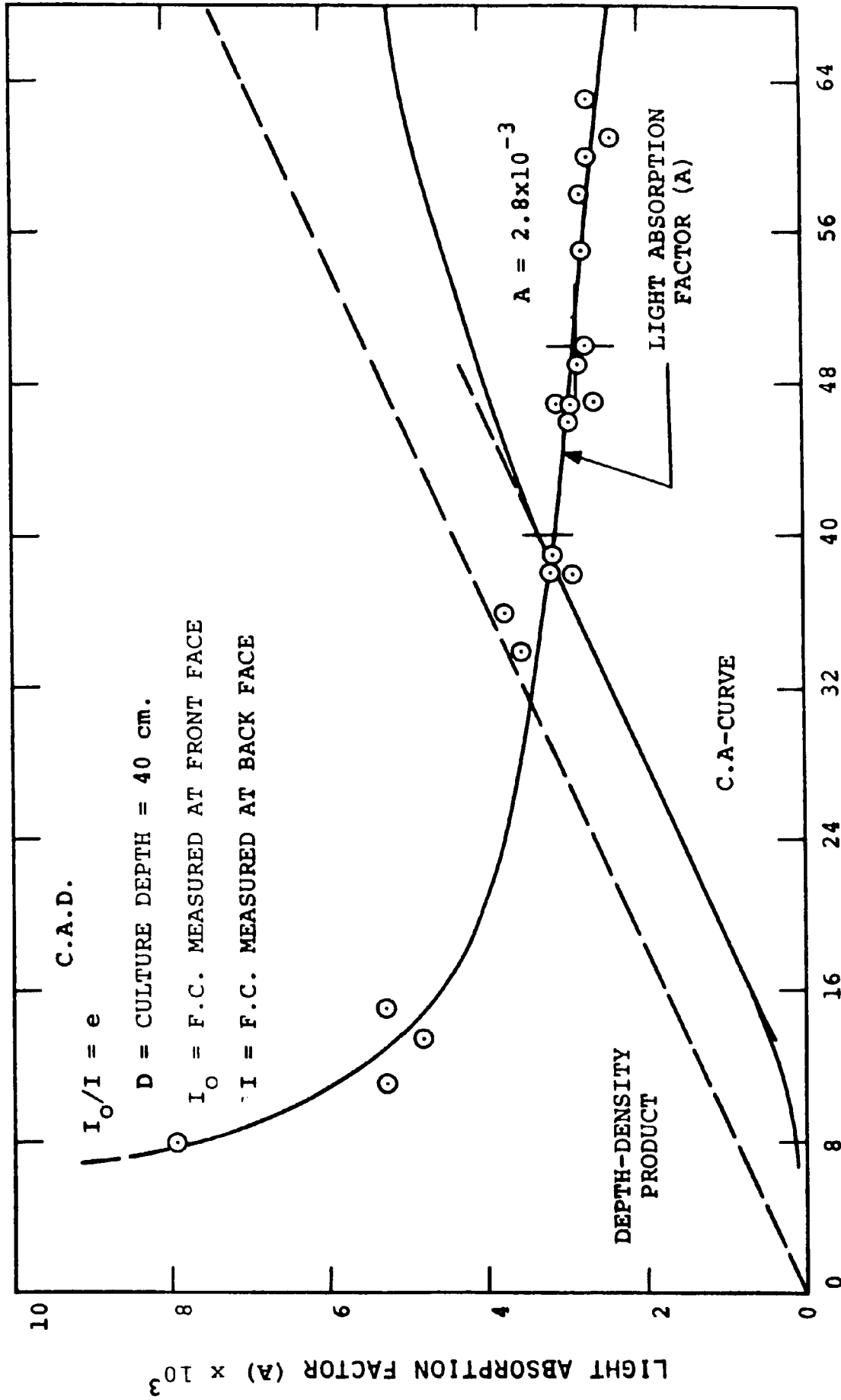


Figure 17. Light Absorption Factor as a Function of the Algal Cell Density

a vertical stratification of phytoplankton, in the manner illustrated in figure 18. Also, it should be realized that the Beer-Lambert equation was not formulated for heterogeneous suspensions (such as is the case here) nor has it been implied that this relationship would be applicable no matter what fraction of the incident light is being absorbed by a given depth of a true solution!

However, in this case it would appear that an exponential relation resembling the Beer-Lambert Law, but quite different in its implications, can be highly useful as described below. In this, as yet empirical expression, the light absorption factor 'A' is a system specific variable that must be determined experimentally and therefore, should not be confused with the light extinction coefficient of the Beer-Lambert expression which is a constant for any given solute. Similarly, the concentration term 'C' of this formula, is dependent not only on the physical and chemical environment surrounding the particular cells but also on their physiology and nutritional behaviors. This being the case, the factor is quite different from the one used in the Beer-Lambert Law. These differences are quite subtle and under no circumstances, can the two formulas be considered identical in their use, implication or interpretation.

5. A NEW METHOD FOR EXPRESSING LIGHT INTENSITY

As illustrated in figures 17 and 18, the empirical formula ($I_0/I = e^{CAD}$) was used to determine graphically

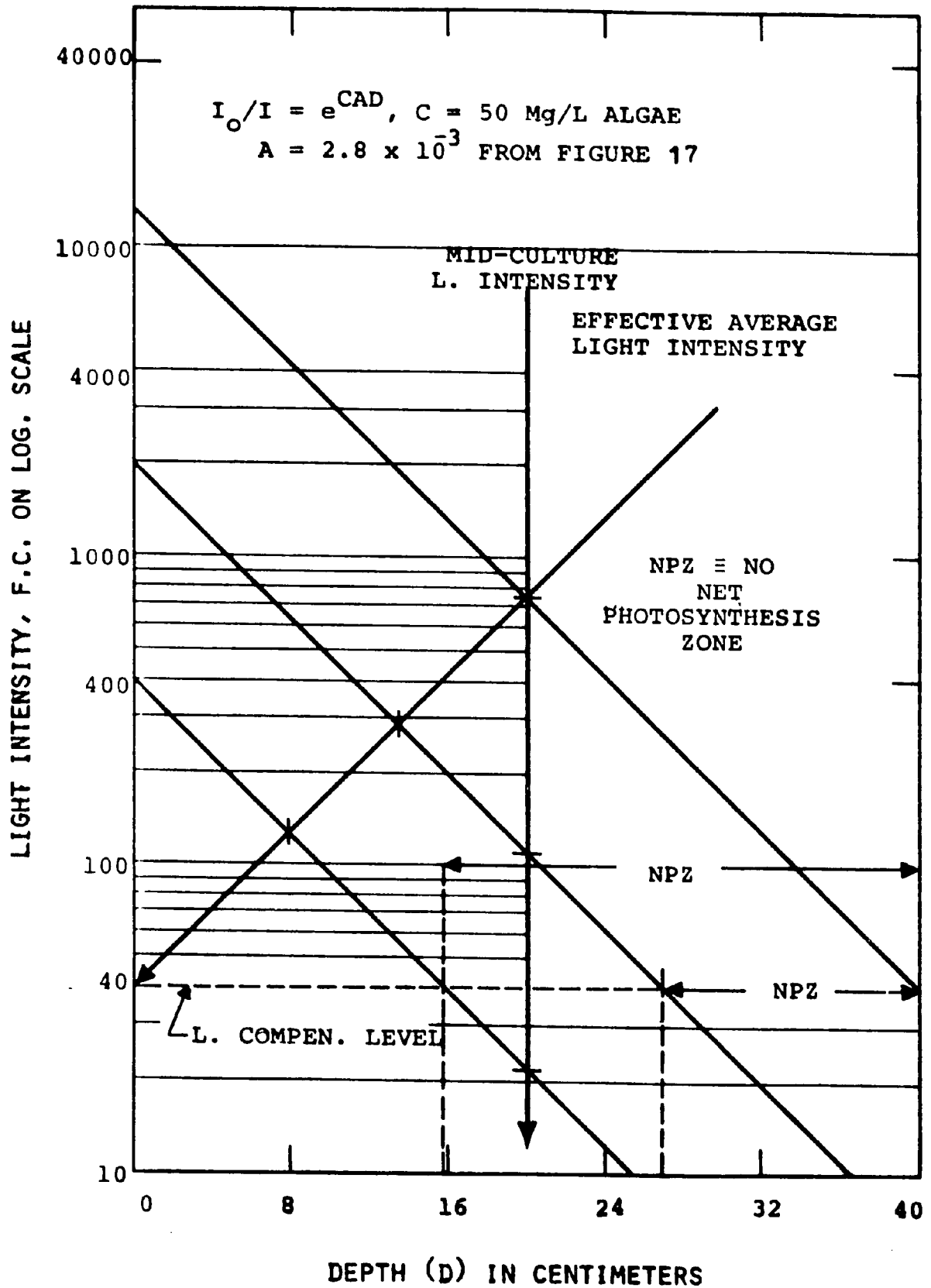


Figure 18. Determination of Average Light Intensity Effective in Net Photosynthesis

the logarithmic average light intensity effective in producing net growth, called 'effective average light intensity' in figure 18. Although the initial reason for this determination was to find answers for the apparent anomalies discussed above concerning the growth curve in figure 16, another significant advantage of this approach was discovered later. This factor is considered below in some detail.

The construction of figure 18 is almost self-explanatory and quite simple. Once a dry weight, algal cell density (C) value is selected (to correspond to the one for figure 16 in this case), the corresponding value of the light absorption factor (A) can be found from figure 17. Then, the only variable to be calculated from the formula is I for a given depth and I_0 . Graphically, it is simpler yet! The process is as follows: Calculate only one value for I for a given set of I_0 , C , A , and D values. Draw a straight line through I_0 and I . The slope of this line on the semi-log paper is now fixed. Within limits of the application of this relation, any number of other lines can be drawn parallel to this one, generating as many values for I at any D as needed corresponding to each value of I_0 , as long as C and therefore, A are held constant. During this study, the factor A , at a given cell density, was not found to vary with I_0 from 800 to 2250 foot candles but this independence may not necessarily hold at higher or lower values of I_0 .

Ignoring the fact that some light energy is required to satisfy the basal metabolism, the logarithmic average light intensity provided for algal growth in the reactor would be

given by the 'Mid-Culture Intensity' line in figure 18. Conceptually however, a certain portion of the culture, receiving less light than the compensation level, can be visualized as a respiratory debt from the standpoint of net growth, which must be compensated by an equivalent amount of photosynthesis in the remainder of the culture. It is almost impossible to define this share of the total precisely, but some 'reasonable' estimates can be made.

When graphically determining the 'effective average' light intensity in figure 18, the net photosynthetic zone was taken to extend up to the depth where the light intensity line crossed the light compensation level, estimated to be 40 foot candles. The rest of the reactor depth, receiving lower light intensities than the compensation level, constituted a zone of no net photosynthesis. The value of light intensity at the middle of the net photosynthetic zone, determined in this manner, was called 'effective average' light intensity.

It would appear from figure 18 that no less than 13,500 foot candles, at the face of the reactor, are needed to make the entire 40 centimeter depth a net photosynthetic zone. The 'mid-culture' and the 'effective average' light intensity values are identical in this case. As can be seen from their vertical and angular paths, indicated by arrows in figure 18, these two values will not be equal in any other case than this for the system used.

When the light intensity at the face of the culture is lower than 13,500 foot candles (as was the case throughout

this study), the value of the mid-culture light intensity is also lower than the one for effective average light intensity. The zone of 'no net photosynthesis' and the difference in values of these two light intensity parameters increase as the light intensity at the face of the culture tank decreases below the 13,500 foot candle mark.

6. SIGNIFICANCE OF THE EFFECTIVE AVERAGE LIGHT INTENSITY METHOD

Among other significances of expressing the light energy, provided to a mass algal culture, as 'effective average' light intensity, one is illustrated in figure 19. All the data points plotted here are the ones reported earlier in figure 16. The illusion of light saturation of the culture and the three distinct regions of the growth rate curve, apparent in figure 16, are no longer there in figure 19! Although more extensive and rigorous data would be required than presented in these figures for a precise quantification of the implicated relations, the value of this approach should be obvious.

Within limits of experimental errors, the growth rate of algae was directly proportional to the effective average light intensity and each increment of 10 foot candles produced an additional 0.056 L/hr. growth rate under the experimental conditions noted on figure 19. The fact that an increment of light produced additional algal growth will be clarified further in the next section. An average effect of light intensity on the critical concentration of phosphorus was noted and the

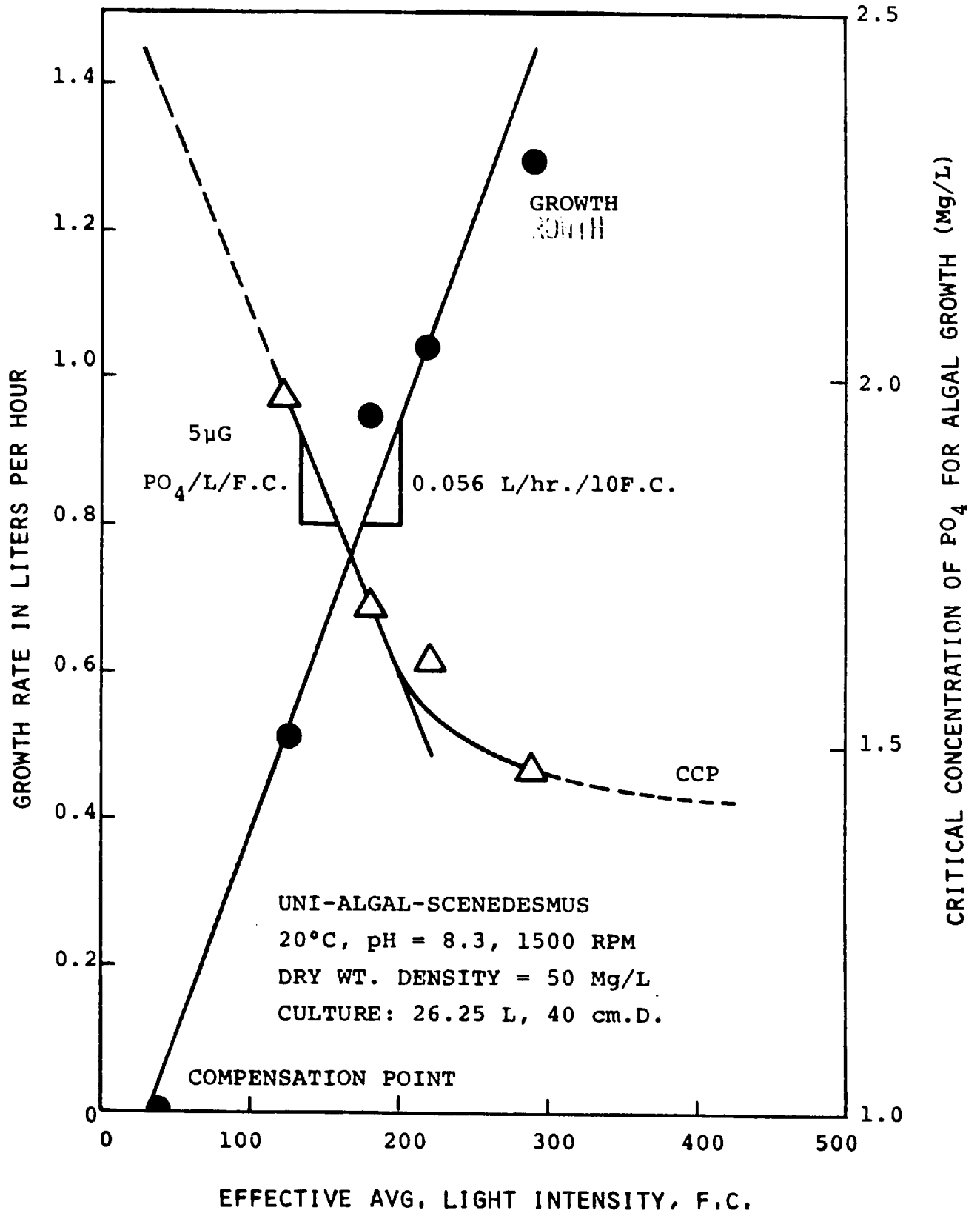


Figure 19. Growth Rate of and CCP for Green Algae as Functions of Average Light Intensity Effective in Net Photosynthesis.

indirect proportionality appeared to break from that of a straight line to the one of a curve at about 200 foot candles on figure 19. It would appear from the slope of this straight line that each decrease of 10 foot candles demanded an additional 0.05 milligram phosphate per liter of the culture for supporting the maximum steady state growth rate of algae under the systemic parameters used.

7. FURTHER EVIDENCE FOR LUP

It should be noted also from figures 11 to 15 that no phosphate appeared in the effluent filtrate when concentrations above the critical level were fed. This would establish further the fact that the algal culture was capable of storage of phosphate above the critical level needed for maximum growth as reported in the last chapter.

8. AN ECOLOGICAL INTERPRETATION

The light intensity effects on the growth rate of algae and on the critical concentration of phosphate (required by the algae for maximum growth under the newly imposed environment) reported in this section are very interesting. Since 'CCP' goes up as light level goes down, a decrease in light intensity not only decreases the growth rate but also the efficiency of the algal cells for extraction of phosphate molecules from their environment.

When other factors are not limiting, the higher the luminous energy available to the cells for growth, the lower

the phosphorus concentration that is required in the ecosystem for supporting maximum growth under these higher growth rate conditions! During the season of algal blooming in a lake, this phenomenon becomes rather unfortunate since this period is also the time of highest light intensity in addition to the presence of other optimal environmental parameters enhancing growth.

8. CELL DENSITY EFFECTS

As pointed out earlier, when other environmental parameters, including the intensity of light, are held constant, a decrease in algal cell density means an increase in the effective average light available to the culture. Similarly, an increase in the cell density would mean a decrease in the available light. This phenomenon is illustrated in figure 20. Taking the line having resulted from $C = 50$ mg/l algae and $A = 2.8 \times 10^{-3}$ (from figure 17) as a reference, it is clear that the effective average light intensity (EALI) for a given value of I_0 (2000 foot candles in this case), increases as the C decreases and vice-a-versa. Similarly, the depth of the zone of no net photosynthesis (NPZ) is directly proportional to the algal cell density as illustrated for $C=68, 50$, and 40 in figure 20.

Furthermore, each increment of EALI produces a proportionate effect on the growth rate of algae. This excellent

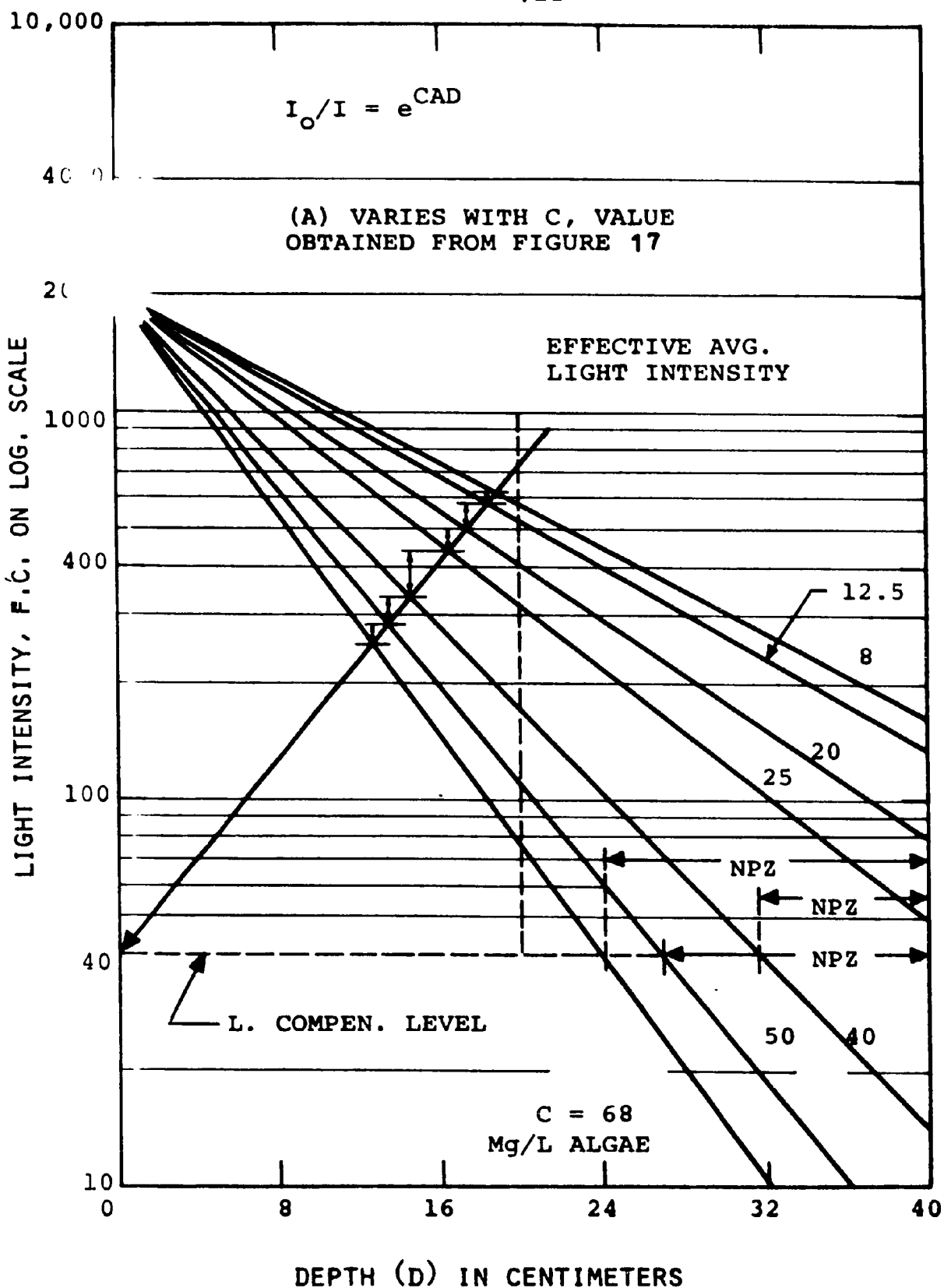


Figure 20. Algal Cell Density Influence on the Effective Average Light Intensity

correlation turns out to be another great advantage of using the effective average light intensity method and will be considered below in adequate detail.

A decrease of cell density in the reactor could possibly influence the CCP value since the collision frequency of the algal cell and the phosphate molecule would be affected. Also, the cell density of the culture which is called algal 'bloom' in a lake generally ranges from less than 5 mg/l to 20 mg/l dry weight of algae in the most active photosynthetic part of the epilimnion. While much denser cultures than 50 mg/l algae may be needed for the nutrient extraction process, where the object is to remove a given quantity of the nutrient from the wastewater most economically, much diluter cultures must be studied to understand the proper nutrient-eutrophication relationships.

1. RESULTS AND DISCUSSION

The results obtained from an experiment in which the constant algal density was lowered to 15 mg/l are presented in figure 21. After the first 33 hours, no phosphate containing substrate was fed to the culture (since the tap water used in the preparation of the synthetic feed always contained some phosphorus, this terminology is relative in its meaning). This feeding schedule continuously depleted the concentration of phosphates in the reactor as shown in this figure. A break in the maximum steady state growth of 2.98 L/hr. occurred when the PO_4 concentration attained a value of 0.48 mg/l PO_4 .

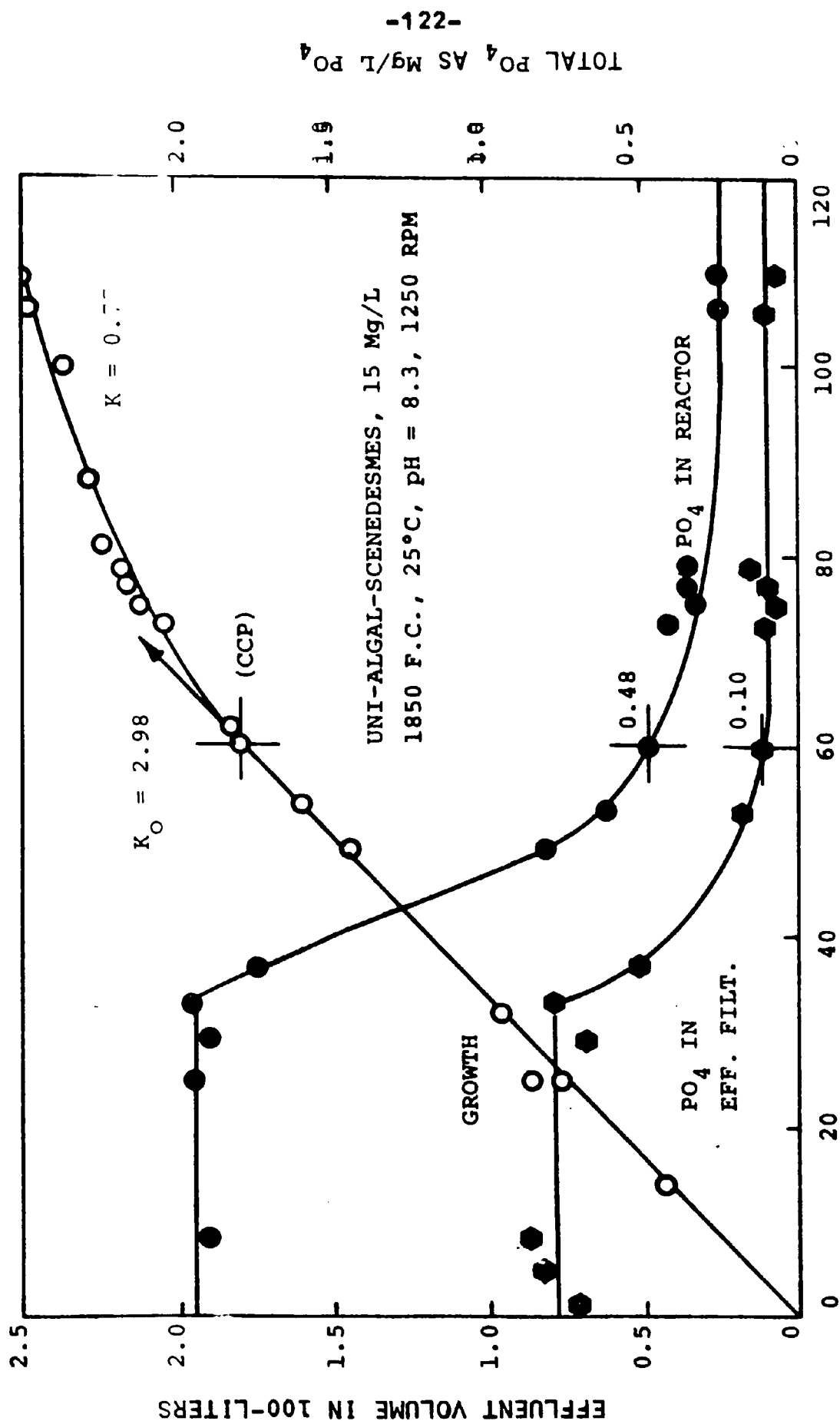


Figure 21. Evaluation of CCP for Algal Growth at Low Cell Density

Thereafter, declines in the growth rate and the PO_4 concentration followed their normal direct relationship .

It should be noted that in response to the constant concentration of PO_4 in the reactor after 96 hrs., the growth rate value of 0.77 L/hr was also stabilized. As will be clear from other data, the attainment of steady state conditions in the turbidostat is possible at any phosphate concentration no matter how low or high. Within reasonable limits of time, the algae will grow at a constant rate which may be much lower than the maximum possible steady state value, depending on the magnitude of the available phosphorus.

One significant difference between the results of this experiment and the results presented earlier is that at CCP, 0.10 mg/l PO_4 was left in the effluent filtrate in this case whereas none was left before. This phenomenon of PO_4 residual in the effluent filtrate at CCP was encountered in all the low cell density experiments and the exact amount of PO_4 in each case is noted on the ' PO_4 In Eff. Filt.,' curves. A discussion on the unavailability of this portion of PO_4 of the total is deferred to the end of this section.

i. Interference by Iron

When the cell density in the reactor was decreased to 10 mg/l algae, the normal color of the synthetic substrate, due to its constituent 5 mg/l Fe, started to interfere with the operation of the turbidostat. Since the algal density had been lowered 5 fold from its previous value of 50 mg/l, the

concentration of Fe in the feed could be reduced. The results of an experiment in which the concentration of Fe was varied are shown in Figure 22.

When 0.5 mg/l of Fe was fed to the 10 mg/l algae culture, signs of chlorosis appeared and the growth of the algae was retarded as shown in figure 22. As soon as the concentration of iron was increased to a 0.75 mg/l Fe level, the growth rate of algae increased and the signs of chlorosis disappeared in a few hours. Further increase in the iron concentration did not improve the growth rate of algae and therefore, 1.0 mg/l Fe concentration was selected for the rest of the low-cell-density studies.

The concentrations of other constituents of the feed, also being in large excess, were lowered 5 fold too without observing any effect on the growth of algae. This was considered desirable from the standpoint of not disturbing the various chemical equilibria and thus, providing the microorganisms their nutrients in about the same ratios as they received when there were 5 times the concentration of nutrients.

ii. Phosphorus Storage and Leaching

A very significant feature of figure 22 is that of the shock loading of the culture with PO_4 at 77 hours. This did not affect the growth of the algae in any discernible manner. However, the cells loaded themselves with phosphate quickly so that they contained 1.05 mg/l PO_4 which represents

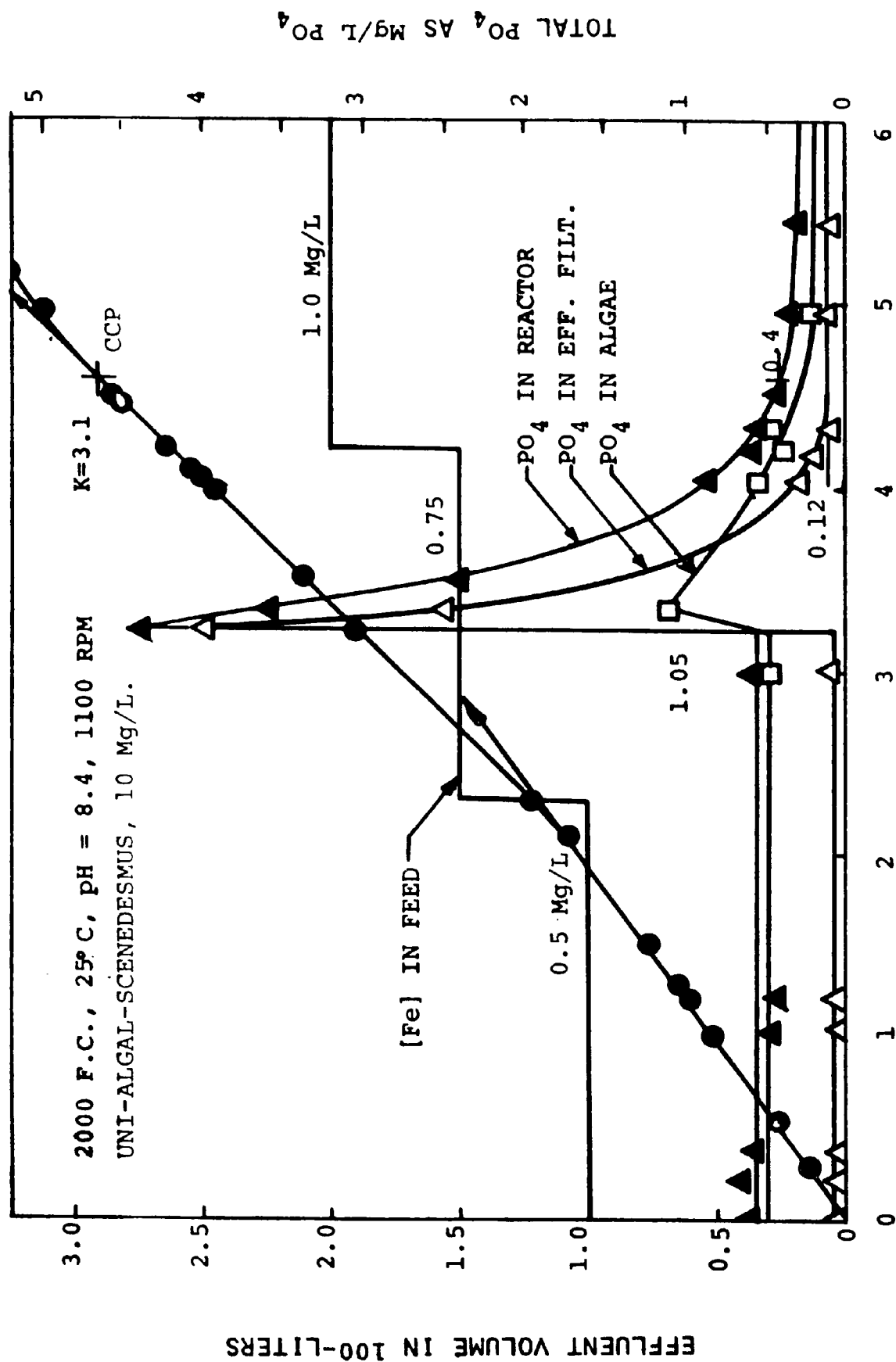


Figure 22. Evaluation of the Minimum Adequate Concentration of Iron and CCP for Algal Growth at Low Cell Density

10.5% by weight of the cells! This would constitute clear and additional evidence for the luxury uptake of phosphorus by algae as a normal process not induced by any changes in the growth environment other than supplying them the phosphate. It is not implied by this data, however, that the PO_4 taken so rapidly from the growth medium was incorporated by the cells as condensed phosphates, ATP, or inorganic - PO_4 .

There is evidence again in figure 23 for the storage of phosphorus by algae as being a normal characteristic. The culture was provided 2.5 mg/l PO_4 at time zero. The algae contained 0.95 mg/l (8.6% by weight) PO_4 after 9 hours at which time less than 0.4 mg/l total PO_4 was present in the growth media (outside the cells as ' PO_4 in Eff. Filt.'). If this phosphorus were stored entirely as inorganic - PO_4 by the cells, the concentration gradient would tend to force the cells to leach their phosphorus out into the media until there was no gradient. Under these circumstances, either the cell must expend energy and adjust its ionic balance in some other way to overcome the osmotic pressure that it must withstand, or convert the internal inorganic phosphorus to some other forms such as ATP, polyphosphates, phytin, guanidino phosphates, and phosphorylated sugars, etc. Probably the latter approach, being more 'expedient' and certain of the two, would be taken by the plankton.

It should be pointed out that the phosphorus rich algae does not later reject the quantity of phosphates that it has picked up once from the medium. In figures 22, 23, 25, 27, and 28, the ' PO_4 In Algae' curves are flatter than the ' PO_4 In

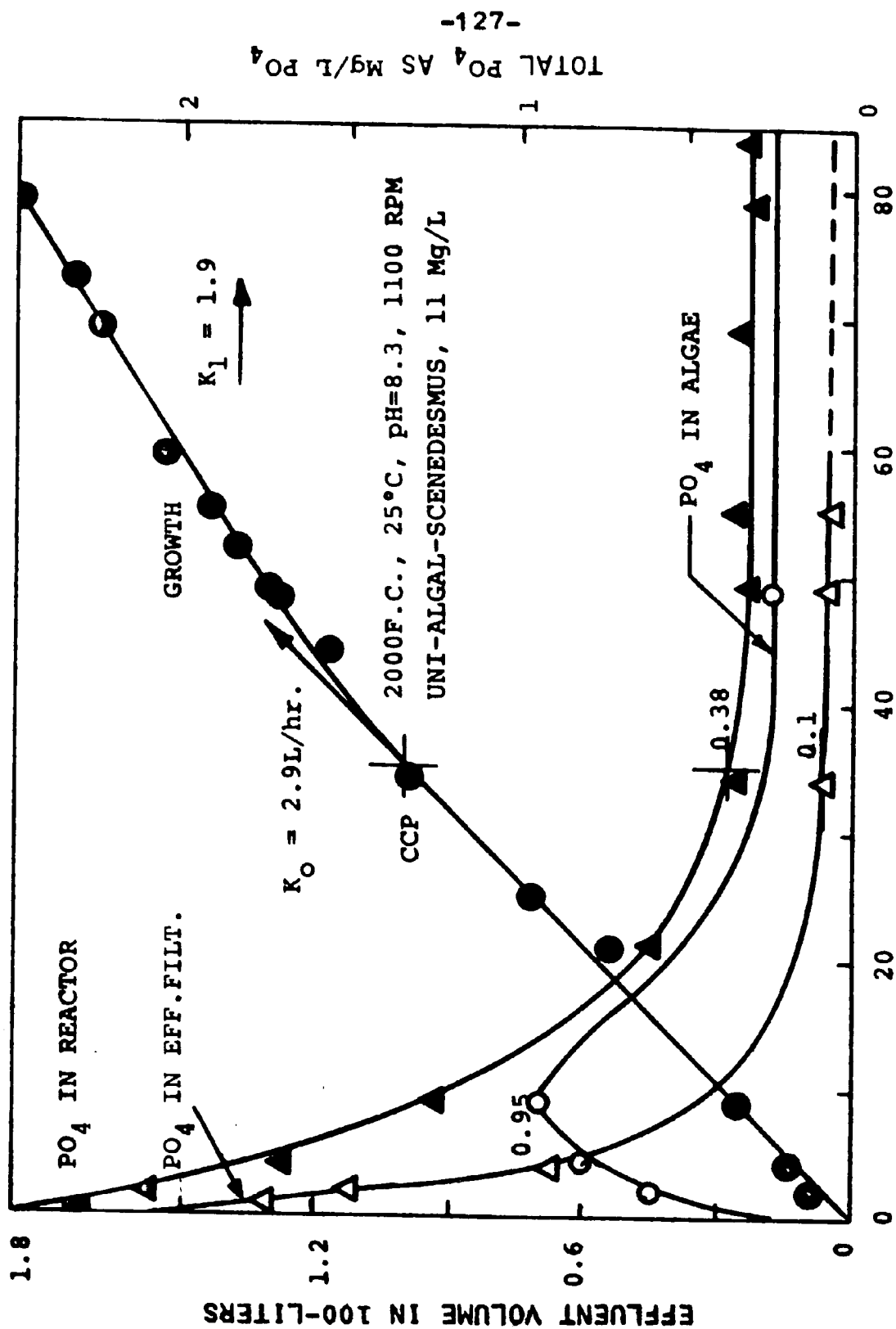


Figure 23. Determination of Luxury Uptake and CCP for Algal Growth at Low Cell Density

Eff. Filt.' curves which supports such a statement. If the algae were subject to such phosphorus desorption, after picking up the relatively large quantity, one would expect a break in the ' PO_4 In Eff. Filt.' curve (but not in the PO_4 in Reactor curve) at the point of phosphorus ejection by algae. The reason that PO_4 in the algae decreases with time is strictly due to the washout phenomenon. Phosphorus-rich cells leave the reactor as the new cells (growing in lesser and lesser phosphorus concentrations) predominate and therefore, lower the total phosphorus concentration present in the 'average' cells.

iii. Algal Growth in River Water

Since the growth characteristics and the P-uptake pattern of algae in the synthetic substrate may not necessarily be the same as in natural waters such as a river water which may contain additional growth factors, some experiments were conducted using the Huron River water collected in Ann Arbor (Island Park site) at the end of July, 1967. The river water was stored in a big storage tank in the laboratory for about a week in order to remove the major portion of its turbidity load. Then, the supernatant from this storage tank was pumped to the regular feed tank where all the nutrients (regularly added to the normal synthetic substrate), were added to it with the exception of phosphorus. Microscopic observations on this particular water revealed that it contained *Chlorella* cells as the predominant autotrophic biota, some

bacteria, and a few protozoa.

The results obtained during the first such experiment are shown in figure 24. After the switchover from the synthetic feed to the river water feed, and at the low phosphorus concentration prevailing in the reactor, the uni-algal-Scenedesmus culture was highly contaminated with bacteria, protozoa and the chlorella cells that were being fed to the culture in the river water. By the time this experiment ended, the biota had been completely converted from a uni-algal-Scenedesmus to a uni-algal-Chlorella type of culture.

In spite of the culture change and the bacterial contamination however, the maximum steady state growth rate value is exactly the same as observed in figure 22. This would appear to be substantial evidence for stating that this Chlorella culture grew at the same rate as the Scenedesmus culture which it replaced. Its phosphorus uptake behavior too seems very similar, if not identical with that of Scenedesmus. The rapid luxury uptake of phosphorus is apparent in figure 24. In figure 25, the CCP and the 'unavailable PO_4 ' values are very consistent with the corresponding values for Scenedesmus cultured in the synthetic substrate.

The experiment with the lowest algal density studied is reported in figure 25. The nutritional pattern of the algae in this case would be similar to that of an algal bloom in a lake disregarding the effects of some obvious environmental differences. Here again, it is apparent that certain portions of the total phosphate in the growth medium are inaccessible to algae when grown in dilute cultures. This same phenomenon

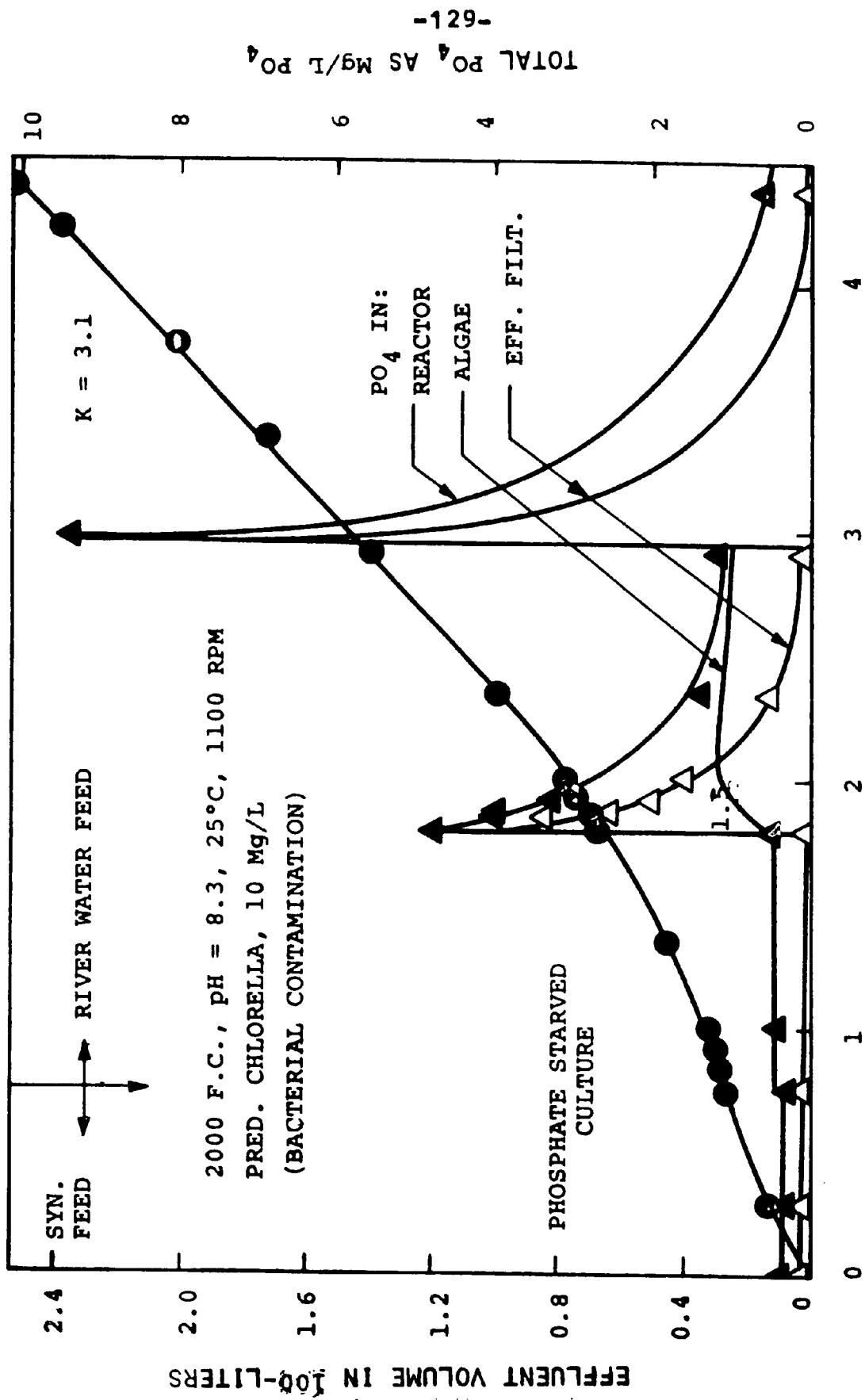


Figure 24. Growth and Phosphorus Uptake Characteristics of Green Algae in River Water Feed

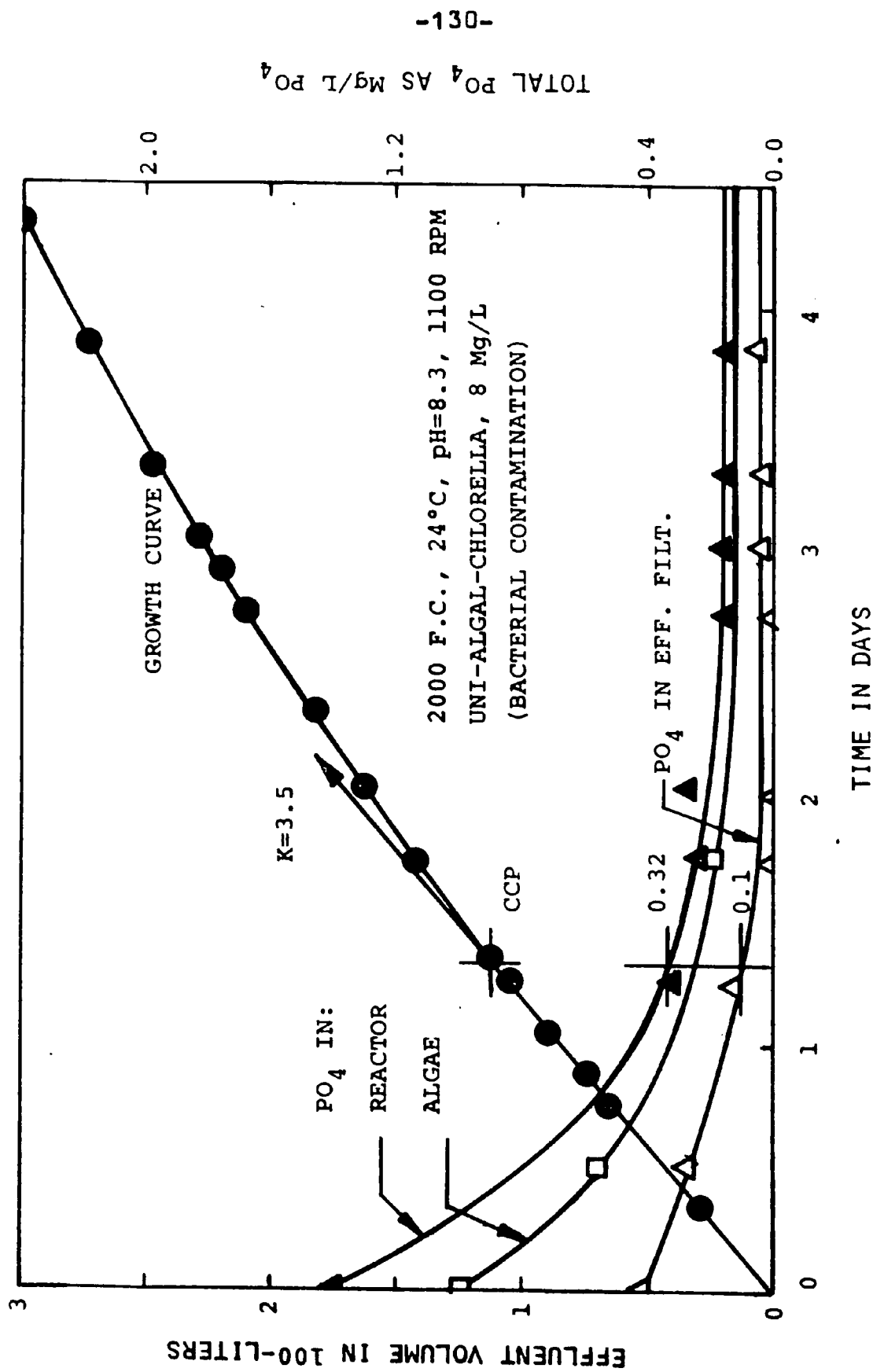


Figure 25. Evaluation of CCP and Growth Rate for Green Algae Grown in River Water Feed

is also frequently the case in a lake or river ecosystem. In figure 25, the PO_4 in effluent filtrate never dropped to zero even when the uni-algal-chlorella culture was starved with respect to phosphates for more than 3 days. In comparing the earlier figures with this one, it would appear that the Chlorella grown in the river water feed was more efficient (on a dry weight basis) in scavenging the phosphate molecules from its environment than the Scenedesmus grown in the synthetic substrate. It can be noted that the unavailable PO_4 values in figure 25 are lower than the corresponding values in figures 21, 22, 23, and 28. On a comparable weight basis there were many more Chlorella cells than Scenedesmus which could account for this discrepancy.

The maximum steady state growth rate value of figure 25 appears to be slightly higher than expected for a uni-algal-Scenedesmus culture. This could not be ascribed to Chlorella alone since their growth rate value in figure 24 is identical to that of Scenedesmus in figure 22. Either the experimental errors, particularly significant in low algal density experiments, were all additive in their effect or it may be that, there is a break in the growth rate pattern of the algae when grown in concentrations below 10 mg/l.

Since a phosphate residual in the effluent filtrate did not exist at an algal density of 50 mg/l and it did exist at or below 15 mg/l, an intermediate cell density of 40 mg/l was selected to verify this finding further. The normal growth pattern with respect to the phosphorus supply, rapid luxury uptake of phosphate, and the critical concentration of PO_4

were observed using an algal cell density of 40 mg/l and river water feed as shown in figure 26. No measurable phosphate was present in the effluent filtrate after the concentration of PO_4 in the reactor had reached the CCP mark.

iv. P-Uptake Pattern at High Cell Density

One experiment was designed to find the luxury uptake, the growth rate pattern, and the CCP at a relatively high cell density. These results are presented in figure 27. The same regular patterns are observed with the exception that the culture absorbed more phosphate (12%) than the customary luxury uptake (about 10% by dry weight). As can be seen in this figure, the culture was P-starved to some degree prior to its exposure to the high concentrations of PO_4 . The phosphorus uptake behavior of P-starved algae is different from the 'normal' cells and will be discussed in the next chapter.

v. A Note on Genetic Potential

The maximum steady state growth rate of 3.1 L/hr (Fig. 22, 24 & 28) means that the theoretical average generation period of these cells was $= \frac{26.25}{3.10} = 8.5$ hrs! Obviously then, the genetic potential of these microorganisms is not utilized to this extent in the experiments where the growth rates are much lower than the values apparent from Figures 21, 22, 23, 24, 25, and 28. For example, the calculated generation time in the case of the 97 mg/l density run (Figure 27) would

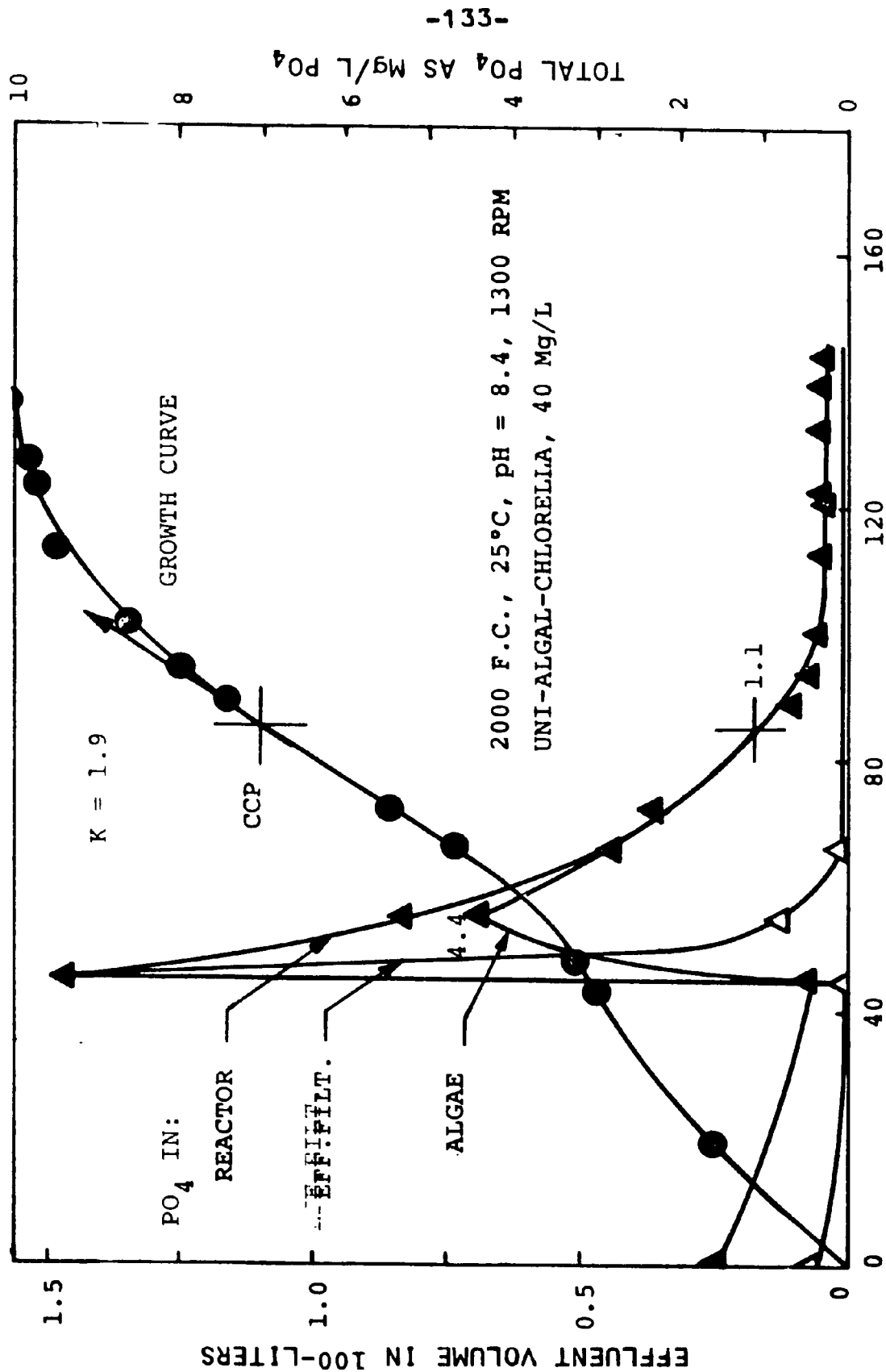


Figure 26. Determination of LUP and CCP for Green Algae Grown in River Water Feed

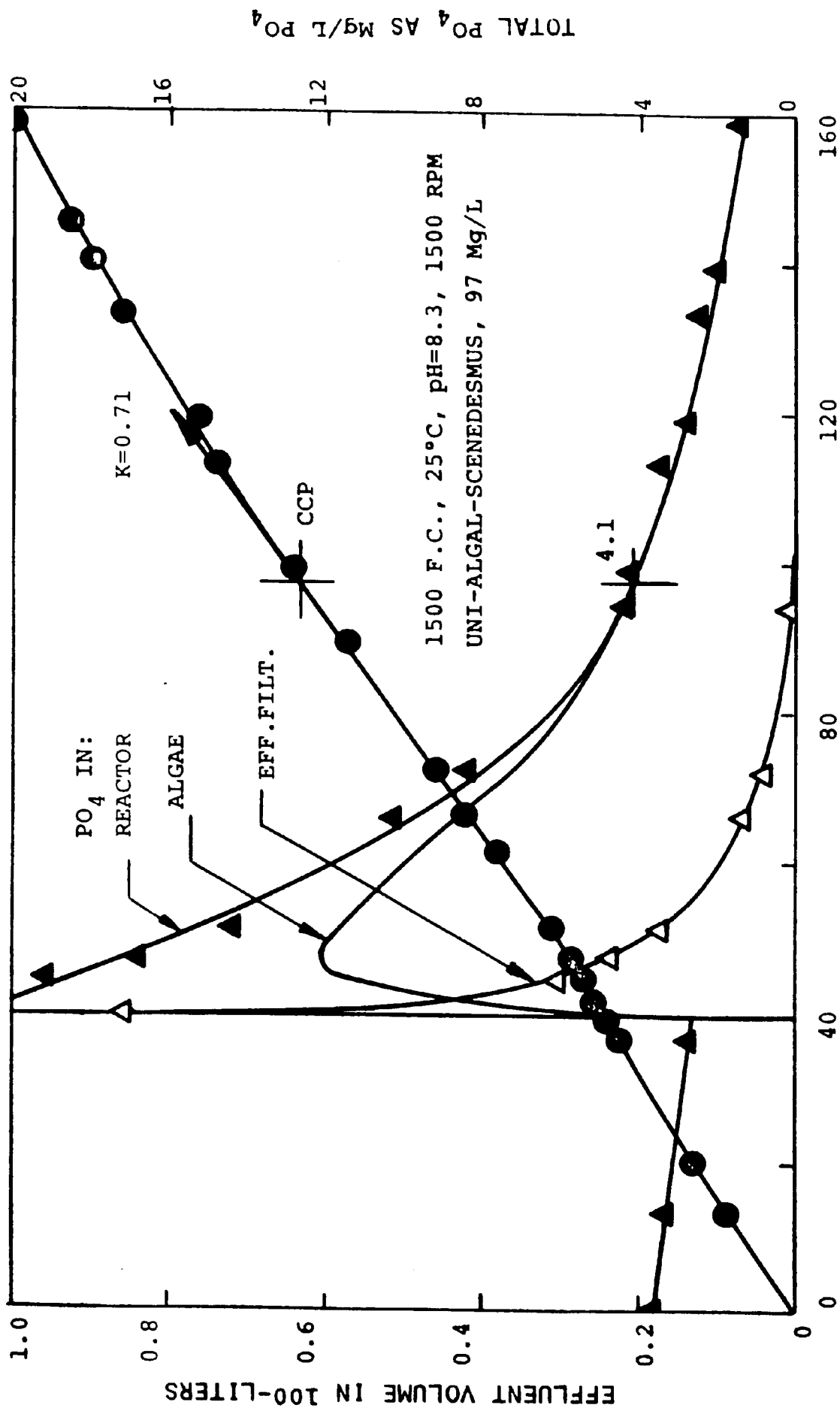


Figure 27. More Than Luxury Uptake of PO₄ and CCP for Algal Growth at High Cell Density

be about 37 hours. It should be pointed out that the 8.5 hour value for the generation time does not represent the maximum genetic potential either. The growth rate value of 3.5 L/hr in Figure 25 would yield a value of 7.5 hours for the average generation period of the culture! It may be concluded that the maximum genetic capability of algae for reproduction was never achieved during these studies. Hence, better conditions for the growth of algae could possibly result in higher growth rates than that observed in this study.

vi. Discussion on the 'Unavailable Phosphate', LUP, and CCP

As pointed out at the beginning of this chapter and later, an appropriate combination of relative concentrations of algal cells and phosphate molecules in the reactor was thought to be crucial for the complete utilization of phosphate by algae. In very dilute cultures, the chances of the algal cell colliding with a phosphate molecule, which was also present in small concentrations, would be greatly reduced. Due to the relatively large 'dilution factors' involved in the low-cell-density experiments, it was speculated that the capability of PO_4 extraction by algae would be reduced, i.e. the cells would never be able to deplete the growth medium entirely of phosphorus under any circumstances. This thinking led to the concept of 'unavailable' phosphate which is illustrated again by figure 28 (also see figures 21, 22, 23, and 25).

In this case, the rate of growth of the algae declined

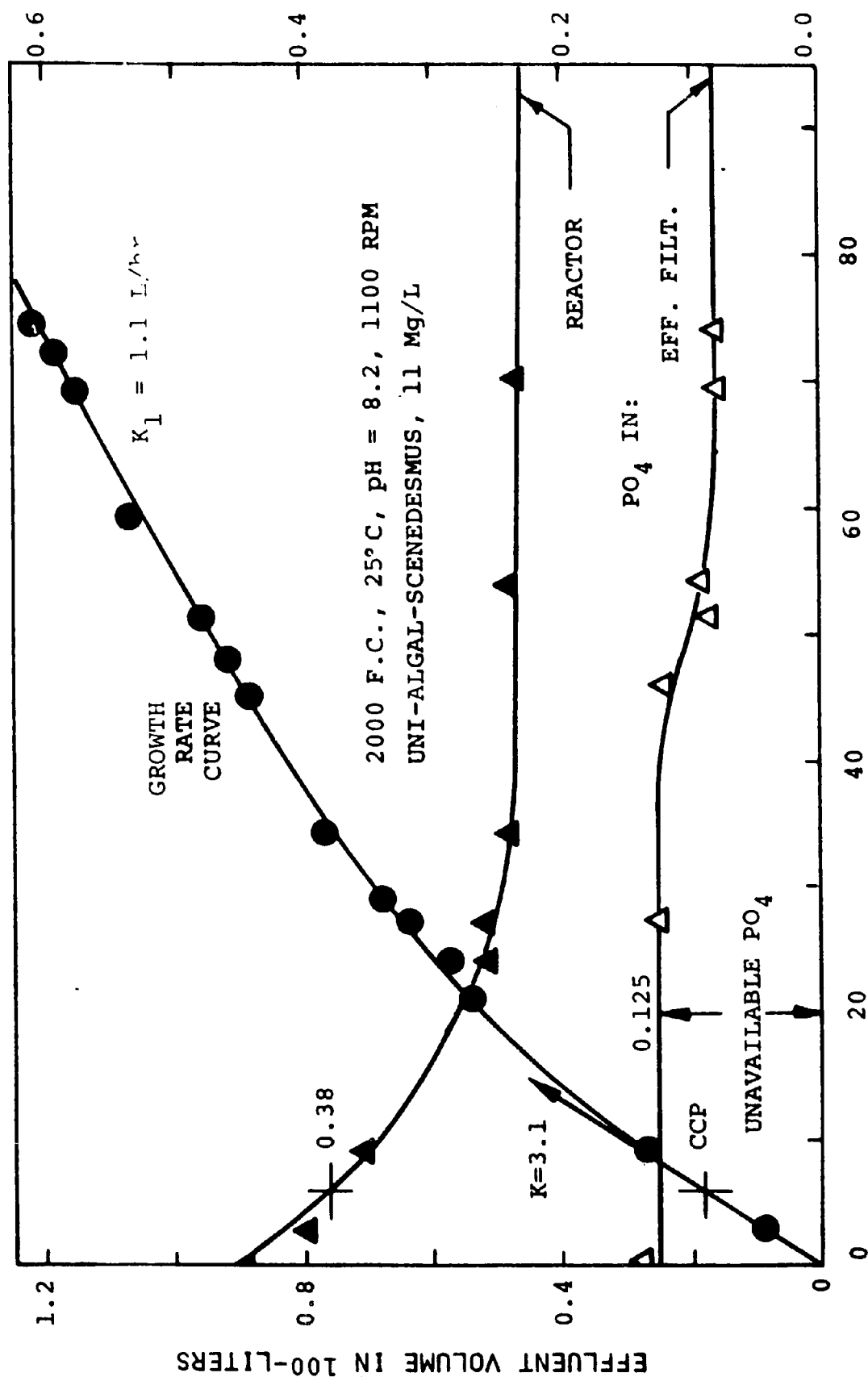


Figure 28. Determination of the "Unavailable" Portion of total PO_4 for Algal Growth at Low Cell Density

to 1.1 L/hr from the maximum steady state value of 3.1 L/hr due to the corresponding decrease in the concentration of PO_4 in the reactor from 0.38 to 0.23 mg/l PO_4 . The unavailable portion of the total phosphate present in the reactor stayed at a value of 0.125 mg/l PO_4 until the algae had developed chlorosis due to an advanced stage of P-starvation. Even then, 0.08 mg/l PO_4 remained unavailable to the phosphate starved algae! This represents about one fourth of the total concentration of PO_4 present in the reactor.

Since this phenomenon of the 'unavailability' of phosphate was observed during all the low cell density runs (algae dry weight density less than 40 mg/l), the attention of the reader to this effect has been invited throughout the 'Cell Density Effects' section.

Figure 29 is a summary of the cell density effects on the unavailable-P, LUP, and CCP values for algae. Since the uni-algal-Chlorella and the uni-algal-Scenedesmus cultures gave a very similar response, the data points on this figure have come from the different experiments disregarding this aspect. Note that the scale for the total phosphate residual in the effluent filtrate is one hundred times expanded as compared to the 'total phosphate extracted' scale. The data points at 100 mg/l dry weight of algae should not be considered rigorously quantitative. During these experiments, the light intensity was only 1500 foot candles instead of the 2000 value for many of the test runs.

It is apparent from this figure that these algae required about 3% PO_4 by weight to grow at their maximum possible rate

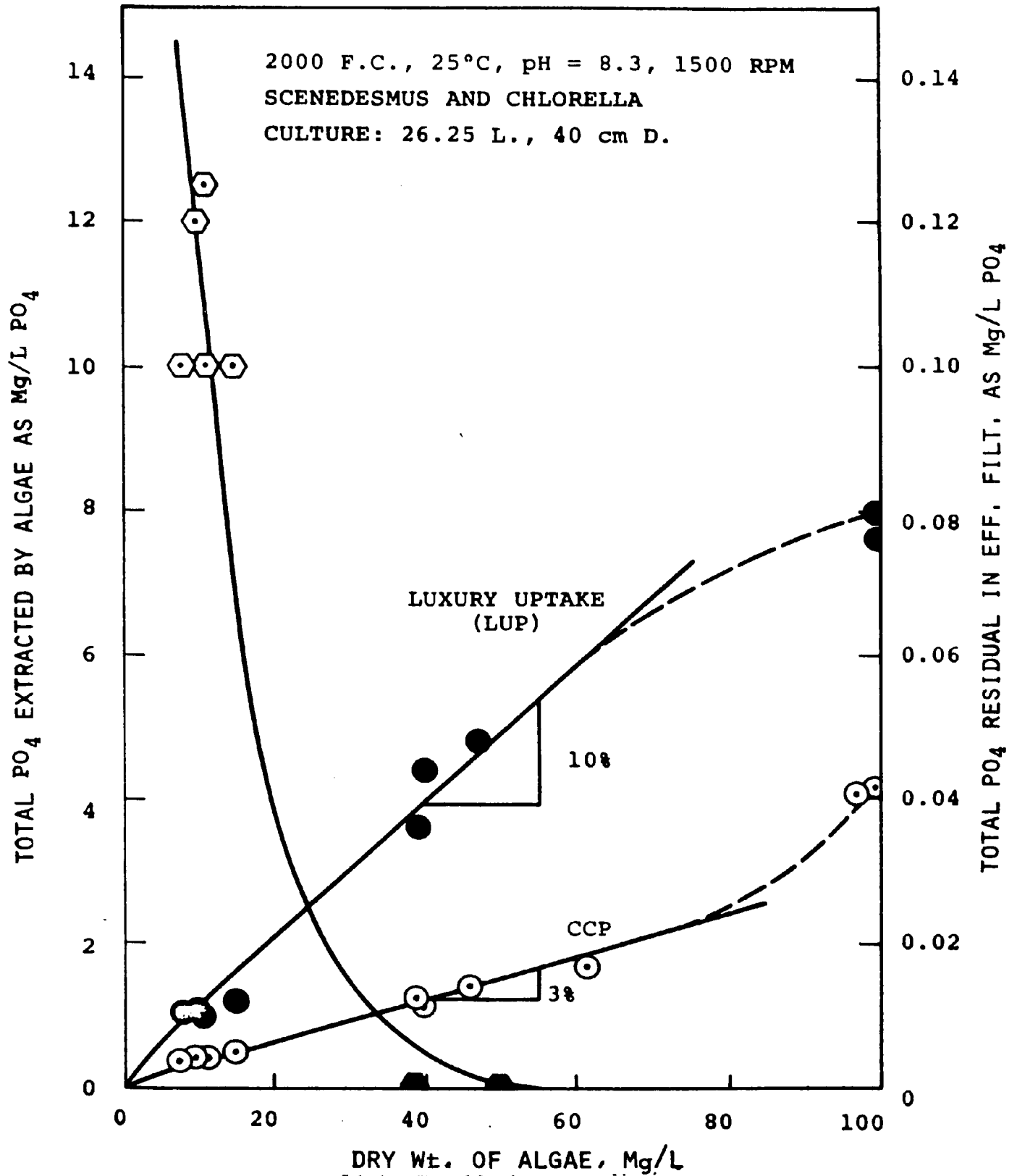


Figure 29. Phosphate Removal and Residual as Functions of Culture Density

under the given environmental conditions and extracted up to 10% PO_4 by weight from their growth media when that amount of phosphates existed. At higher cell densities, the effective average light intensity available to the culture decreases. Therefore, the CCP value is seen to increase beyond 60 mg/l algal cell density. This observation is in good agreement with the one illustrated in figures 16 and 19.

Since phosphorus storage by the cell against a concentration gradient or storage as condensed phosphates, etc., would require energy, the autotrophic microorganisms must depend on the intensity of light available for the ability to store phosphates in maximum amounts. Hence, the decline in the 10% value for the LUP under conditions of higher algal densities, as is apparent in figure 29, would seem very reasonable.

At or above a cell density of 40 mg/l, the algae leave no detectable quantities of phosphates in the effluent filtrate as long as there is less than 10% PO_4 by weight of the microorganisms in the reactor. However, at lower densities than 40 mg/l, the phosphate residual rises very rapidly with a corresponding decrease in the dry weight density of algae.

The 'unavailability' of phosphorus would be a highly significant argument for justifying the approach of controlling eutrophication by limiting the nutrient input. Some authorities have argued against the possible effectiveness of phosphorus control on the premise that even if all the domestic and industrial phosphorus supplies were diverted from the receiving waters, there would still be enough

phosphorus in these ecosystems (due to the agricultural run off, phosphate rocks, and due to the phosphates in the rain water) to support the nuisance level of algal growths! The phenomenon of unavailable phosphate, defined during these experiments, would be quite helpful to water resource management experts.

To illustrate the point, assume that an ecosystem culture exists with an algal density value of 10 mg/l. Based on the light intensity results reported in the last section, this culture should not require more than about 0.25 mg/l PO_4 for its maximum growth since under these relatively high illumination conditions, the CCP value would not be expected to exceed 2.5% by weight of the microorganisms. Assuming again mixing is ideal and that all available nutrients are present, would the normal growth of a 10 mg/l algal bloom be supported, if 0.25 mg/l PO_4 were added to the ecosystem? Obviously, not! Referring back to figure 22, the normal growth of a 10 mg/l culture could not be supported unless at least 0.4 mg/l PO_4 were present in the growth medium. It can be seen from Figure 28 that either the growth rate value of such a bloom would be reduced to about one-third the maximum possible rate or only about 3 mg/l algae could grow, if the actual phosphorus supply were limited to 0.25 mg/l PO_4 . When there are only 3 mg/l algae growing, the phosphate residual curve of figure 29 would indicate that, the unavailable portion of the total phosphate in the ecosystem would rapidly increase further and hardly any growth could take place! Without belaboring this point further, it may be concluded that any possible decrease

in the concentration of PO_4 in an ecosystem, particularly in the parts per billion ranges, must retard the pace of eutrophication and do so rather dramatically.

C. INTERRELATION OF CELL DENSITY AND LIGHT INTENSITY EFFECTS

Since independent studies of the effects of light intensity and of the cell density on the growth rate of algae already have been reported, the method that predicts the latter may now be discussed. The experimental maximum steady state growth rates, observed during the different algal cell density runs, are plotted in figure 30. The slope of the line passed through these points (-0.043) was used for normalizing the growth rate data where needed.

The cell yield curve was generated arithmetically by multiplying the abscissa and the corresponding ordinate values. It is interesting to note that the 40 mg/l density produced the most weight of algae per unit time and on this basis, could be called the optimum cell yield point for these particular conditions. Although the theoretical significance of this particular cell density is still obscure, it was concluded back in 1949 by Ketchum et al (123) that for every population of unicellular algae, there is an optimum density which produces a maximum yield. The results obtained by Gates and Borchardt (26) also showed that a particular algal cell density, under the given environmental conditions, produced

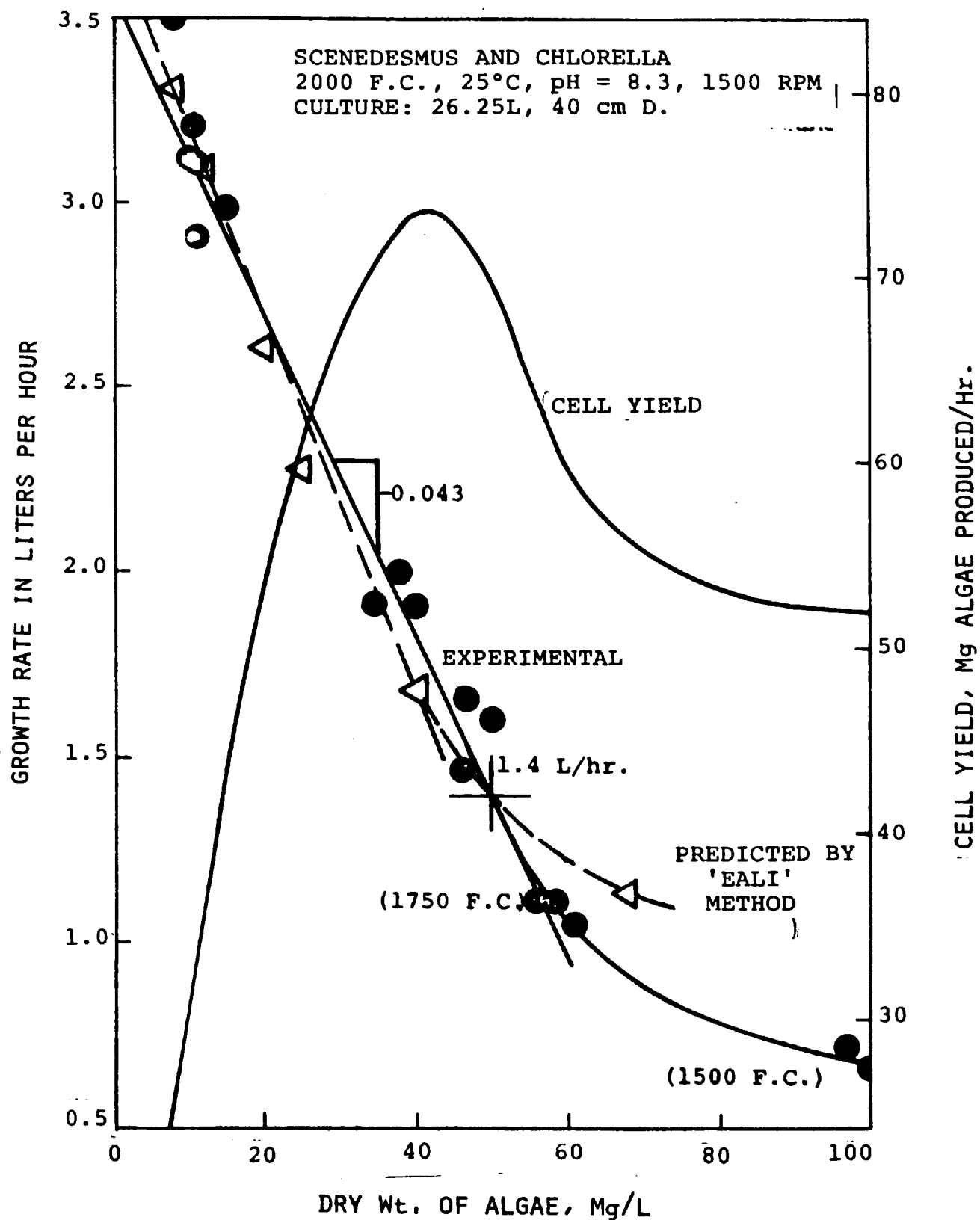


Figure 30. Cell Yield and Growth Rate of Green Algae as Functions of Culture Density

more cells/unit time than any other density and that, this density depended on the depth of the culture tank (along the path of light penetration).

There are some experimental findings in this study that would tend to relate the optimum yield point to the characteristics of the effective average light intensity available to a culture. Referring back to figure 17, the product of the light absorption factor (A) and the algal cell density (C) started deviating from its parallel with the depth-density product line exactly at $C = 40$ mg/l. This was, of course, due to the characteristics of the light absorption factor curve which appears to be a straight line from $C = 40$ onwards but is a curve before this point.

From figure 19, one would predict a corresponding increase of 0.056 L/hr in the growth rate of the 50 mg/l constant density *Scenedesmus* culture, for each increment of 10 foot candles of the effective average light intensity, as long as the algae does not become light saturated. As illustrated in figure 20, a decrease in algal cell density (holding all other parameters constant) produces a corresponding increase in the effective average light intensity. Taking the 50 mg/l density as a reference point, one can easily determine the decrease or the increase in the effective average light intensity (EALI) caused respectively, by raising or lowering the algal cell density in the reactor within the limits of $C = 8$ to $C = 68$ (figure 17).

Now suppose that only one growth rate (for the 50 mg/l density culture) determination were carried out for the

given set of environmental conditions indicated in Figure 30 and that the other growth rate values had to be predicted. Using the light intensity - growth rate relationship (0.056 L/hr growth for each 10 foot candle increment of EALI) and the information on figure 20, the growth rate values for C=8, 12.5, 20, 25, 40, and 68 were calculated and plotted on figure 30. The dotted line plot, predicted by this method, leaves no doubt that the technique works.

For illustration purposes, assume that the 50 mg/l constant density culture is known to grow at a rate of 1.4 L/hr under the environmental conditions noted on figure 30. Now pose the question: What will the growth rate value be if the culture density were reduced to 12.5 mg/l without changing any other factor? For answering this question, go to figure 17 and find the appropriate value for A which is 5.4×10^{-3} . Now calculate the value of I in the empirical relation $I_0/I = e^{CAD}$ where C = 12.5, A = 5.4×10^{-3} , D = 40, and $I_0 = 2000$. Locate this point (134 F.C.) on the right ordinate and draw the line indicated for C = 12.5 in figure 20. The effective average light intensity works out to be about 580 F.C., whereas it is seen to be about 280 F.C. for C=50. Hence, we have increased the EALI value 300 F.C. by lowering the algal cell density to 12.5 mg/l. This should provide us an additional growth rate of $0.056 \times \frac{300}{10} = 1.68$ L/hr if the light intensity - growth rate relation is valid. When this figure is added to the growth rate actually obtained at C = 50 mg/l, we get a theoretical growth rate value of 3.08 L/hr for C = 12.5. Similar calculations were carried out for

all the 6 points through which the dotted line is passed in figure 30.

It must be emphasized that although the 'EALI' predicted the algal cell density growth rate data very well, no universal application of this exact relation is implied. However, the approach appears promising for practical applications and should be checked out systematically for possible modifications.

D. DIURNAL CYCLE EFFECTS

Light being the principal driving force for all photochemical reactions, its periodic absence from an algal culture would be expected to reduce the overall anabolic efficiency of these photosynthetic microorganisms. Obviously, these cells will not be able to store energy in the form of organic - phosphates in the dark as they do in the light. Furthermore, the microorganisms would be forced to draw upon their previous reserves of energy for respiration during darkness and their mass growth should decline considerably, if the conditions for heterotrophic growth are not adequate.

The metabolic uptake of phosphorus from the growth medium should also decline, if not be entirely stopped during darkness. Since the dark reactions of the photosynthetic microorganisms can go on even when the supply of light is cut off, the cells may divide themselves during darkness but

the total biomass of the culture would not be expected to increase very much, if at all.

Giese (44) has described quite authentically the basic ideas relative to the role of light in the persistent daily rhythm of organisms. Such information need not be repeated here, but according to this author, "rhythm" is a factor of photosynthetic capacity rather than of the photosynthetic process per se.

With cells kept on light-dark ratios of 12/12 (hours), the maximum in photosynthetic capacity may approximately be at the middle of the light period. A common speculation that variation in photosynthetic capacity is caused by changes in photosynthetic pigments may not be true since no diurnal variations in either chlorophyll or carotenoid content were found with *Gonyaulax* cells (44). Changes in the concentration or activity of enzymes involved in dark reactions of photosynthesis possibly could induce rhythmicity but no such change has actually been demonstrated.

Whatever the biochemical and biophysical details, the basic concern of this research was to define the impact of L-D, 12:12 diurnal cycle, on the growth of, and P-uptake by, unicellular green algae. Although continuous high intensity (more than 1000 F.C.) lighting of algal cultures destroys their natural periodicity, the original rhythms are restored simply by placing these cultures in the dark or in dim light (44).

Figure 31 illustrates a typical response of the turbidostat algal culture to such a lighting cycle. There are

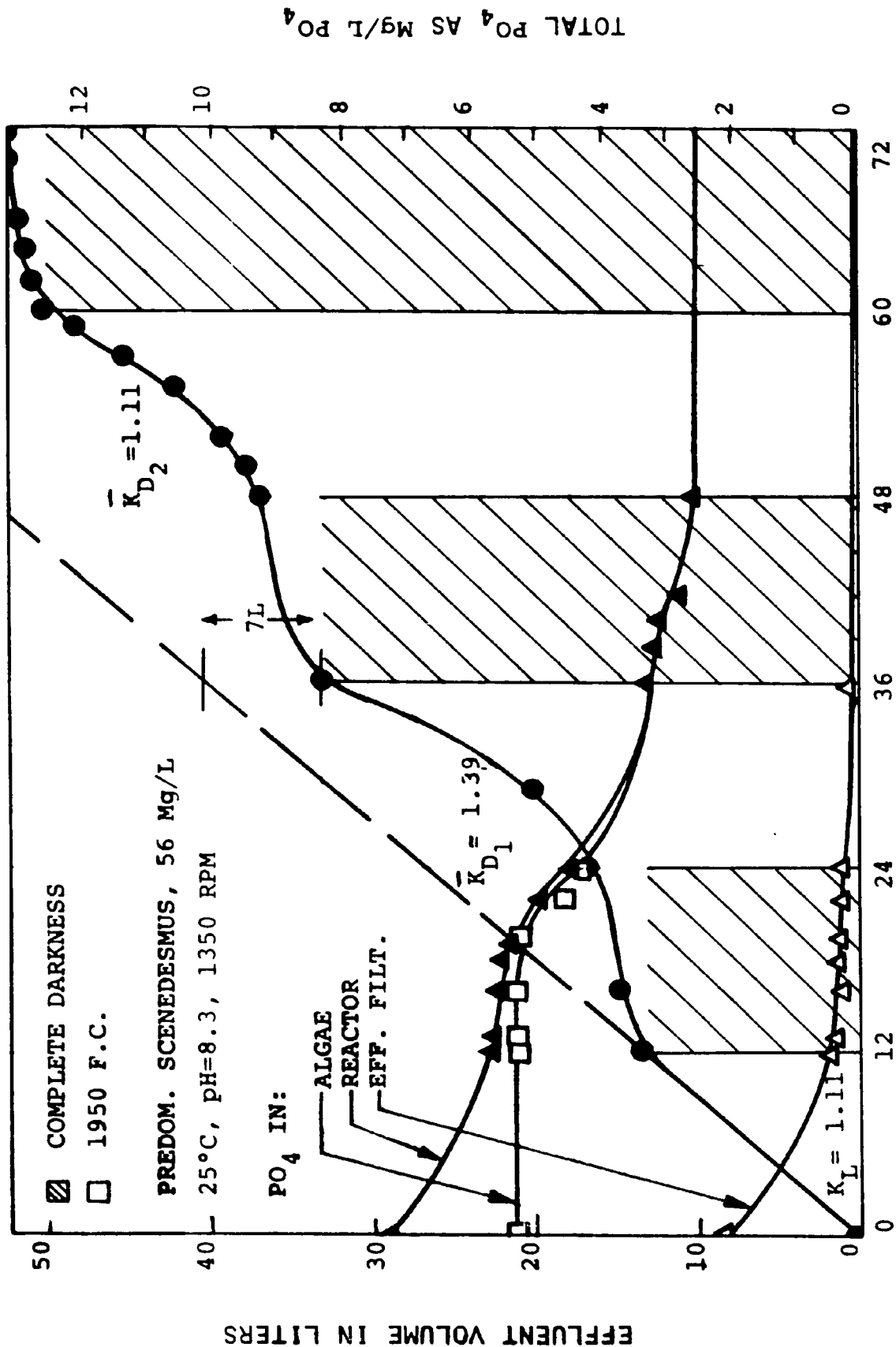


Figure 31. The Diurnal Cycle Effect on the Growth and the PO₄ Uptake Process of Predominantly Scenedesmus Algae

several features worth noting. Firstly, the growth rate and phosphorus uptake during darkness do decrease to low levels as speculated. Secondly, the average growth rate for the lighted period, after the first dark period, is seen to be higher than the previous maximum steady state growth rate. This factor apparently disappears after the second dark period since the average growth rate value of 1.11 L/hr is identical to that of the maximum steady state growth rate under continuous lighting conditions. Thirdly, none of the phosphorus stored by algae leaches into the substrate during the first dark period. The cells contained 9.5% PO_4 by dry weight. At the onset of the second dark period, the algae contained about 6% PO_4 , while the substrate contained only traces, but again no stored phosphate leached out of the cells.

Fourthly, the algal cell numbers increase during darkness as illustrated in Figure 32. It is apparent from this figure that the relative *Chlorella* counts increase much more than a corresponding increase in the *Scenedesmus* counts. Within the limits of the accuracy of counting, at the end of each lighting period the cell counts approximately decrease to their original values observed under continuous lighting conditions. Note also from Figure 33 that the cell numbers increase during darkness without an accompanying increase in the dry weight density or a decrease in the per cent transmittance of the culture. An unusually high scatter in the dry weight and per cent transmittance data apparent in figure 33 was due to the manual feeding of the culture during the dark periods since the turbidostat apparatus became nonfunctional in the absence of light.

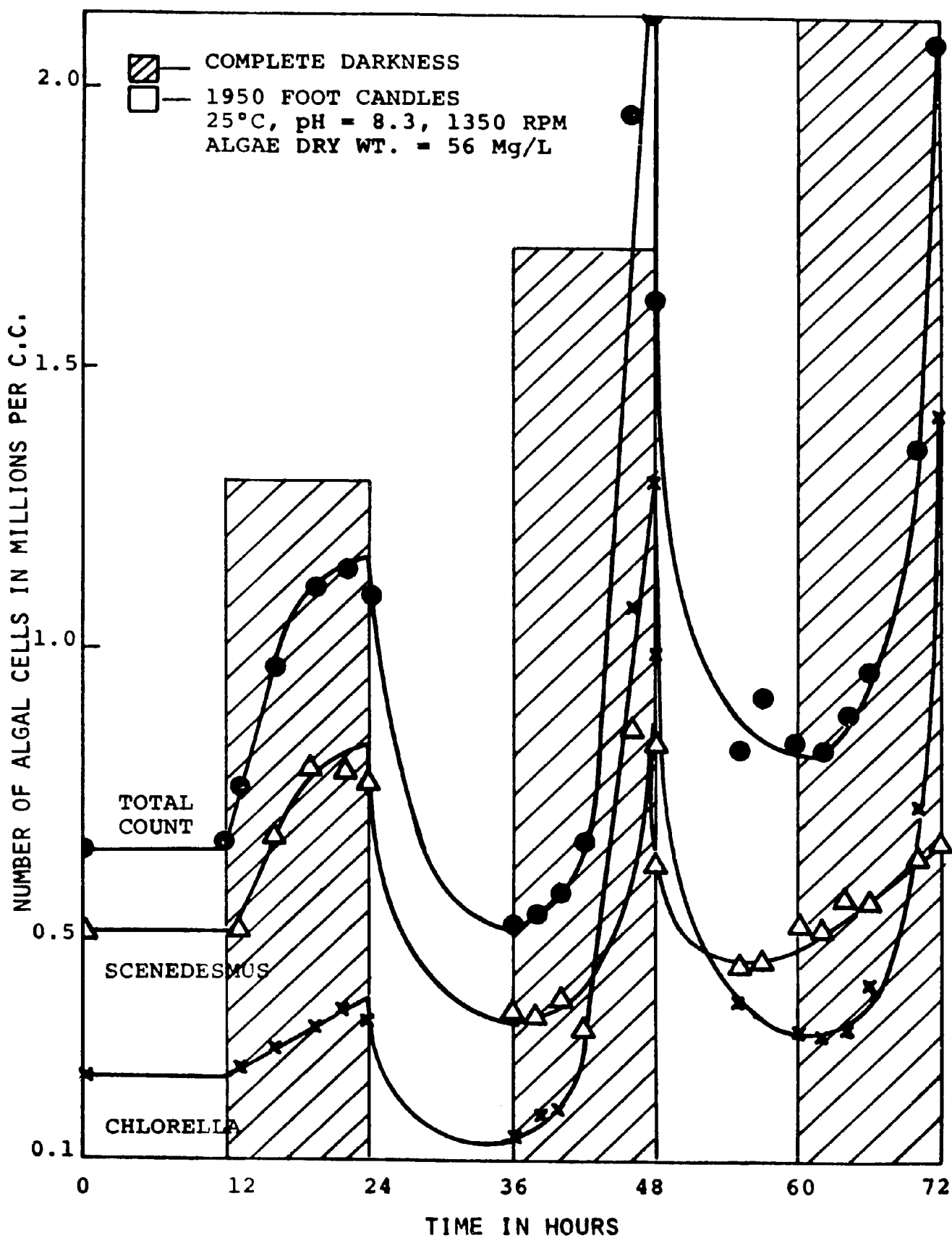


Figure 32. The Diurnal Cycle Effect on the Cell Division of Chlorella and Scenedesmus

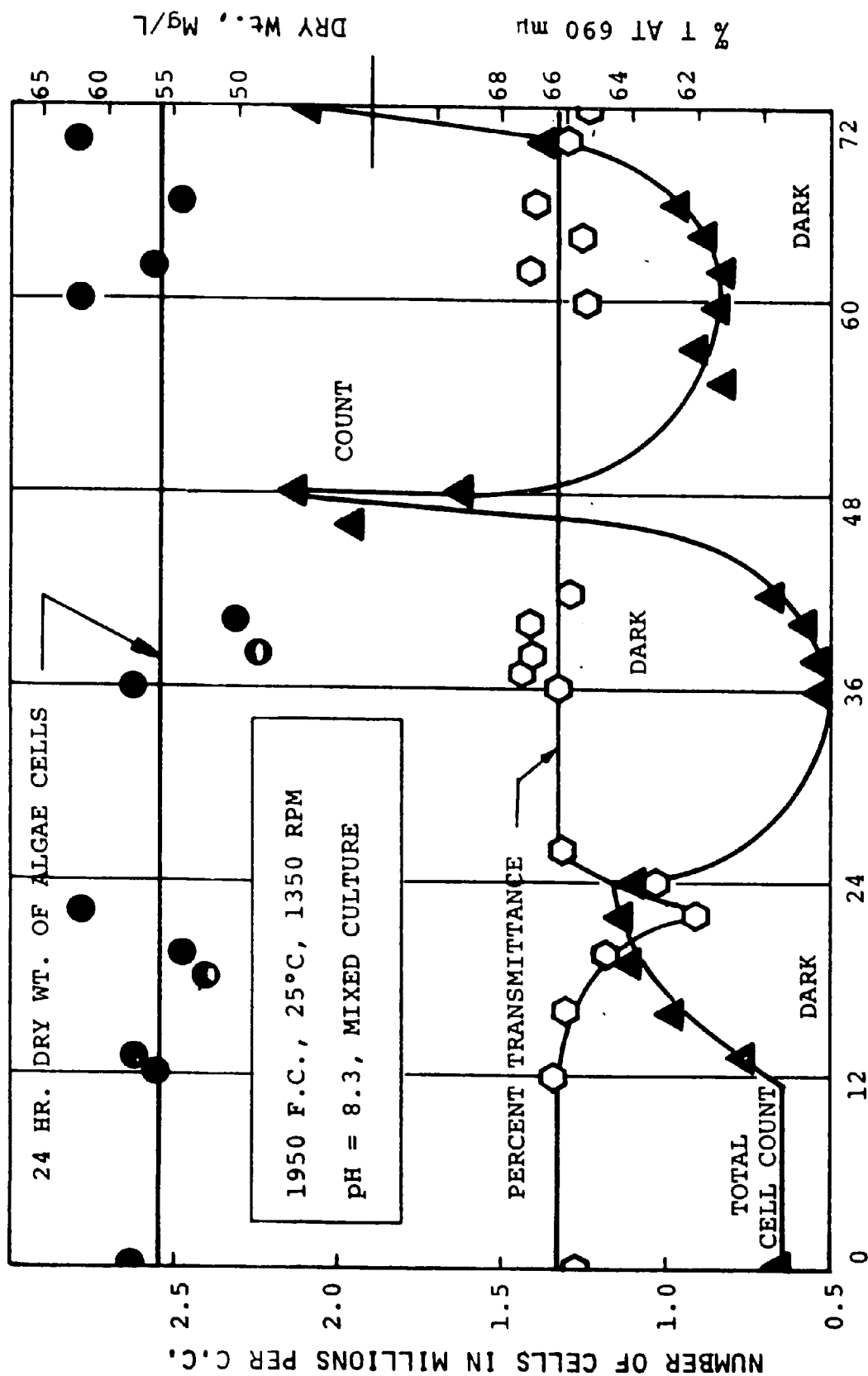


Figure 33. The Diurnal Cycle Effect on the Relationship among Dry Wt., % T, and Cell Count of Green Algae

These observations were verified further using the pure *Chlorella* culture and batch experiments illustrated in Figures 34 and 35. It should be recalled that high % T means low algal cell density. The starting value of 97% T in figure 34 would correspond to about 2.5 mg/L dry weight of algae and the terminal 68% T represents approximately 30 mg/L algae - a twelve fold increase in biomass. In figure 35, the initial dry weight density was close to 20 mg/L whereas the experiment ended at a cell density above 200 mg/L.

These experiments clearly support the turbidostat observations reported in the earlier paragraphs. It is apparent from figures 34 and 35 that the cell density, within the limits of these experiments, does not influence the characteristic response of the culture to the diurnal cycle. No significant phosphorus uptake and biomass increase is observed in darkness while there is a dramatic increase in the cell numbers. As reported before for the observations presented in figure 15, the cell numbers increase at the expense of the average cell size and this was confirmed clearly by microscopic observations where the cells in the actual process of division could be seen.

It may be concluded from these studies that although no phosphorus storage by the green algae is possible during darkness, the experimental evidence demonstrates that no stored phosphorus leaks out of the cells either. No extraction of phosphorus from the medium is observed and the cell mass growth of algae is greatly reduced during darkness. Obviously, the overall efficiency of phosphate removal by algal cultures

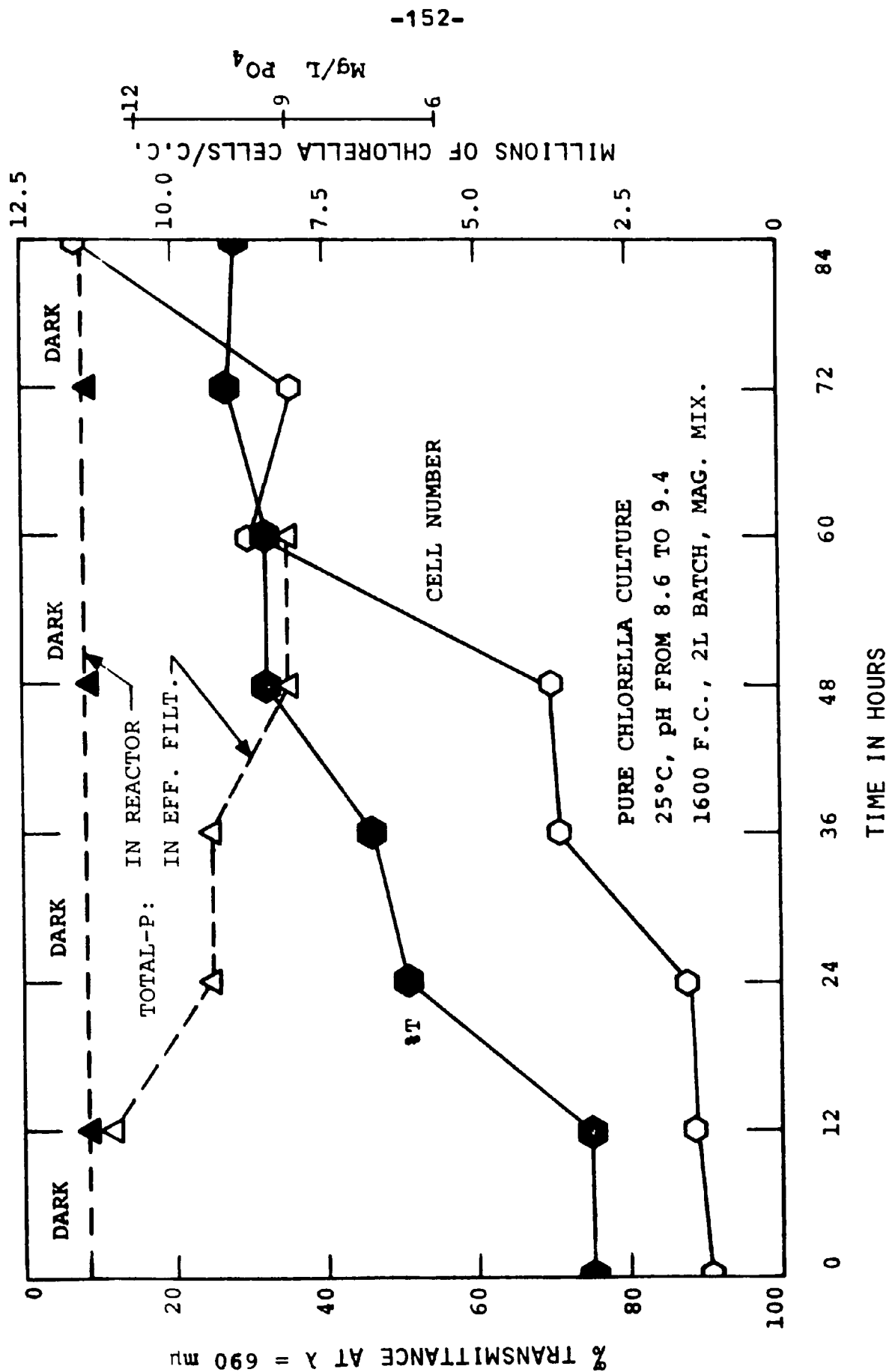


Figure 34. Diurnal Cycle Effect on % T, Cell Count, and Phosphorus Uptake in a Batch Experiment

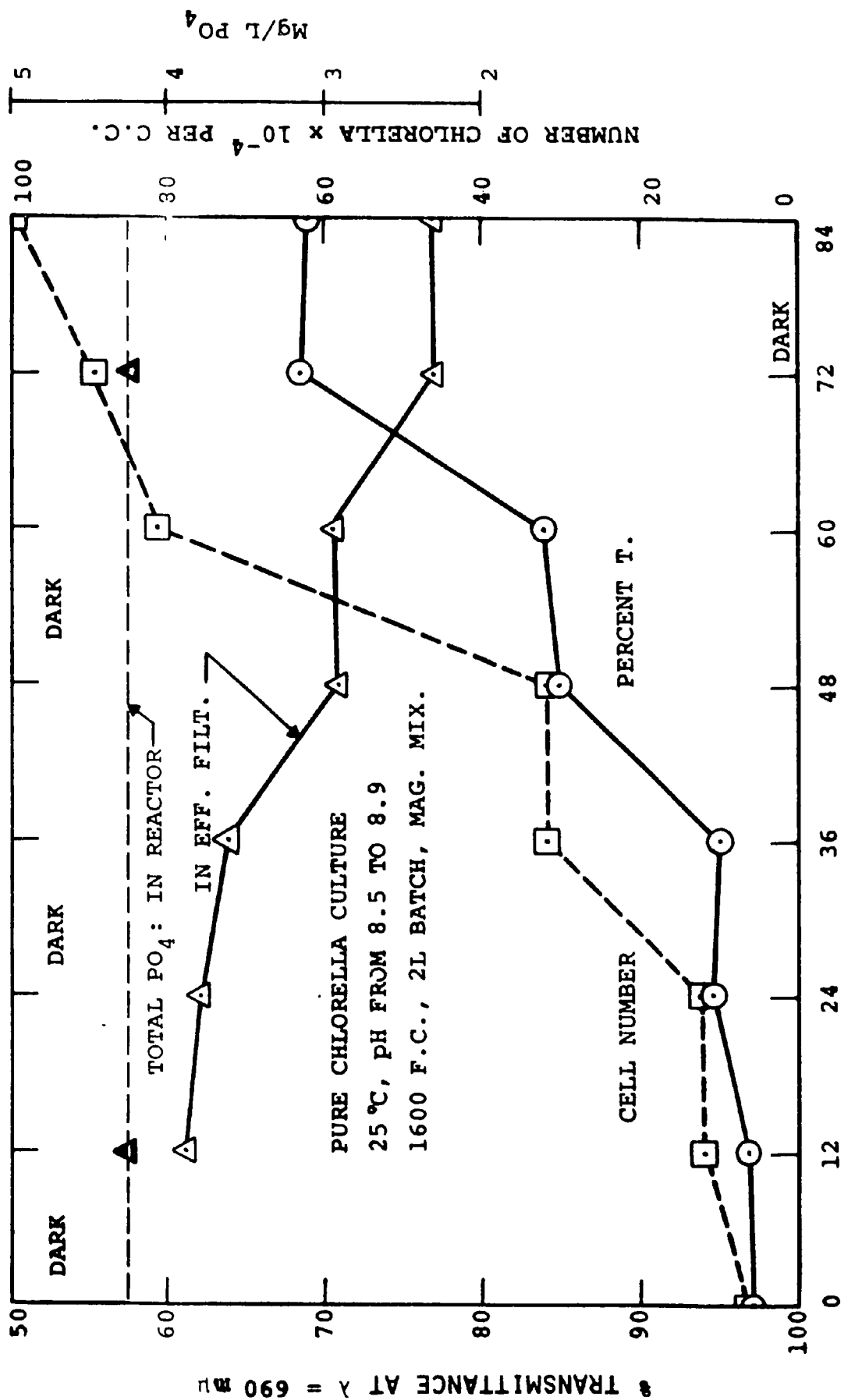


Figure 35. Diurnal Cycle Effect on % T, Cell Count, and Phosphorus Uptake in a Batch Experiment

would diminish, when used in a natural light-dark environment, from the normal high levels experienced under continuously lighted conditions. Furthermore, phosphorus removals would be in a direct proportion to the reduction in the total growth. As a first approximation, an efficiency factor of 64% in the removal of PO_4 is apparent from the period 36 to 60 hours in figure 31 for green algae similar to *Scenedesmus* and *Chlorella*.

These results are in substantial agreement with the ones obtained by Luebbers and Parikh (120). They observed no net oxygen production during darkness using 6,8,10,12,14,16, and 18 hours day and in fact, observed net oxygen consumption during this period in every combination of hours for day and night.

Tamiya et al (124) conducted similar studies using a batch culture apparatus and the organism *Chlorella ellipsoidea*. They reported that the packed cell volume per liter increased significantly only in light, the cell numbers generally increased during darkness (without an accompanying increase in the packed cell volume), and the mean cell diameters were smaller after the dark periods than they were when the darkness ensued. Although their cell number data was relatively inconsistent, their conclusions were similar to those developed in this study.

L. TEMPERATURE EFFECTS

1. GROWTH LIMITING MECHANISM PREDICTED BY E_A

As stated at the beginning of this chapter, temperature and mixing can both be important factors in transporting the nutrient molecules from the bulk liquid phase to the inner cell boundary. Figure 36 illustrates the results of calculating the energy of activation (E_A) for algal growth from the experimental growth rate values observed at different temperatures. The value of E_A (5.96 K cal) calculated from the growth rates actually observed at 15, 20, and 25°C is in the range of values where diffusion 'may be' the operational growth limiting mechanism. Hence, the energy of activation would provide some support for the speculation that temperature enhances the nutrient diffusion into the cells and thereby, affects the growth rates.

2. GENERAL CONSIDERATIONS

Ecologists find by inference that temperature affects the distribution of algal species in time and space. Physiologists are intrigued by the complex biochemical and biophysical mechanisms that permit microorganisms to flourish under extremely different temperatures. Some useful discussion of the various possible effects of temperature on microorganisms can be found elsewhere (125).

Very briefly, temperature can affect the growth of microorganisms through its action either on the external medium,

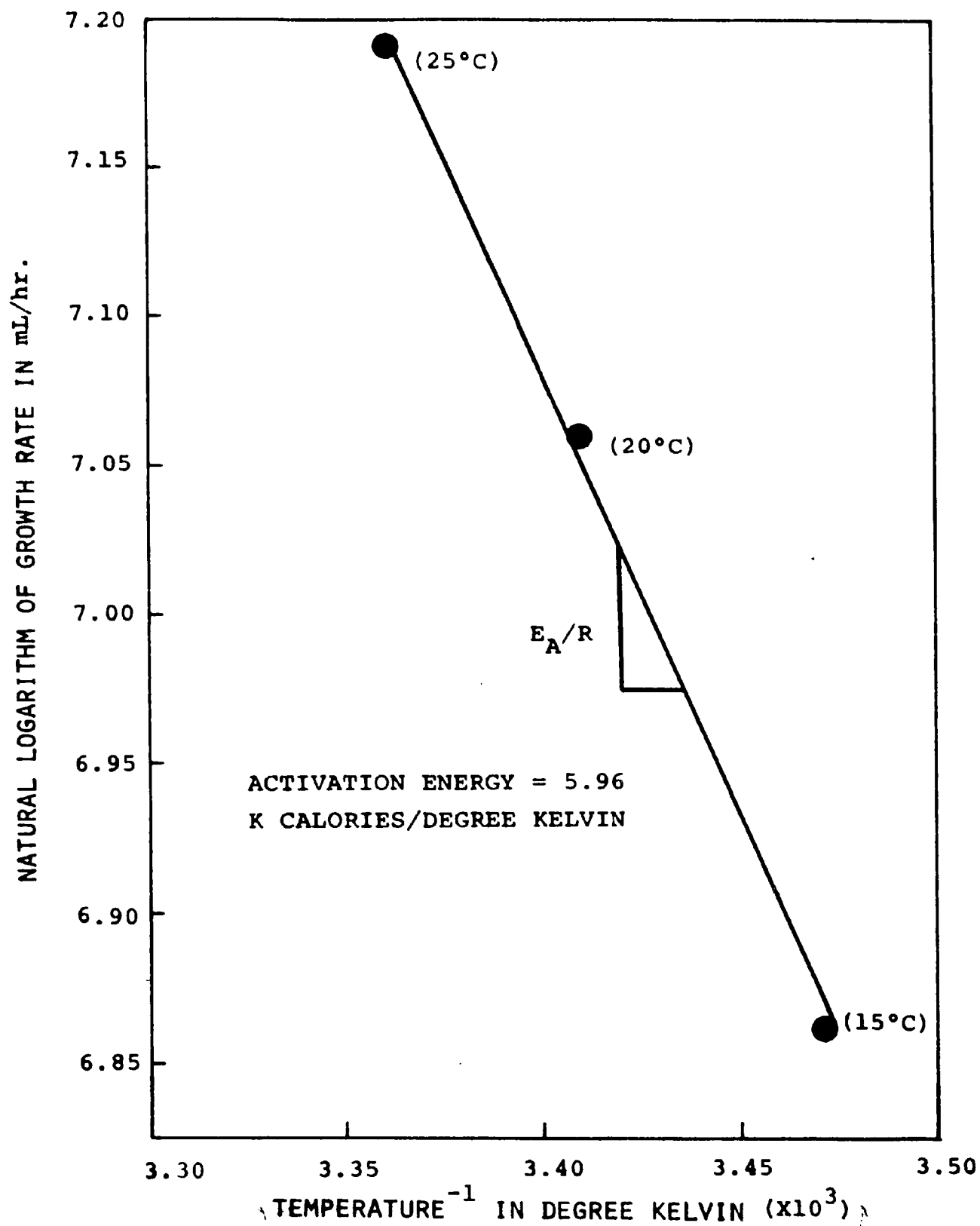


Figure 36. Calculating Energy of Activation for Algal Growth from Temperature Data

or directly on the cells, or on both. Solubilities of important gases in water are temperature dependent and this factor alone could limit growth. The solubility coefficient of CO_2 is reduced from 0.76 to 0.36 and of O_2 from 0.028 to 0.019 with a corresponding increase in temperature from 25 to 60°C (125). Temperature acts directly on the cells by speeding up or slowing down the different biochemical reaction sequences. The concept of thermolability of proteins and other macromolecules is widely recognized. At temperatures close to 0°C, the chemical reactions in the cell almost come to a halt and the protoplasm may not survive mechanical damage caused by the freezing and thawing of intracellular liquids. In the case of frost resistant snow algae, a high osmotic pressure of their cell sap can be correlated with a lowering of their freezing point as is the case with the flagellate Dunaliella salina which can maintain some motility even at temperatures as low as -15°C (125).

When discussing the effect of temperature on the growth of microorganisms, perhaps the terms "Stenothermy" and "Eurythermy" also should be explained. Stenothermal is a term used to describe those cells whose biological activity may be impaired by a change of a few degrees above or below their optimum temperature for growth and reproduction. On the other hand, the organisms with a wide limit of temperature tolerance (mainly Chlorophyta and Cyanophyta) are referred to as eurythermal.

The green algae *Scenedesmus* and *Chlorella* employed in this study grow well in medium temperatures and are therefore,

mesophilic. Since they are known to grow under a relatively wide range of temperatures, these algae would be called eurythermal. Not all eurythermal microorganisms are mesophilic but many mesophils are eurythermic.

From the nutrient control and bio-extraction standpoints, this investigator was interested in determining the effects of temperature on the growth rate and on the critical phosphate level that must be present in the medium to satisfy the metabolic needs of the algae. Phosphorus uptake reactions being endergonic, the rate of uptake would be expected to decline with a decrease in temperature or to increase with an increase in temperature, up to a point. At higher temperatures where growth rates decrease, beyond the optimum temperature for growth (OTG), the temperature effects on CCP may not be easily predicted however, since a concomitant decrease in growth rate with increasing rate of P-uptake is somewhat difficult to comprehend.

3. EXPERIMENTAL RESULTS

Considerations of these factors led this investigator to the design of experiments for exploring this phenomenon in the most practical range of temperatures, i.e. from 15 to 30 degrees centigrade. Figures 37 to 40 show the results of four such experiments at 15, 25, 25, and 30°C, respectively. Similar information obtained at 20°C has been presented earlier in figures 11 and 12. These are the same familiar illustrations showing the CCP points, maximum steady state

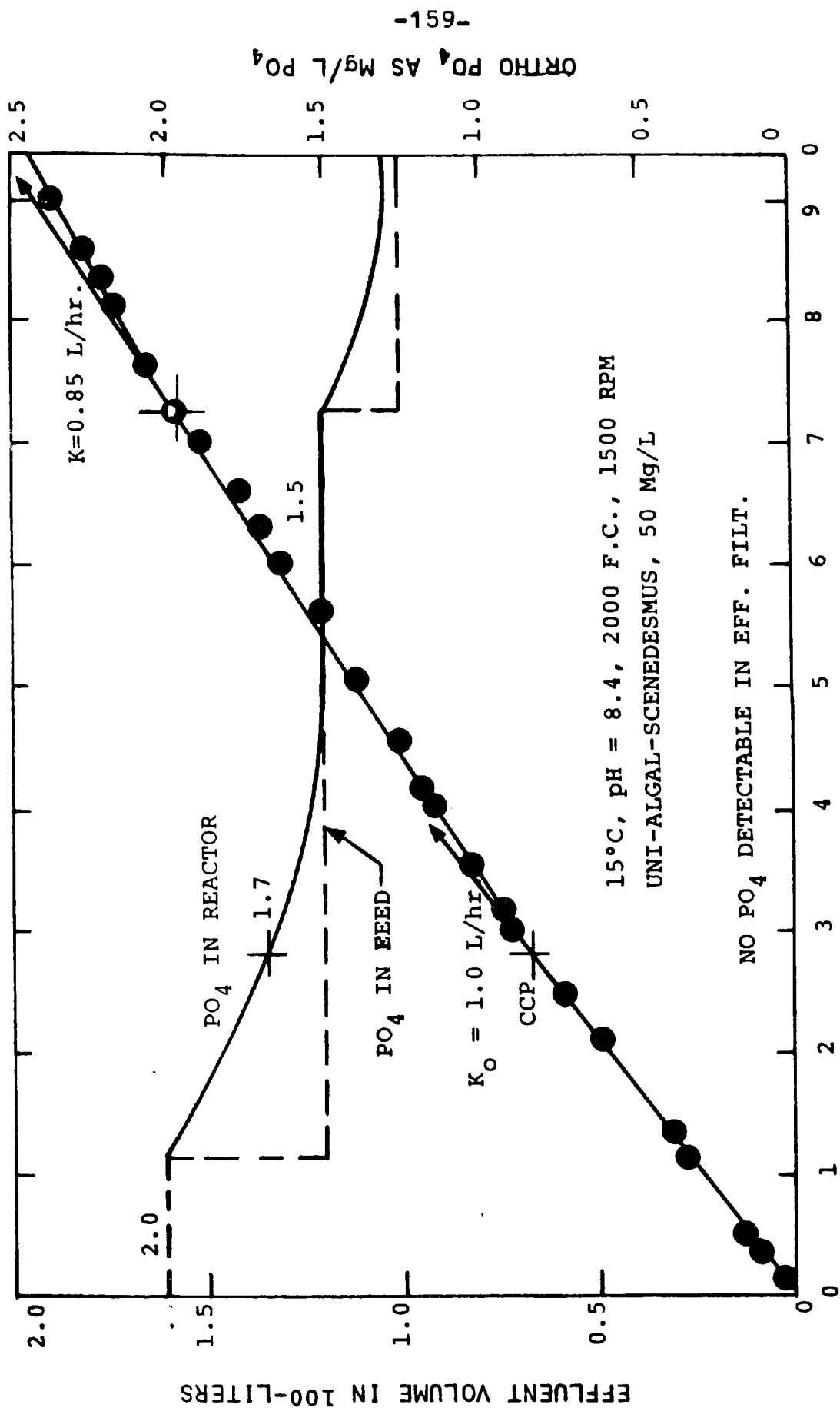


Figure 37. Effect of a Stepwise Reduction of PO_4 in Feed on the Growth Rate of Green Algae at 15°C

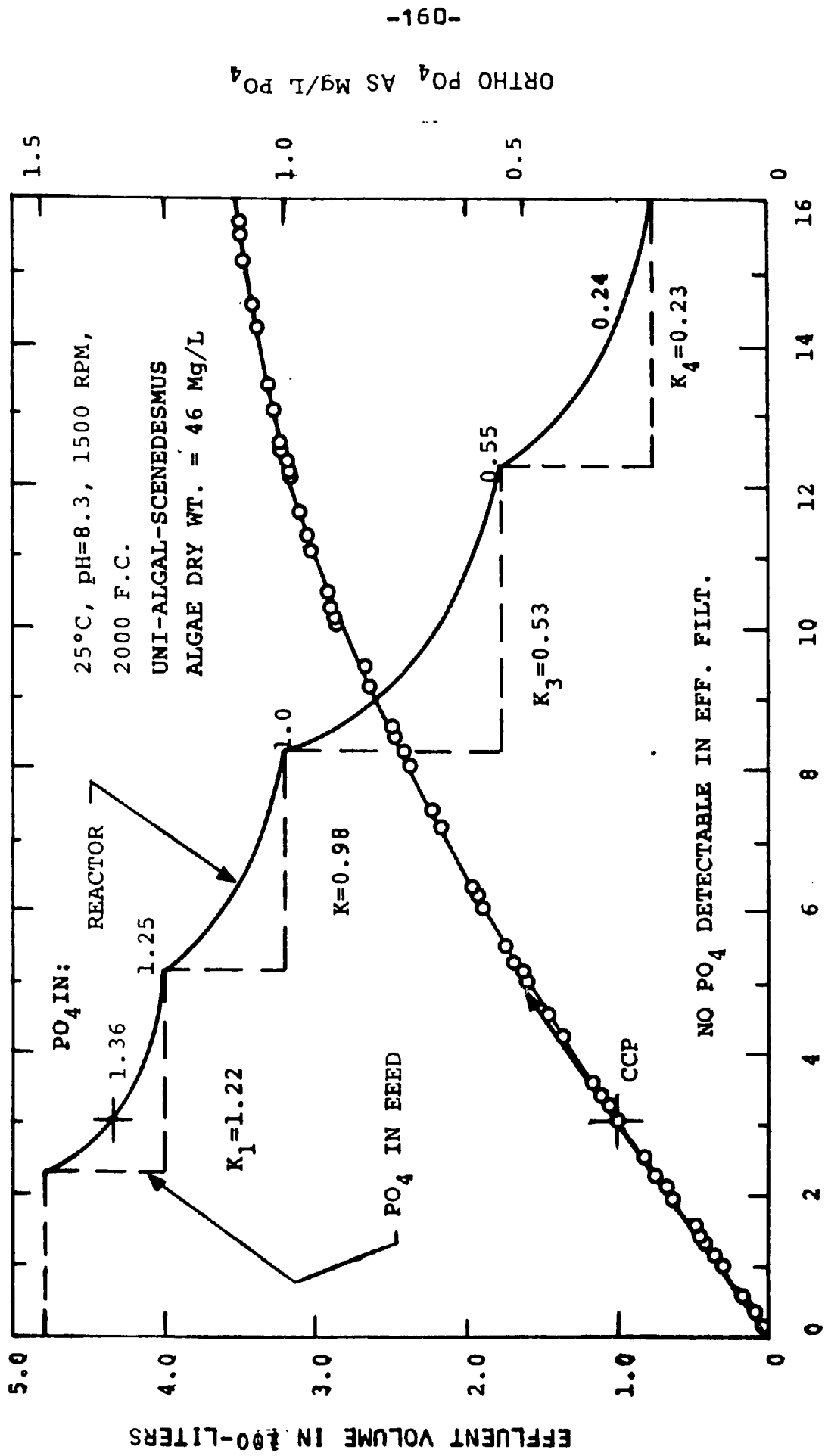


Figure 38. Effect of Stepwise Reduction of PO_4 on the Growth Rate of Green Algae at 25°C

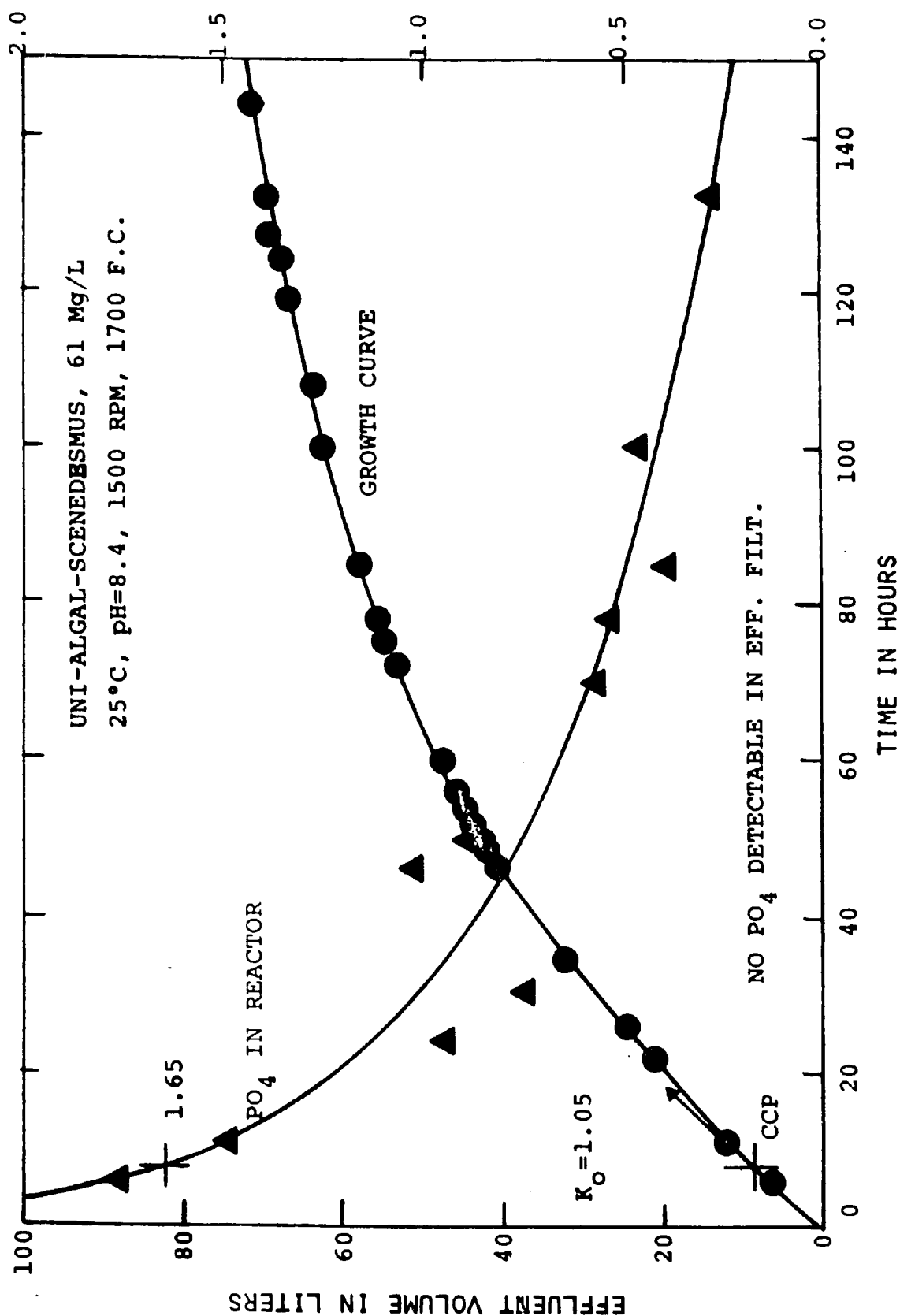


Figure 39. Effect of a Continuous Degradage in PO₄ on the Growth Rate of and CCP for Green Algae at 25°C

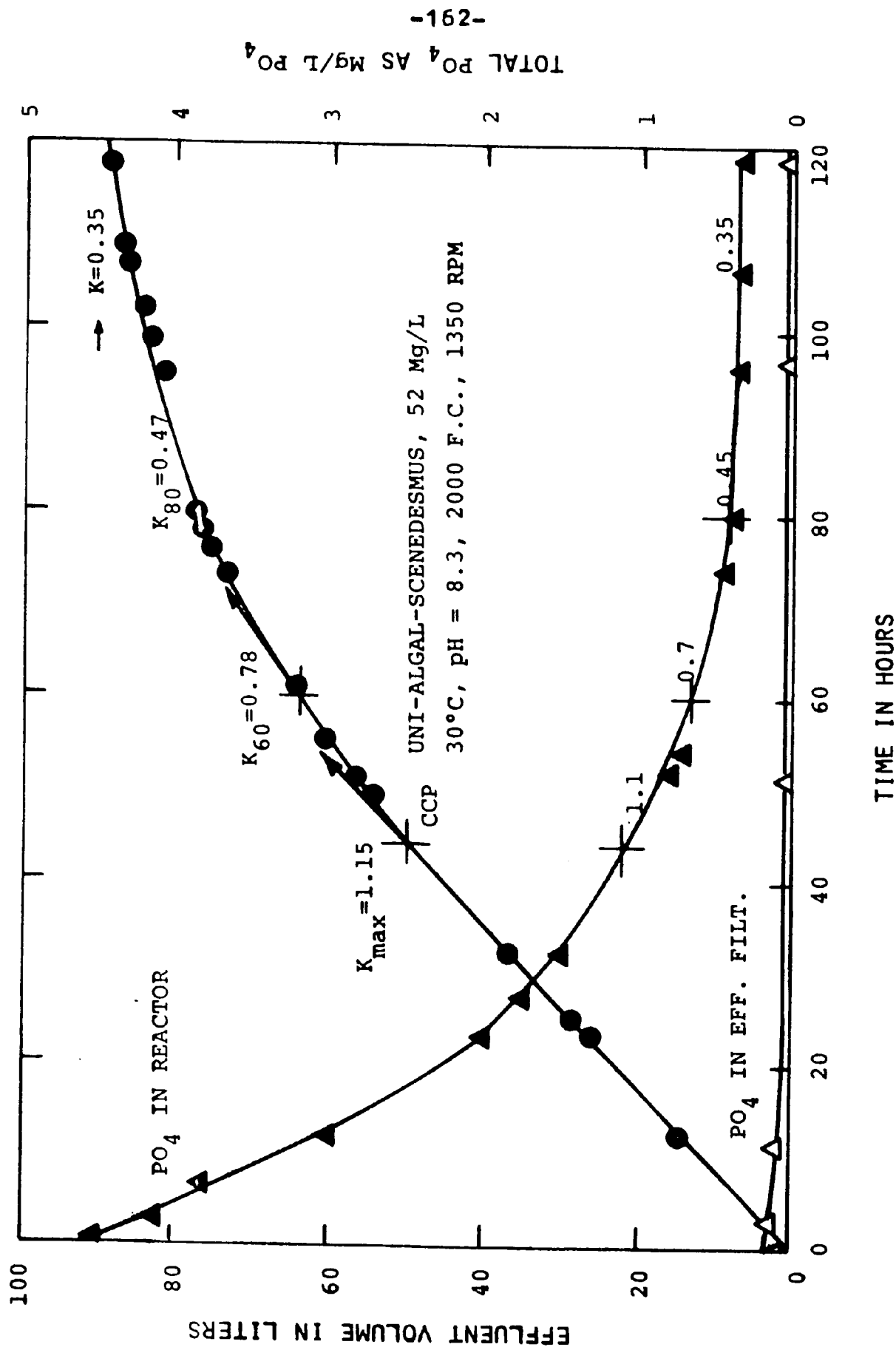


Figure 40. Effect of a Continuous Decrease in PO₄ on the Growth Rate of and CCP for Green Algae at 30°C

growth rate values, and the effect of decreasing concentration of phosphate on the growth rate of algae under the given environmental conditions.

i. P-Residual at High Temperature

It should be noted that there was some phosphate (typically a low algal cell density phenomenon) residual left in the effluent filtrate at 30°C, but none was detected at any other temperature. This may mean a very high turnover of phosphates at high temperatures.

ii. Discussion of Temperature Effects

A summary graph of the effect of total phosphates concentration in the reactor on the growth rate of algae, at different temperatures, is presented in figure 41. It is apparent from this figure that temperature influences the CCP value as well as the maximum steady state growth rate of algae. The fact that lower growth rate values, in all cases when extrapolated from CCP backwards, intersect the abscissa at zero, demonstrates the fact that the data was highly reproducible and consistent from run to run. It does not constitute evidence however, for stating that the rate of growth of the microorganisms was zero at zero temperature.

Figure 41 illustrates atleast two very important concepts. Firstly, concentration of phosphorus beyond the CCP point (although not shown in the figure, concentrations up to 24 mg/l PO_4 were studied) do not affect the maximum steady state

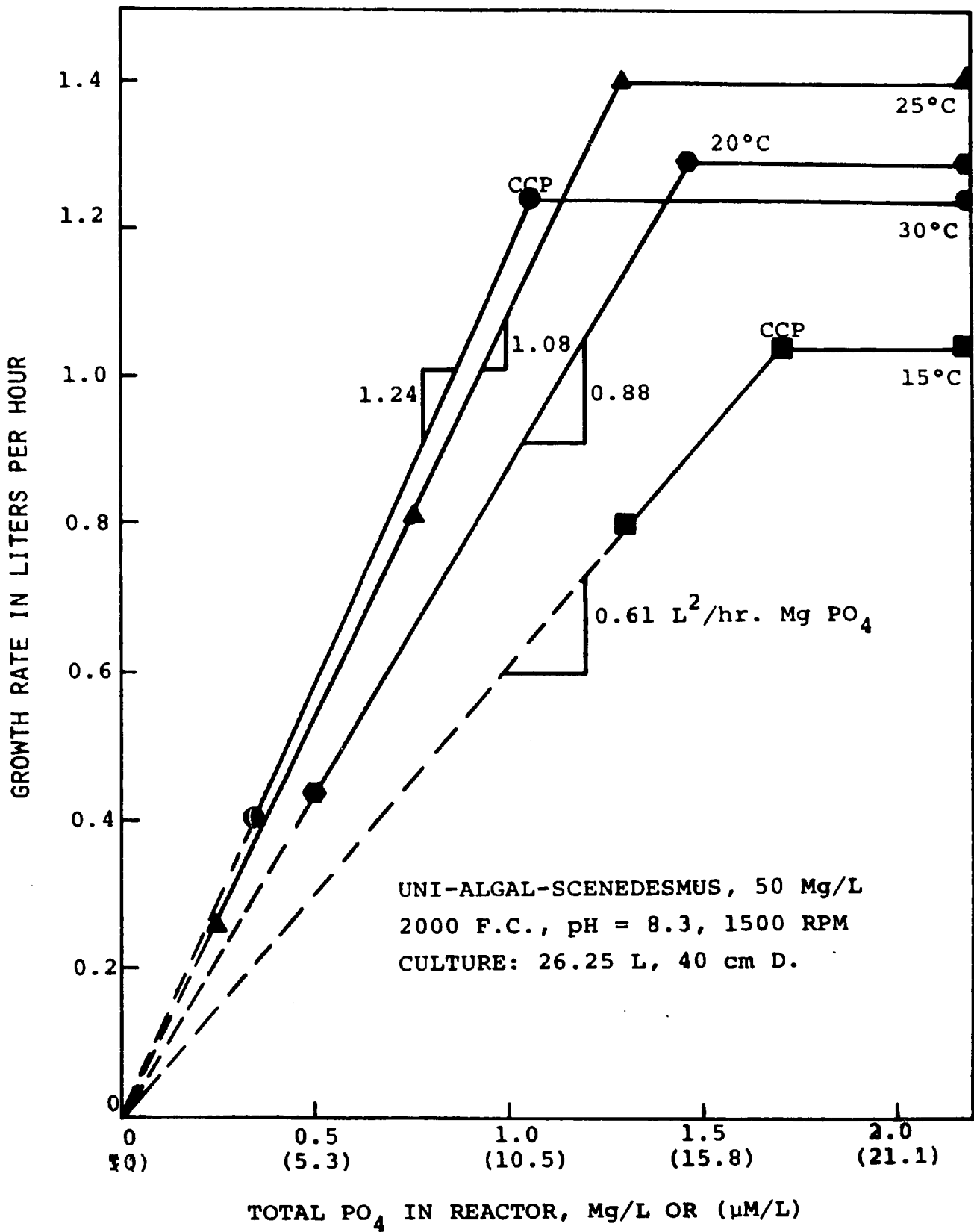


Figure 41. Dependence of the Growth Rate of Green Algae on Phosphorus at 15, 20, 25, and 30°C

growth rate value at any temperature, atleast from 15 to 30°C. Secondly, there is a sharp break in the growth rate dependence on the concentration of PO_4 in the medium right at CCP and not a curvilinear response. Furthermore, below the CCP value, there is a direct and quantitative proportionality between the concentration of phosphorus in the medium and the growth rate of algae as indicated on figure 41 for various temperatures.

The effect of temperatures on the growth rate of and CCP for green algae is illustrated further in figure 42. All the data points in this summary graph (as in the figure 41) came from the figures 11,12,37,38,39, and 40. The scatter in the two growth rate values at 25°C is atleast partly due to the different light levels used. Some error may have been introduced by the normalization process used for calculating the growth rate values for a constant density of 50 mg/l algae from the corresponding experimental values of 46 and 61 mg/l algae. The curve is passed through a growth rate value of 1.4 L/hr which is consistent with the maximum steady state growth value shown for this density in figure 30.

It is clear from figure 42 that CCP varies linearly with temperature irrespective of the growth rate response of the culture to the temperature. Within a range of 10°C to 35°C, 0.047 mg PO_4 / L/°C value may be used for quantitative estimates of minimum concentration of phosphorus required, at any temperature, for supporting the maximum growth of a 50 mg/l constant density culture. Similarly, the value 0.068 L/hr/°C could be used for growth rate prediction purposes. Also,

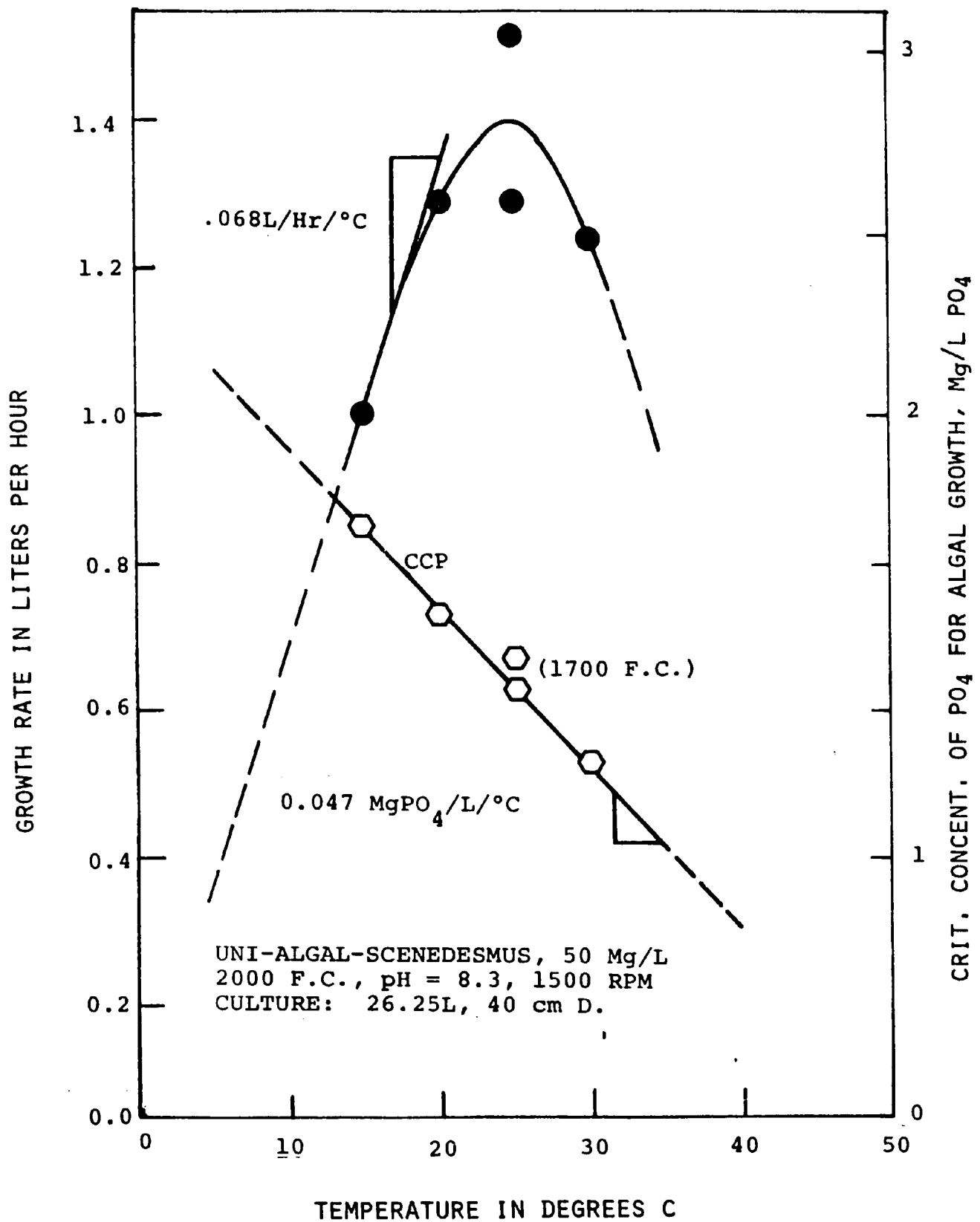


Figure 42. Effect of Temperature on the Growth Rate of and CCP for Green Algae

semi-quantitative and qualitative estimates for natural systems could be made using these values and the ones reported earlier relating the growth rates, algal cell densities, and the light intensities.

Apparently, higher phosphorus concentrations must be present to grow the same mass of protoplasm at lower temperatures and conversely, much lower PO_4 concentrations are required at higher temperatures. Such a relationship may explain partly the blooming of plankton after a warm spring day or after a warm rainfall. It should be recognized however, that algal blooms are known to occur under ice cover and the temperature relationship may not explain all situations.

F. MIXING EFFECTS

The rate of a chemical reaction is given by the following Arrhenius expression:

$$\text{Rate} = A \cdot e^{-E/RT} \dots\dots\dots 9$$

where, E is the Arrhenius energy of activation, R is the universal gas constant, T is the absolute temperature in degrees Kelvin, and A is a constant referred to as a frequency factor. From the standpoint of collision theory, the rate of a chemical reaction may be expressed (126) as:

rate = collision frequency x energy factor x probability factor..... 10

Collision frequency in this relation and the frequency factor 'A' of Eq.9 not only depends upon the concentrations of the reactants and how large they are but also are functions of how fast they are moving. It is the agitation of the algal cells and the nutrient molecules that is affected by mixing when the temperature and the reactor contents are fixed. Hence, the higher the rate of mixing the higher the collision frequency factor would be.

Another important way in which mixing may effect the growth of algae is that at high cell densities, a turbulent suspension exposes the cells to intermittent periods of light and darkness. The cell may be able to integrate (thus utilizing more effectively) the available light to some extent under these conditions and therefore, grow faster under more turbulent conditions. The sheer force applied in this manner to the cell may have other consequences which are largely unknown at present. The growth of batch algal cultures was studied by Gates (105) and he reported that the total algal growth increased with increasing mixing speeds. Some experiments, designed for determining the mixing effects on the growth rate of and phosphorus uptake by a constant density continuous algal culture, are illustrated in figures 43 to 47.

The algal cell density in different experiments ranged from 34 to 100 mg/L, the luminous energy varied from 1600 to 1900 foot candles (at the face of the reactor), the pH was held in the range from 8.1 to 8.3, and the temperature was held constant throughout the study at 25°C. Unfortunately,

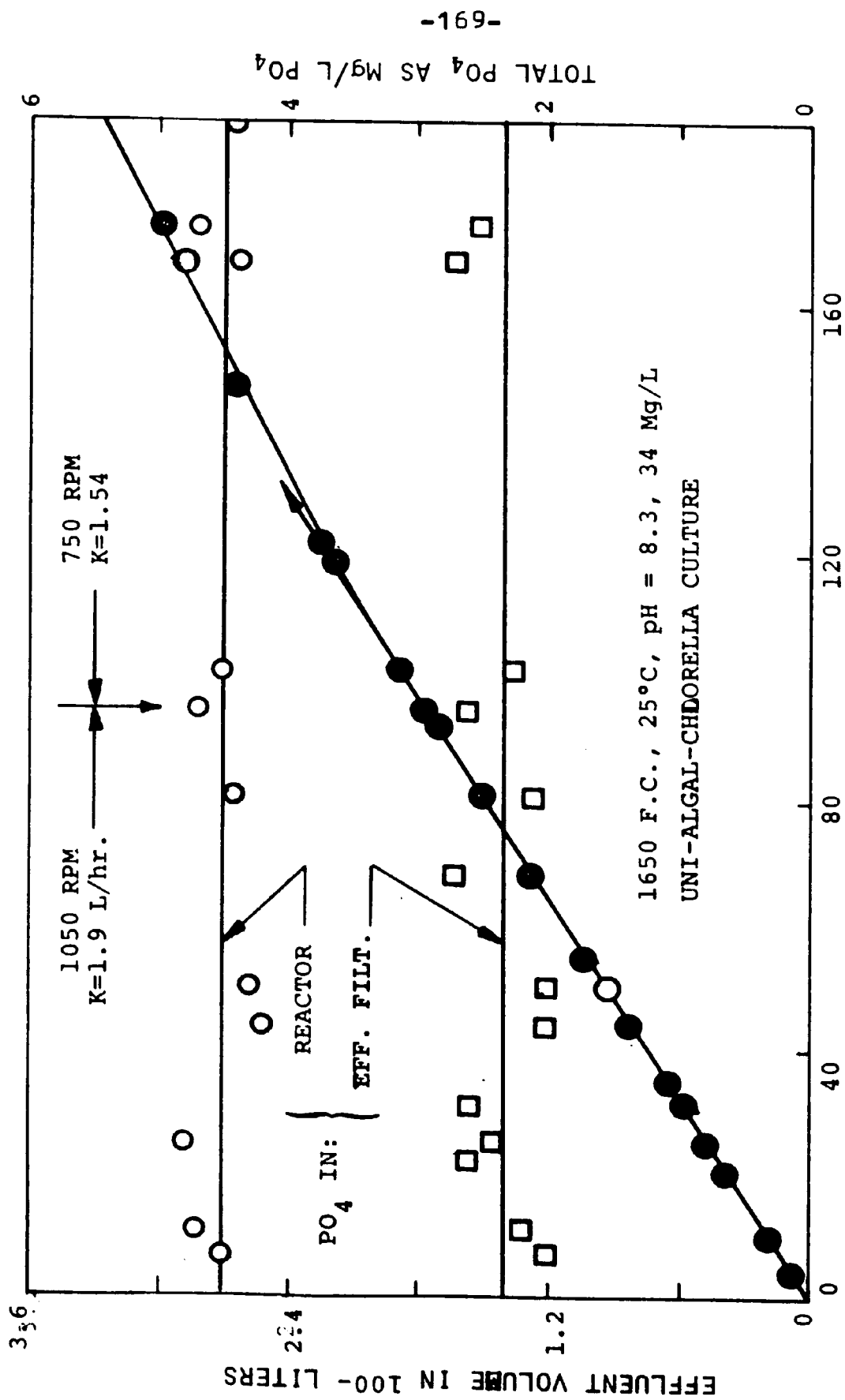


Figure 43. Effect of Mixing Speed on the Growth Rate of Chlorella Grown in Synthetic Substrate

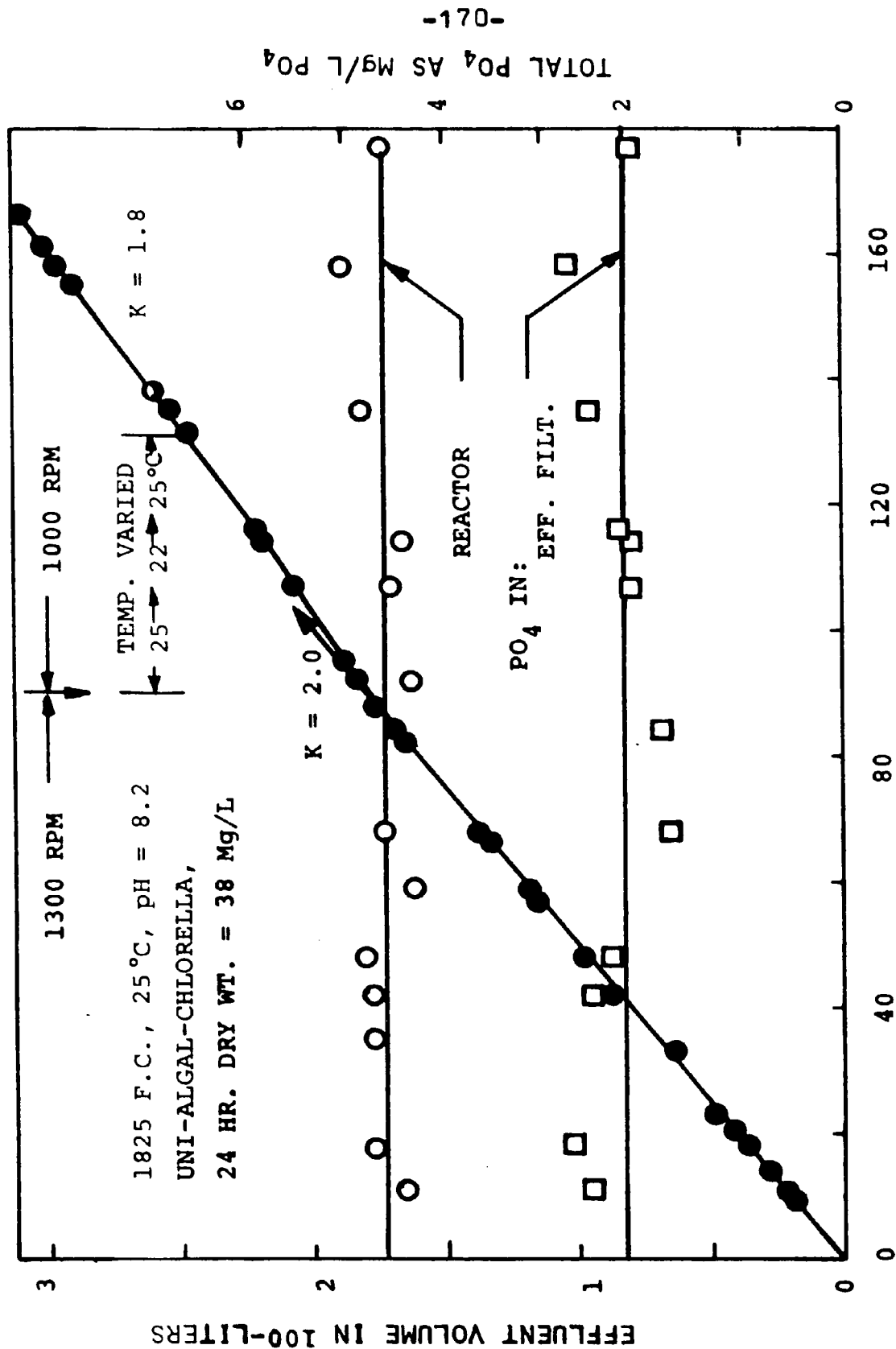


Figure 44. Growth Rate of Chlorella Grown in Synthetic Substrate as a Function of Mixing Speed

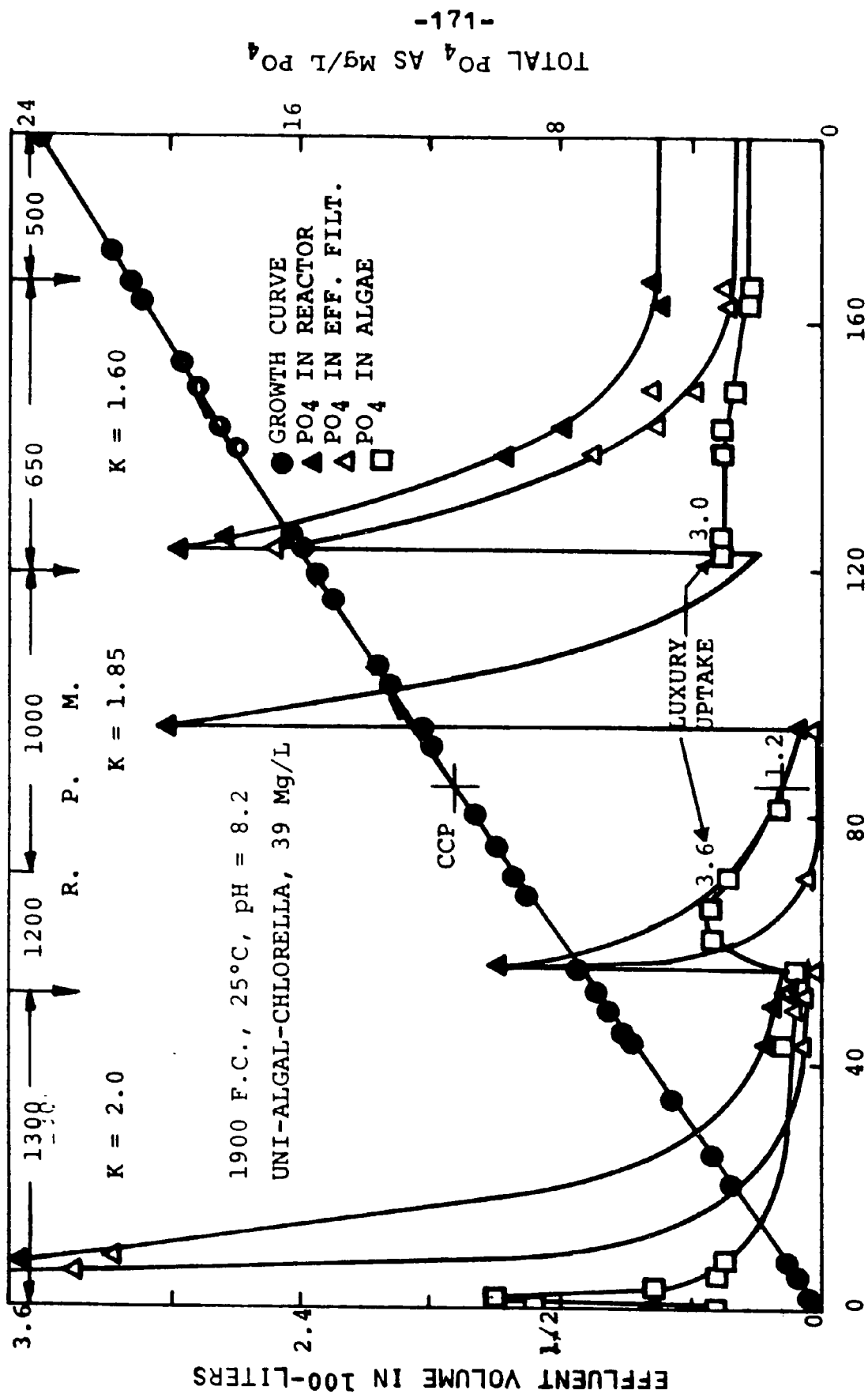


Figure 45. Dependence of the Growth Rate of Algae Grown in River Water Feed on the Speed of Mixing

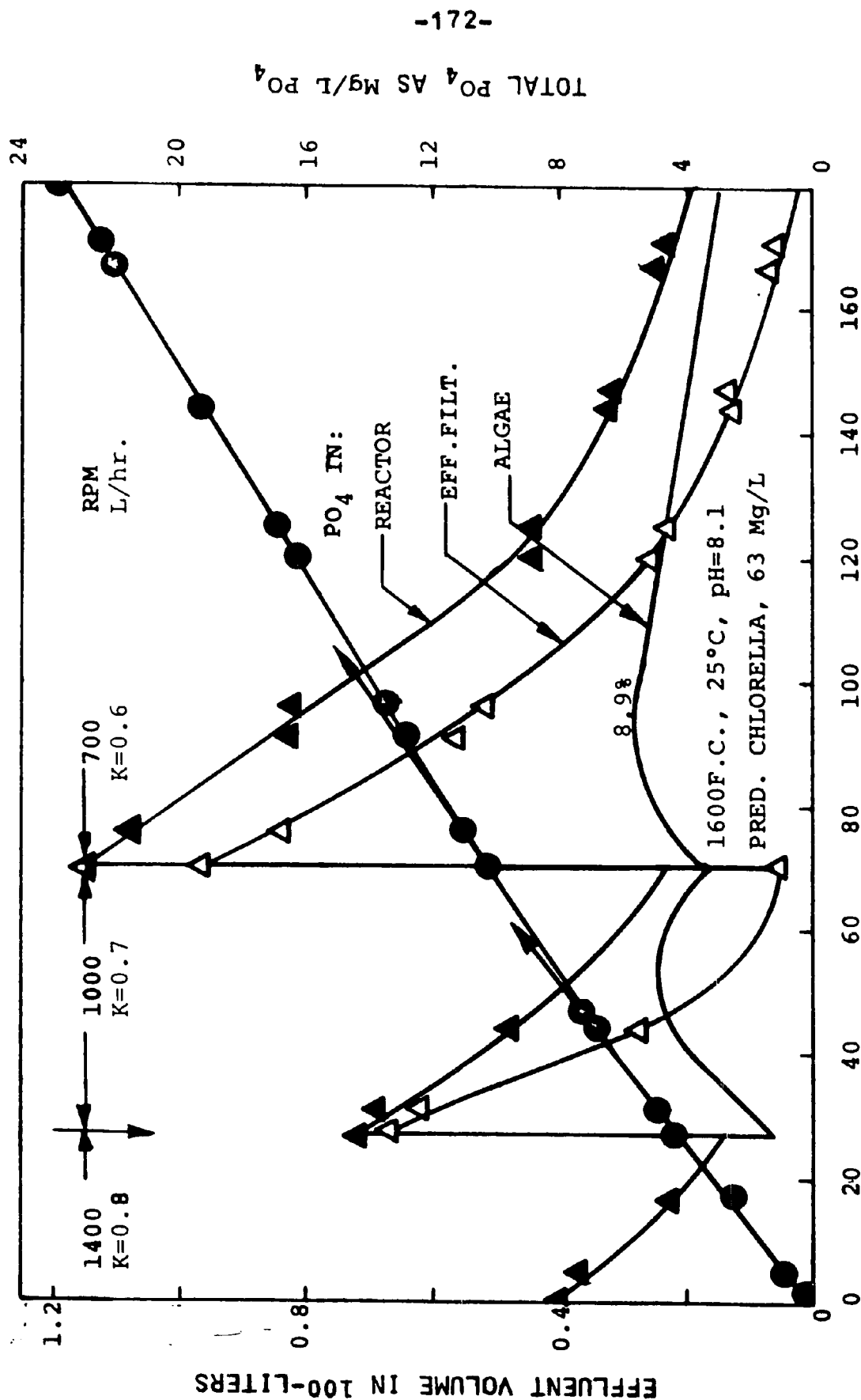


Figure 46. Mixing Effects on the Growth Rate of Predominantly Chlorella Culture Grown in Synthetic Substrate

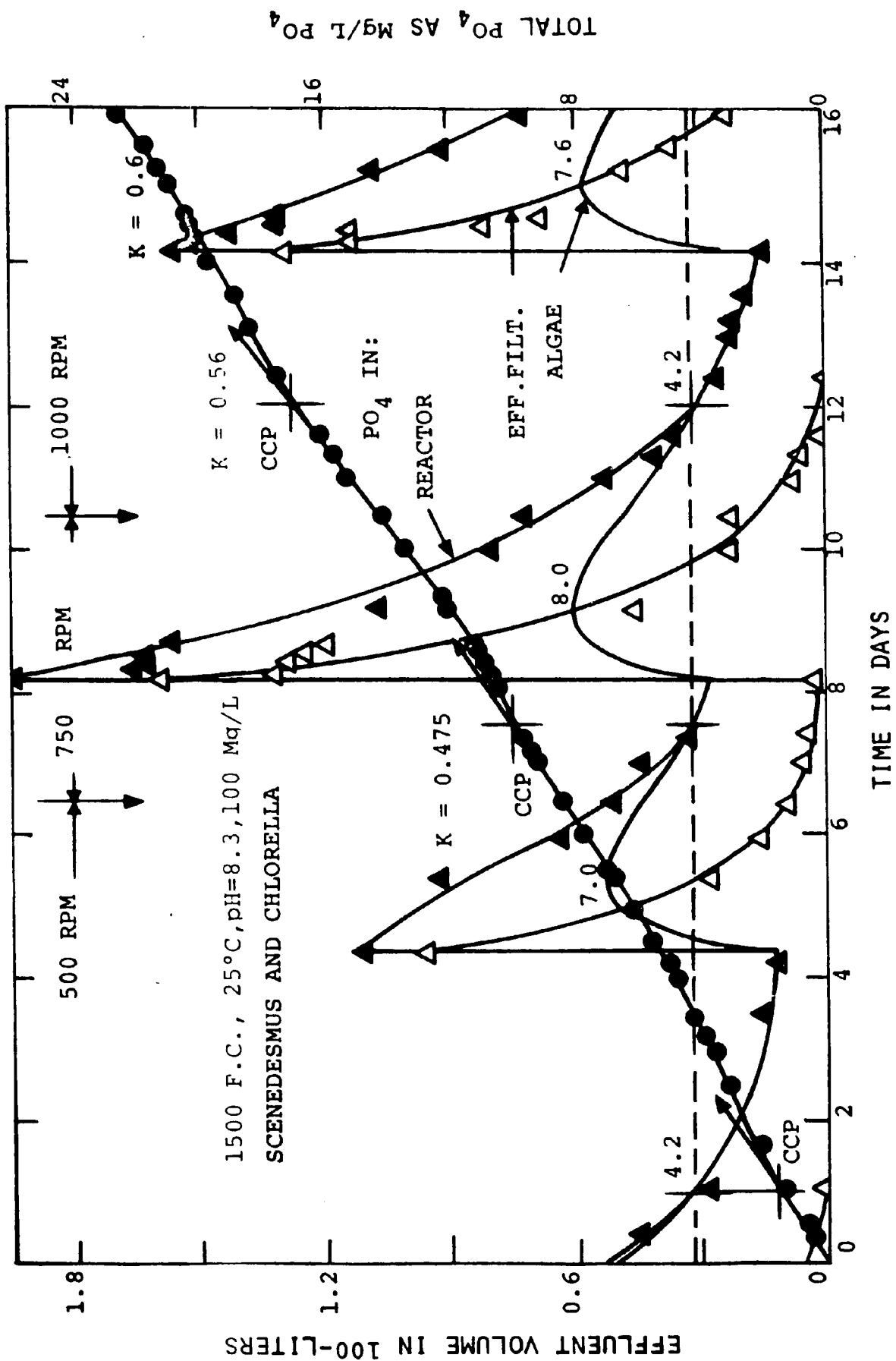


Figure 47. Mixing Effects on the Growth Rate of Green Algae

the revolutions per minute (RPM) could not be increased beyond 1400 without interfering with the operation of the turbidostat due to air entrainment in the culture medium. Reduction of RPMs below 500 could not keep all the cells in suspension at all times and therefore, provided unreliable results. Hence, the range of 500 to 1400 RPM constituted the logical limits of the turbidostat apparatus without further modifications in the design of the impeller and/or the photo-electric sensing system. Any changes of this type were not considered desirable from the standpoint of being able to correlate the mixing data with the rest of the study already conducted.

Figures 43 and 44 show the results obtained under a constant supply of phosphorus to the uni-algal-*Chlorella* cultures. A break in the growth rate curves but not in the ' PO_4 ' in effluent filtrate' curves is seen at the point of change of RPM. The phosphorus content in the substrate during the experiments reported in figures 45 to 47 was intentionally varied from a large excess to that concentration which 'might prove growth limiting' under each RPM condition.

As usual, the maximum steady state growth rate value corresponding each RPM value studied, the CCP point, and the luxury uptake amounts are indicated on these figures. The magnitudes of CCP in figures 45 and 47 appear to be independent of the corresponding RPM values and are consistent with what has been reported earlier.

The luxury uptake of phosphorus apparently decreased from 3.6 to 3.0 mg/L, i.e. from 9.25% to 7.7% by dry weight

of algae, when the revolutions per minute were reduced from 1200 to 650 in figure 45 even when there was a favorable phosphorus concentration in the reactor during the 650 RPMs. This phenomenon does not seem to exist however, in figure 46. Actually, the luxury uptake appears to have better correlation with the phosphorus concentration in the reactor than with the RPMs in figure 47. Hence, a definite conclusion is not possible from this data as far as the luxury uptake of phosphorus is concerned.

A summary of the effect of the rate of mixing on the growth rate of algae is presented in figure 48. The data points have been taken from figures 43 to 47. In spite of the several variables and the use of normalization process, 'a line of best fit' through the points, calculated from different experiments, clearly shows the trends. The value $0.05 \text{ L/hr/100 RPM}$ however, is very system specific and is not recommended for extrapolations to other systems.

Furthermore, the expression of the rate of mixing in revolutions per minute of a specific impeller held at a particular point in the reactor in itself leaves much to be desired. It is apparent to this author that more rigorous studies on the mixing aspects than reported here should prove useful since the trends are well established by this study. Also, some other measure of mixing than RPM should be established so that the information gained will serve a much more universal purpose.

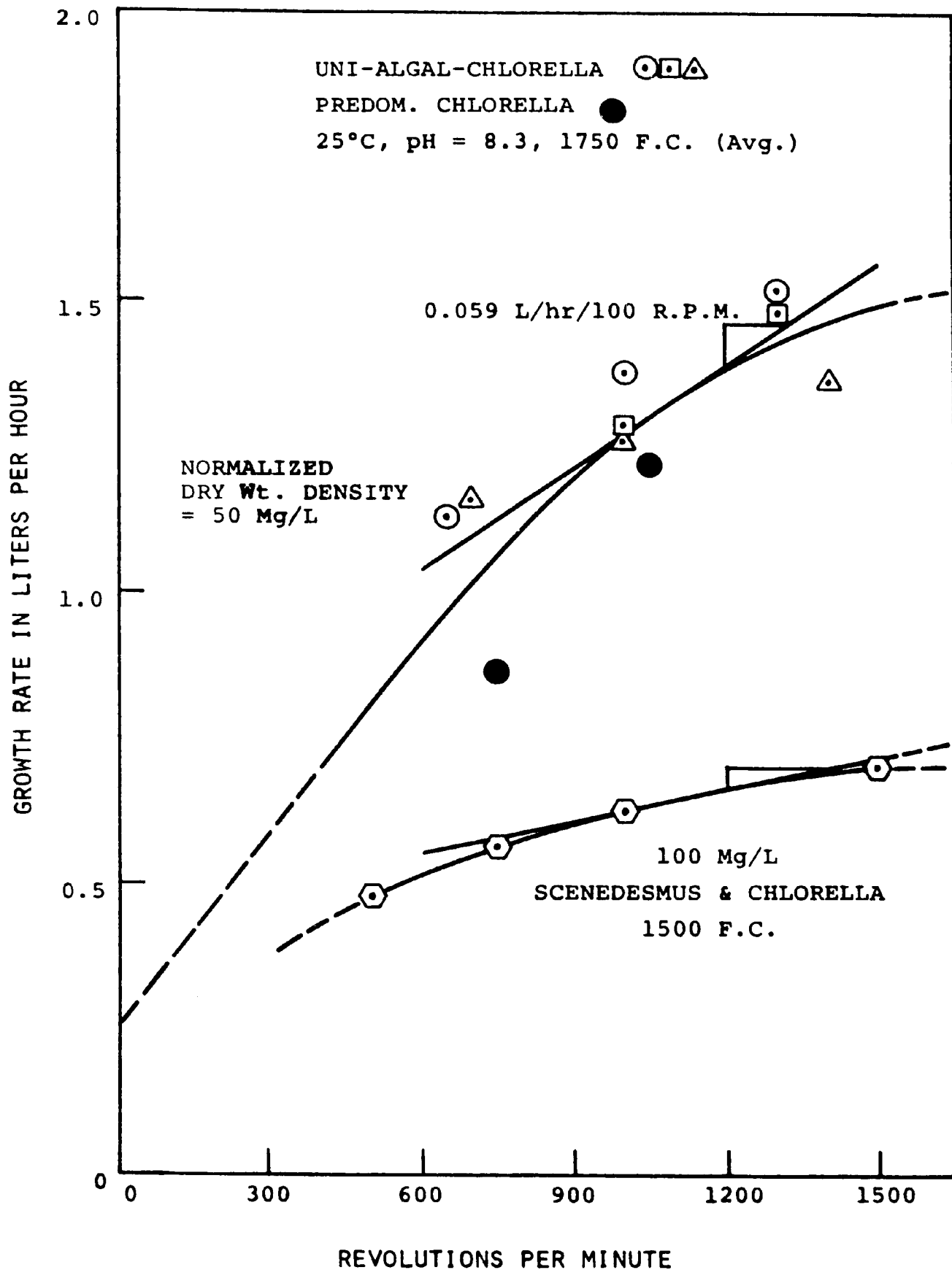


Figure 48. Summary of the Effect of Mixing on the Growth Rate of Green Algae

CHAPTER IX

PHOSPHATE UPTAKE BY P-STARVED ALGAE

A. MECHANISMS OF METABOLIC REGULATION

Some understanding of the metabolic regulation of microorganisms is necessary for proper appreciation of the results to be presented in this chapter. Very briefly, microorganisms turn the synthesis of their enzymes catalyzing any metabolic sequence on or off, depending upon the demands for that specific sequence. REPRESSION and FEEDBACK INHIBITION decrease, while INDUCTION increases the amount of one or more specific proteins (enzymes) as the result of exposing the cell to some regulatory substance. In general, repression by end products (or structurally related compounds) is the rule for biosynthetic sequences whereas induction of enzymes is frequently observed in catabolic pathways involving carbon and energy sources (40). The inducibility or repressibility of enzymes is controlled genetically. Further discussion on these important concepts can be found in any advanced textbook on biological chemistry or microbial metabolism.

Once the general regulatory mechanisms are understood, it is easy to visualize how the amounts of some of the microbial enzymes can be manipulated by changing the nutrient composition of the substrate. When there is an excess of phosphorus in the growth medium, the microorganisms will pick up some maximum amount depending upon the environmental conditions and dictated by their biochemical needs, plus some

extra for storage (Luxury Uptake), but no more. An additional supply of phosphorus, under these conditions, would not raise the internal phosphorus concentration of these already phosphorus-satisfied cells.

Why don't these cells pick up more phosphorus? Inorganic phosphorus accumulation in the cells represses the biosynthesis of enzymes responsible for the phosphorus uptake reactions. The higher the internal content of phosphorus is, the greater the repression of these enzymes will be. As a consequence of excess (above the metabolic needs) phosphorus in the cell, the level of the specific enzymes drops so low that no significant uptake of phosphorus is possible from the medium. When some of the internal inorganic phosphorus is combined with organic molecules and is used up in metabolic reactions, the level of this phosphorus in the cell drops. This leads to a reduction in repression of the enzymes responsible for more phosphorus uptake from the medium and consequently, an additional quantity of phosphorus is extracted from the substrate.

Now, assume the question is asked, Can the level of the specific enzymes, responsible for the phosphorus uptake process, in the cells be raised to abnormally high levels by reducing the internal phosphorus content of the cells below their normal metabolic requirements for this element? If the answer to this question is yes, then these cells must develop a tendency to pick up abnormally high (more than the luxury amount) quantities of phosphorus from their growth medium and within limits, independently of the prevalent environmental conditions. This, in fact, is verified by the results to be

reported below.

B. DEFINITION OF P-STARVATION

Reducing the internal phosphorus content of the cells, below their normal metabolic requirements for this element, is referred to as "phosphate starvation". This term is encountered quite frequently in references to nutritional studies on many organisms, including bacteria, yeast, and algae. The relative meaning of this term and mechanism of producing phosphate starvation would appear to be extremely important in the nutrient-cell interaction.

When the unicellular organisms multiply, they share their internal phosphate resources with their offspring. A continuous culturing technique, such as the turbidostat used in this study, when operated with a substrate containing no phosphate, will deplete the culture of its stored phosphate through dilution. This phenomenon is called washout (see figure 5) and the time to produce a given chemical concentration in the culture can be checked experimentally and predicted mathematically.

Below the critical phosphate level, which appears to be quite well defined, the growth rate declines in direct proportion to the phosphate present in the cell (see figure 41). The culture which contains half the critical level of phosphate, grows at half the maximum rate. Strictly speaking, any phosphate concentration less than the critical level, is indicative of phosphate starvation.

C. MEASUREMENTS FOR DEGREE OF P-STARVATION

As the cells continuously reduce their phosphate level below the critical, they become progressively more and more P-starved. Hence, the degree of phosphate starvation of an algal culture of given density, may be indicated by the ratio of the actual phosphate concentration in the P-starved cells (P_S) and the critical concentration of phosphate (P_C), i.e. P_S/P_C . Since the value of CCP for a relatively constant temperature, light intensity, and pH has been found to depend only on the biomass; it can be expressed as per cent by dry weight of the cells. Furthermore, CCP can be treated as an experimental constant as long as the environmental factors do not vary greatly. A value of 0.03 would appear reasonable for this constant from what has been reported earlier. Then, P_C would be numerically equal to the product of this factor and the dry weight density of the algal culture. In equation form, this can be expressed as follows:

$$P_C = \text{Experimental Cell Density, mg/l} \times 0.03 \dots\dots\dots 11$$

If the algal cell dry weight density and the total phosphorus concentration of a P-starved culture (P_S) is determined experimentally, the ratio P_S/P_C can easily be calculated. A value of 1.0 for this ratio ($P_S = P_C$) would mean NO starvation whereas zero would indicate the extreme when $P_S=0$, or a case of absolute depletion of phosphorus from the cells. Within these two extremes, a decreasing value of P_S/P_C would mean an

increasing degree of phosphate starvation.

Qualitatively, the degree of phosphate starvation of green algae can be observed visually by comparing the colors of the P-rich and P-starved algal cultures, if other factors are known to be adequate for growth. During this study, the visual observation of the color of algae proved useful and complemented the P_s/P_c ratio technique for a direct determination of the degree of P-starvation. Although this author did not follow the algae color spectrophotometrically, such a technique might prove quantitative as far as discerning the degree of starvation, due to any single nutrient producing chlorosis, is concerned.

A third method of determining the degree of P-starvation might be the enzymatic analysis approach. As discussed above, the phosphate starved algae should synthesize a greater amount of alkaline phosphatase than the enzyme synthesized by the P-rich algae. Fitzgerald (127) reported that there was a 20-to 40 - fold difference in the alkaline phosphatase content of algae, depending upon whether the supply of phosphorus in the medium was limiting or in excess. Since alkaline phosphatase can extract PO_4 from a wide variety of compounds, including the condensed and the organic phosphates, it is reasonable to expect that this enzyme will combine with the PO_4 group of the p-nitrophenylphosphate molecule and liberate the p-nitrophenyl anion in solution. This anion produces yellow to pink color in the solution but the molecule p-nitrophenyl- PO_4 is colorless.

Fitzgerald (127) suggested that an algal suspension to

which some p-nitrophenyl- PO_4 or the commercial, alkaline phosphatase pill, has been added, may be incubated at 37°C and pH 9 for about 30 minutes. Accordingly, if the solution contained a yellow to pink color at the termination of this test, an evidence for the presence of alkaline phosphatase would have been obtained. It would follow that phosphorus might be limiting for algal growth. It may be cautioned here that under alkaline conditions, there is bound to be some hydrolysis of PO_4 from the p-nitrophenyl- PO_4 molecule and consequently, further refinement of this test should be considered.

A pigment content analysis of the P-starved cells would represent a fourth method of determining the degree of P-starvation. During the progression of phosphorus or nitrogen starvation (or starvation with respect to almost any nutrient that produces chlorosis) of green algae, the relative chlorophyll content decreases while the yellow pigments (carotenoids) increase. Either the follow up of the carotenoid content or of the chlorophyll content of the cells with respect to the supply of phosphorus to the culture medium may lead to quantitative relationships that can be used in defining the degree of P-starvation of cells.

D. RESULTS AND DISCUSSION

Once the degree of P-starvation (and therefore, the level of enzymes responsible for the removal of phosphate from the medium) of given algal cells is defined, an estimate of the potential of these microorganisms for removing phosphorus

from a P-rich waste water should follow. The major emphasis of this research was to correlate the degree of P-starvation of green algae with the maximum amount of phosphorus uptake by these cells, when provided with a sudden slug of phosphates.

Figures 49 to 55 show the response of P-starved algae to the shock loading of a culture with phosphates at time zero. The environmental conditions and the degree of P-starvation (P_s/P_c) are indicated on these figures. Also, note the two important phosphate concentration values written on each. The first of these two numbers, represents the actual concentration of phosphates in the P-starved algal cells (P_s) just before the slug of phosphorus was added. The second number refers to the maximum amount of phosphorus removed by the constant density algal culture from the solution phase in the reactor.

As indicated earlier, total phosphorus determinations were carried out on the reactor contents and on the effluent from which algal cells were removed by using a 1.2 μ pore size high grade filter paper. A 'Phosphate in algae' curve was then generated by taking the difference between the ' PO_4 in reactor' and the ' PO_4 in effluent filtrate' curves.

The fourth curve appearing on each figure shows the characteristic growth of the constant density culture. In these figures, the initial growth rates vary from almost zero to values much lower than the maximum steady state growth rates. The maximum growth is obtained only after the culture has absorbed its maximum amount of phosphorus from the medium. When the maximum steady state growth rate is extrapolated

back to intersect the abscissa, the value for the 'LAG PERIOD' results. The longer it takes to resume maximum growth of the culture, the more 'inactive' the culture must have been at the beginning of each experiment. The lag period determined in this manner is correlated below with the degree of P-starvation and with the maximum phosphorus uptake by the P-starved culture.

1. P-REMOVAL POTENTIAL OF P-STARVED ALGAE

During the experiments reported in figures 49 and 50, the algae had absorbed the maximum amount of phosphorus at about 9 hours. Note that 70% of this maximum amount was extracted from the medium during the first three hours and it took 6 additional hours to pick up the remaining 30%. In the case of figure 49, 38 milligrams of well starved algae ($P_s/P_c = 0.30$) were exposed suddenly to 25 mg of phosphate. The maximum amount of P-uptake by the culture was 8 mg which represents ($8/38 =$) 21% dry weight of algae. A similar response of the P-starved *Scenedesmus* is indicated in figure 50 where 36 mg of algae extracted 7.2 mg PO_4 which is 20% by weight. The lag period in figure 49 is seen to be 9 hours, whereas it is about 12 hours in the case of figure 50. Apparently, the higher the degree of P-starvation, the greater the value of the lag period to be expected.

It was not the aim of this research to determine the metabolic mechanisms by which the cells are able to absorb such a high quantity of phosphate, but it was ascertained that no

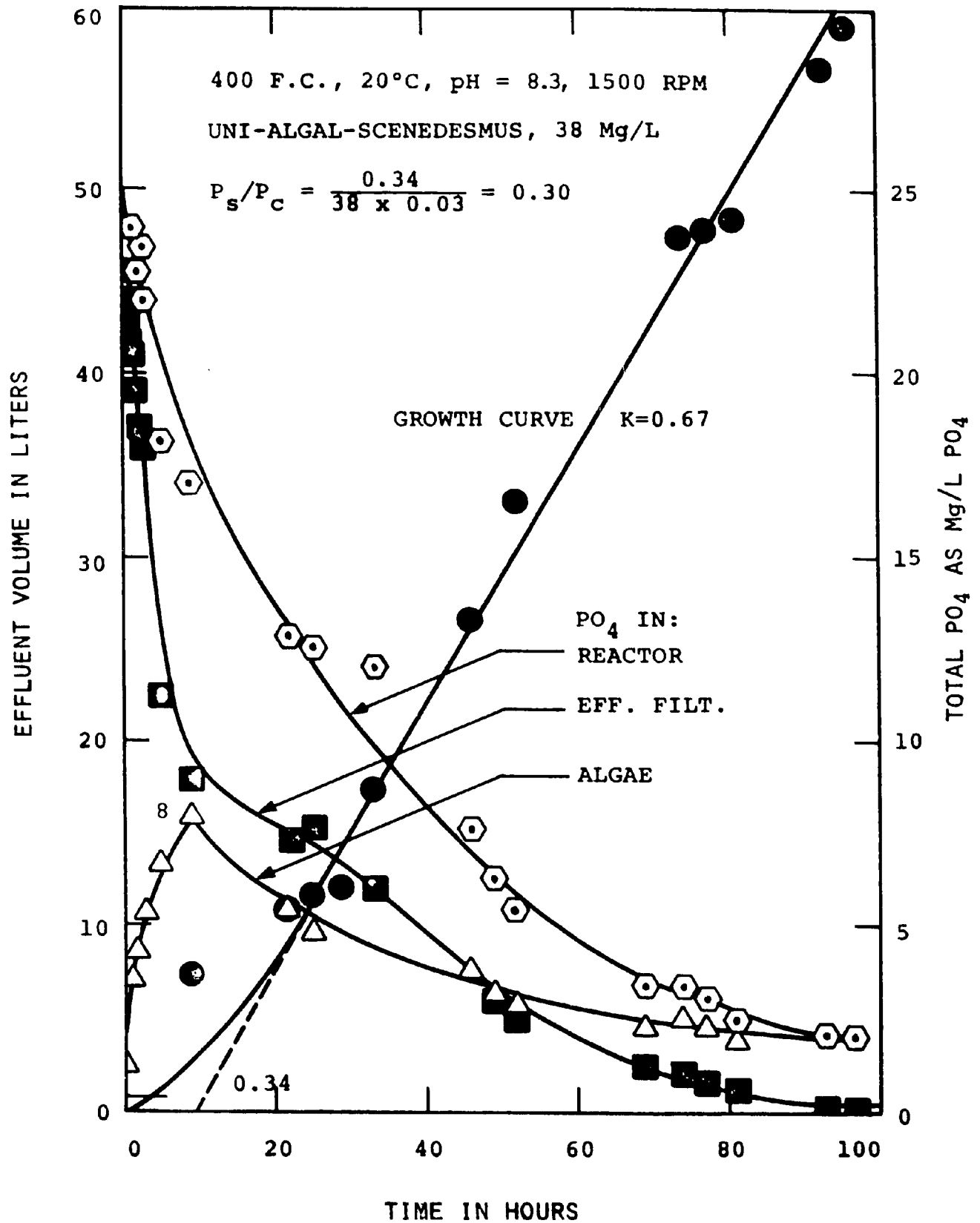


Figure 49. High PO₄ Uptake by P-Starved Algae at 400 F.C. and 20°C

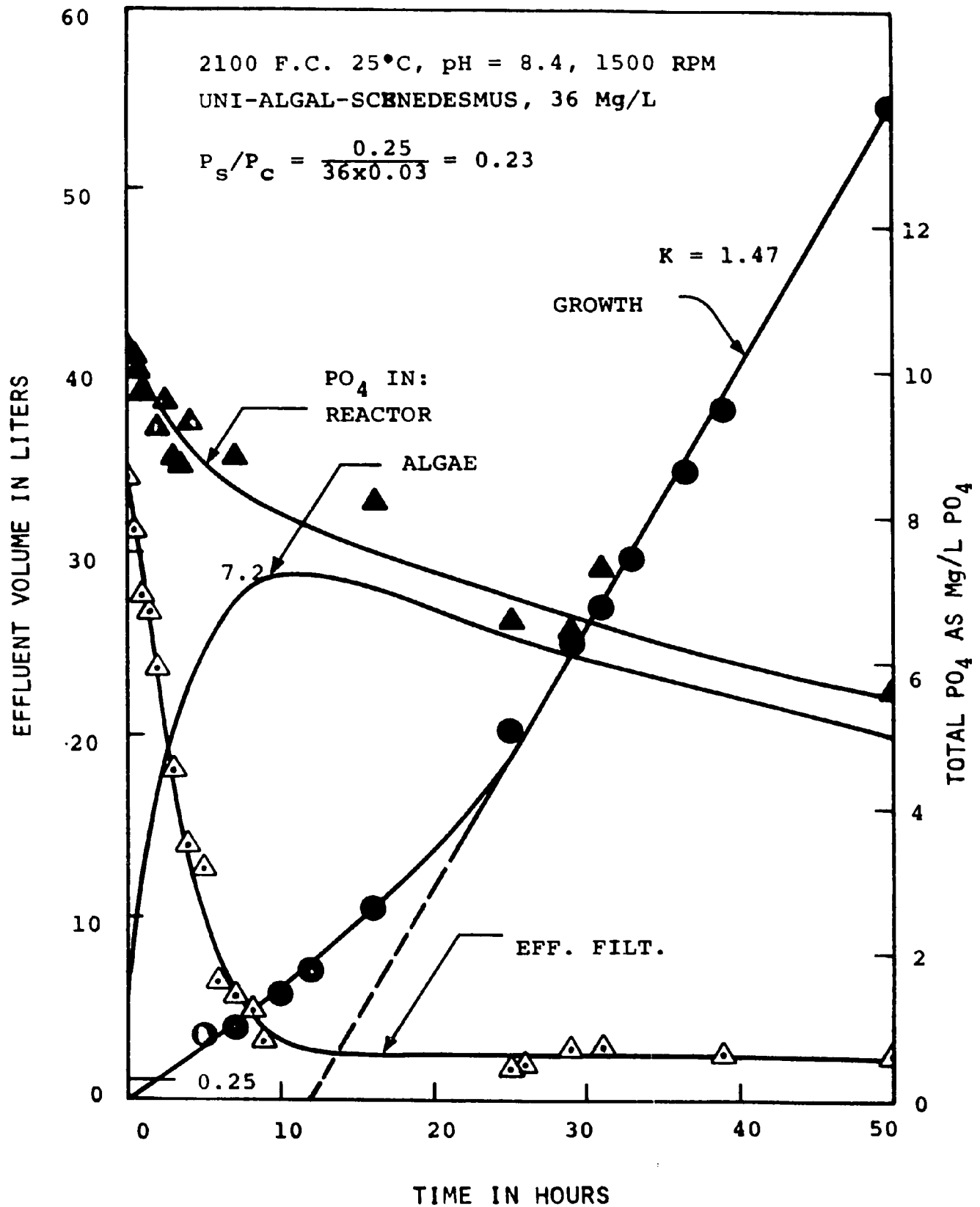


Figure 50. High PO₄ Uptake by P-Starved Algae at 2100 F.C. and 25°C

subsequent leakage from the cells of the absorbed phosphates occurred. One may ask a series of questions such as: In what form are these high quantities of phosphates stored? Does this phosphate actually get incorporated into the cells so rapidly? How can the cell have so much internal space for the storage of phosphate which in this case amounts to 1/5th of its dry weight? From what source did the P-starved cells get the energy to store such high levels of phosphorus? All of these questions and many more, are both interesting and practical. A rigorous biochemically oriented study would appear capable of answering some, if not most, of these questions. A few speculations along these lines may be possible but no exact answers to the foregoing questions should be expected from the results reported here.

From an engineering standpoint, it is significant to know that a P-starved algal culture can extract more than twice as much phosphate as is possible by P-satisfied algae under the most favorable conditions (LUP). If the algae were not P-starved at time zero in figures 49 and 50, the maximum possible P-uptake could not have been more than 8 to 10% by weight of the cells, compared with the actual uptake of 21% by weight in figure 49. Furthermore, it is apparent that the phosphorus uptake process of P-starved algae is not very sensitive to some of the environmental conditions. Between the experiments reported in figures 49 and 50, the temperature varied from 20 to 25°C and the light intensity from 400 F.C. to 2100 F.C. (at face), but no effect of this variation on the rate or the maximum amount of P-uptake was observed.

2. INSTANTANEOUS UPTAKE PHENOMENON

Another phenomenon is indicated by the results illustrated in figures 51, 52, and 53. Instead of the comparatively slow rate of P-uptake by the P-starved algae during the experiments reported in figures 49 and 50, the maximum amount of phosphorus uptake by the algae in these experiments took place almost instantaneously. The results show that within the time of processing the first sample (time zero on the figures), essentially full capacity of the cells for phosphorus extraction was attained.

As can be seen from figure 51, it took the 13.5 mg of algae only one hour to pick up 2.8 mg of phosphate from the medium which is equivalent to 20.7% by dry weight of the cells. This result and the one reported in figure 25 (8 mg/l Algae) were the only two experiments in which the ' PO_4 in algae' curve showed a steeper slope, after the maximum absorption point, than the corresponding slope of the ' PO_4 in effluent filtrate' curve. If the phosphate in algae declines more rapidly than the phosphate in the effluent filtrate does, an interpretation of phosphate release from the cells is possible. Whether this phenomenon is entirely due to the diluteness of these cultures remains to be established.

It is apparent from figures 52 that the dramatic change in the kinetics of P-uptake by the P-starved algae cannot be accounted for by the degree of phosphate starvation alone. The value of this parameter changed from 0.25 in figure 51 to 0.85 (slightly starved) in figure 52, but the maximum uptake point

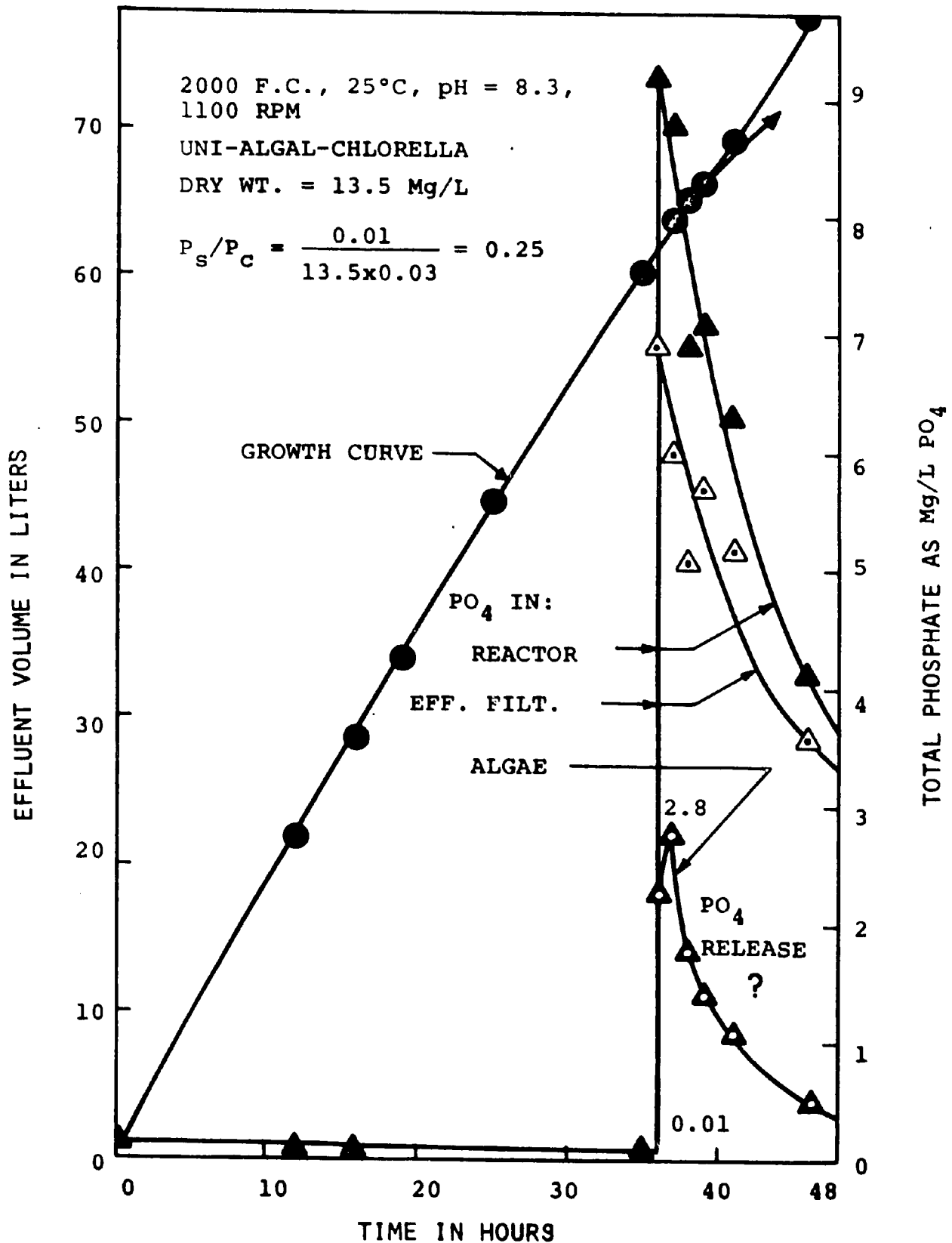


Figure 51. Instantaneous PO₄ Uptake in High Light by P-Starved Uni-Algal-Chlorella Culture at Low Cell Density

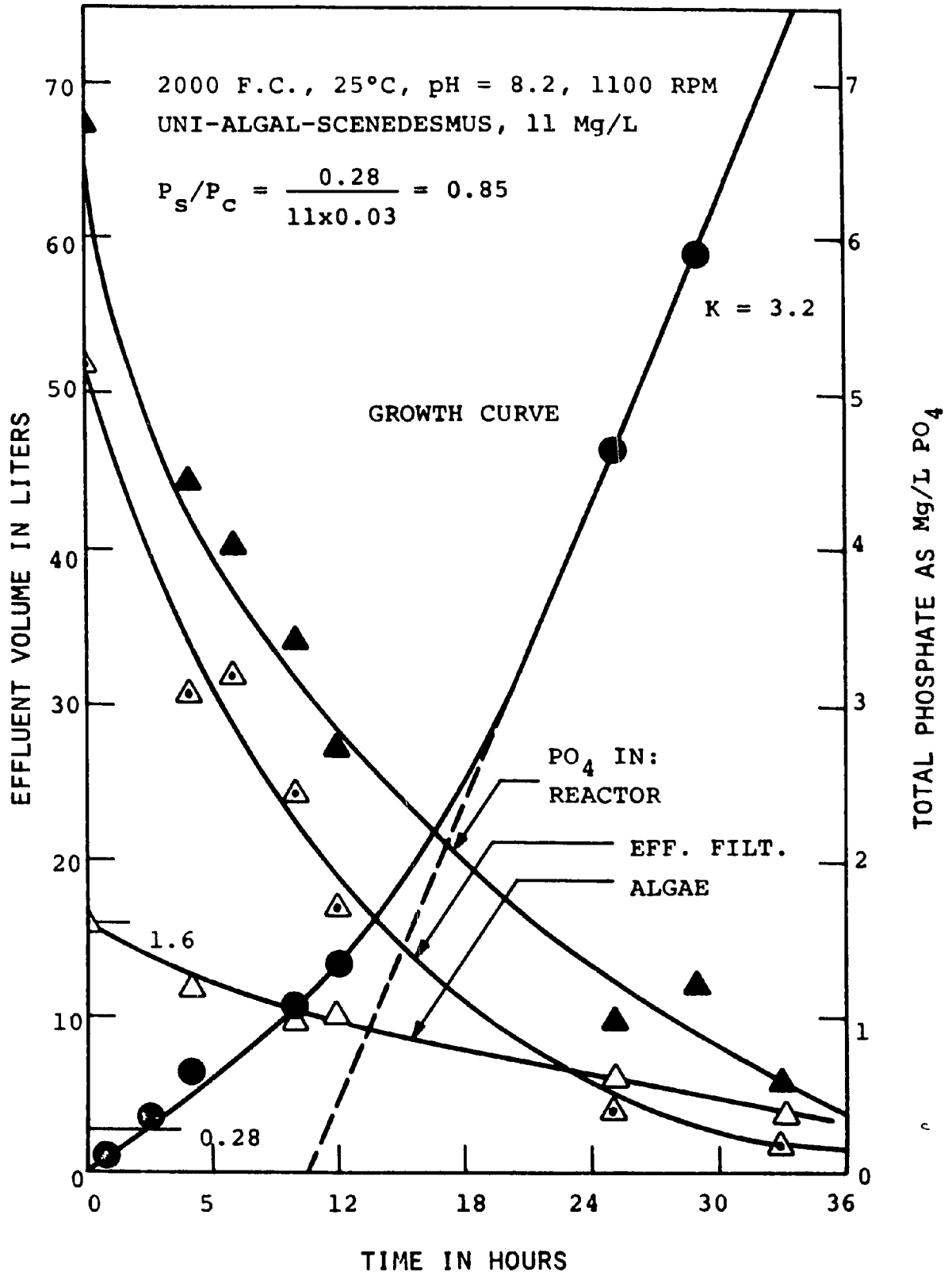


Figure 52. More Than Luxury Uptake of PO₄ by Algae at Low Cell Density

in the latter case actually moved toward zero time. It was postulated earlier that the amount of P-uptake should depend upon the degree of P-starvation. In the experiment illustrated in figure 52, P_s/P_c was = 0.85 and the maximum phosphate uptake was only $(\frac{1.6}{11} =)$ 14.5% by dry weight of the cells instead of the 21% value reported in figure 49 which supports such a hypothesis.

3. CONCEPT OF OPTIMUM STARVATION

A high degree of P-starvation can produce more active algae for the uptake of phosphorus only to a point. If the P-starvation is too advanced, the cells may suffer some irreversible losses and therefore, become less inactive in the maximum uptake of phosphorus from their growth environment. This point is illustrated in figure 53 where the P_s/P_c is equal to 0.10 - the highest degree of P-starvation produced in algae during this study. Although instantaneously, 58 mg of algae picked up only about 10 mg phosphate which represents 17.4% phosphate by dry weight of cells. Furthermore, even when the maximum amount of phosphorus was absorbed by the culture at time zero, the lag period is seen to be 16 hours - the maximum observed in the continuous culture study.

It would appear from this evidence that the algal cells had been 'overly' starved in this case and there is an optimum point of P-starvation from the standpoint of maximum extraction of phosphate employing algal cultures. Starvation beyond this point is unnecessary and wasteful.

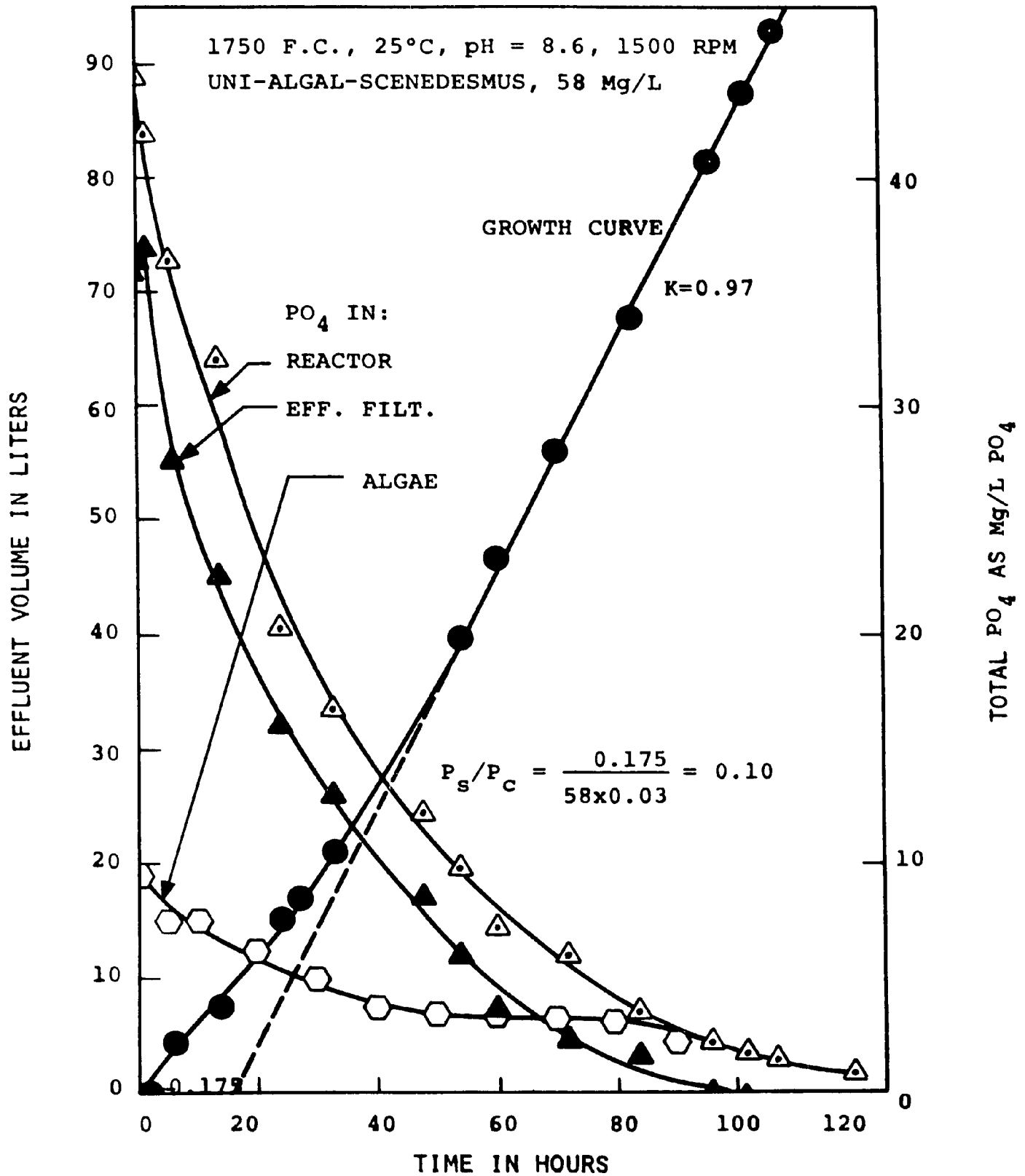


Figure 53. Instantaneous PO₄ Uptake by 'Overly' P-Starved Algae When Shocked with a Relatively High Initial PO₄ Dose

4. MAXIMUM P-UP TAKE IN DARK

Since the variation in light intensity from 400 to 2100 F.C. (figures 49 and 50) did not affect the rate or the amount of phosphorus uptake by P-starved algae, special experiments were designed to discern if the P-uptake process of P-starved algae depended on light at all. The results obtained during two such experiments are presented in figures 54, 55, and 56.

It is apparent from the ' PO_4 in algae' curve of figure 54 that the absence or presence of light neither affects the rate nor the maximum amount of phosphorus uptake by the P-starved *Scenedesmus*. Complete darkness was maintained in the culture for the first 6 hours during which the cells picked up about 87% of their maximum amount of phosphate. When the lights were turned on at 6 hours, no break or discontinuity in the P-uptake (PO_4 in algae curve) process was observed.

The maximum potential of the P-starved algae for P-uptake was attained at about 10 hours. It is clear that 52 mg of algae extracted 11 mg of phosphate which is 21% by dry weight of cells - the exact percentage observed under continuous lighting conditions during the experiment illustrated in figure 49. The kinetics as well as the equilibrium values of phosphorus uptake are very similar in figures 49, 50, and 54 inspite of the drastic differences in the light intensities available to the culture, varying from zero during darkness to a continuous value of 2100 F.C. in figure 50.

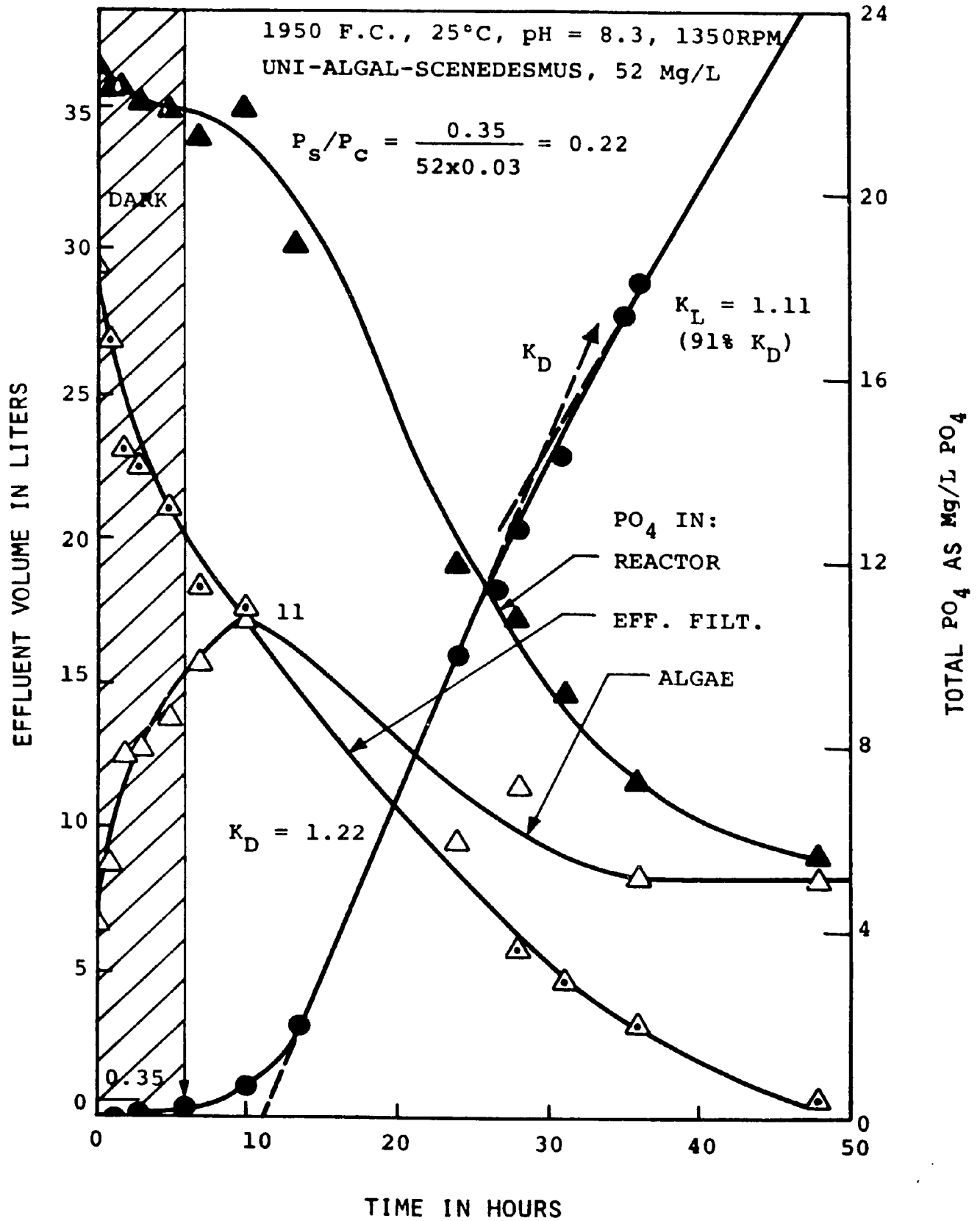


Figure 54. Regular High PO₄ Uptake by P-Starved Algae in Dark

5. RAPID CELL DIVISION OF P-STARVED ALGAE IN DARK

The dark period certainly affected the growth resumption pattern however, of the P-starved algae. Essentially no growth was observed during darkness in the case of figure 54 and then, upon proper illumination of the culture, the growth rate was higher than the eventual maximum steady state growth rate. This is reminiscent of the diurnal cycle effects on the growth rate of P-satisfied algae.

This phenomenon is illustrated further in figures 55 and 56. In this case, the algae was less starved with respect to the phosphates ($P_s/P_c = 0.55$) than was the case in figure 54 ($P_s/P_c = 0.22$) and as a result, some growth was observed even during the dark period. The dark grown cells again showed a higher growth rate than the cells observed under continuous lighting conditions for the same algae.

Figure 56 would support the statement that a rapid cell division of P-starved *Chlorella*, but not of *Scenedesmus*, takes place during darkness. The dramatic increase in cell numbers of P-starved *Chlorella* under zero light conditions is similar to the results reported for P-satisfied cells in the Diurnal Cycle studies. It can be seen from figure 55 that the P-starved culture had absorbed its maximum amount of phosphate within the time of processing the first sample. So, during the cell division process in the dark, *Chlorella* could have used the newly absorbed phosphate to regenerate its internal polyphosphate supply. This would be in line with the statement made earlier in this manuscript that algal cells

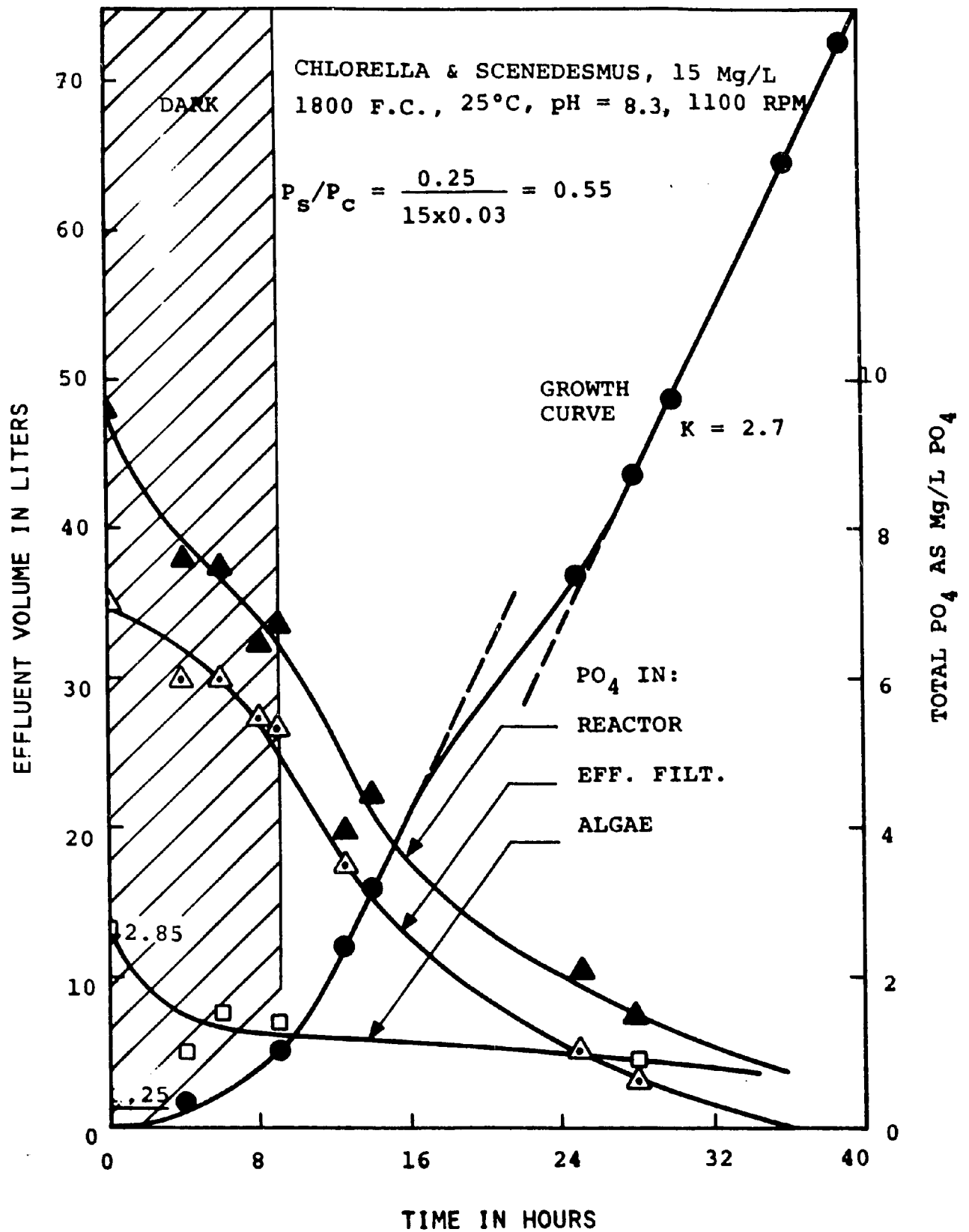


Figure 55. Instantaneous PO₄ Uptake in Dark by P-Starved Predominantly Chlorella Algae at Low Cell Density.

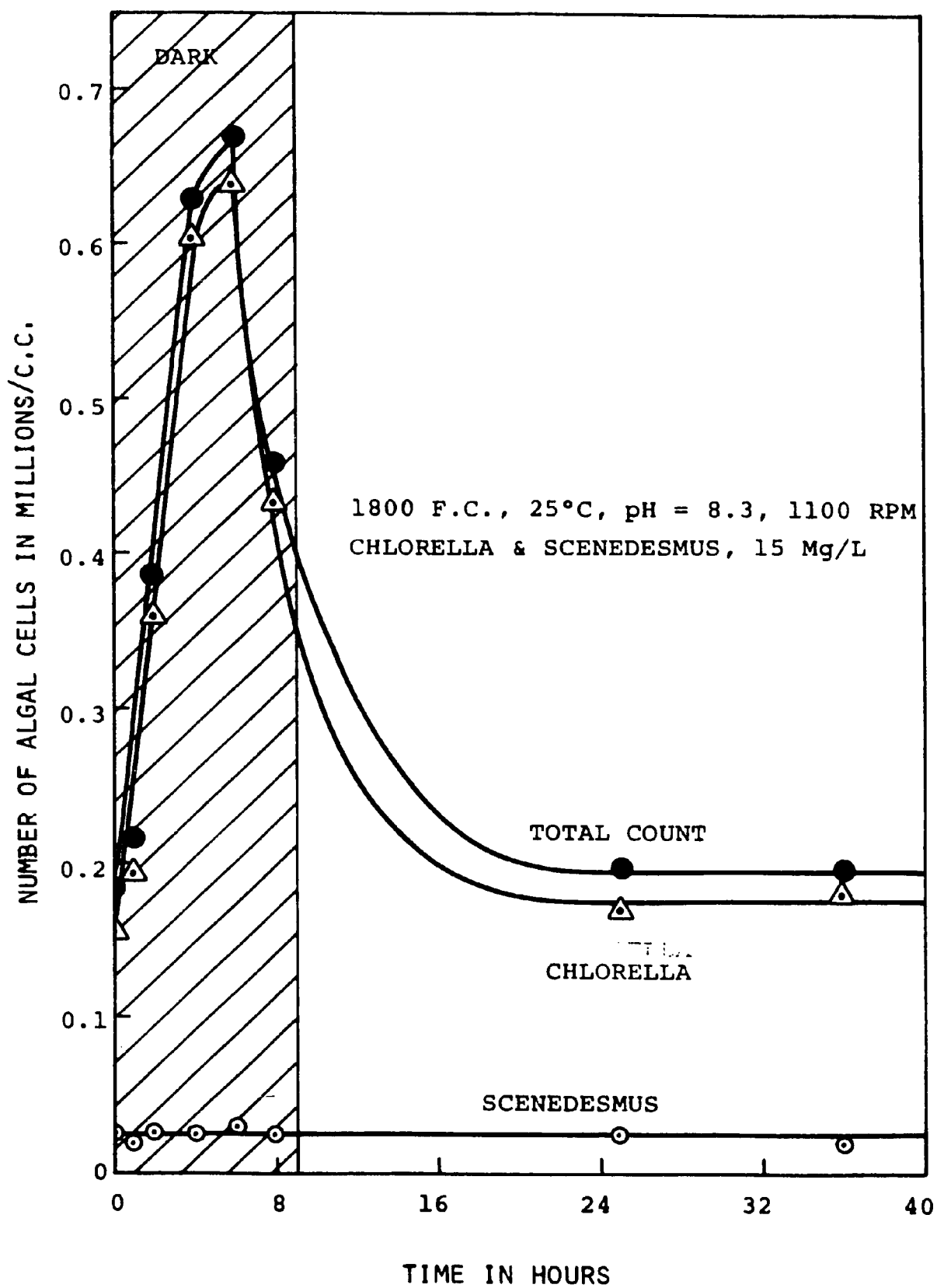


Figure 56. Rapid Cell Division of P-Starved Chlorella in Dark When Slug Dosed with PO_4

containing no polyphosphate cannot reproduce.

6. SUMMARY OF TURBIDOSTAT RESULTS

A summary of the pertinent results reported in this chapter is presented in figures 57 and 58. The maximum phosphate uptake by a P-starved algal culture is seen to be independent of the algal cell density and of the other environmental conditions, at least within the range of values indicated in figure 57. When the degree of P-starvation is fixed, the slope of the line which results from plotting dry weight density of algae versus the total PO_4 uptake, gives the per cent PO_4 uptake by dry weight of algae. Figure 57 is a plot for the approximate conditions of optimum phosphate starvation .

The variations in the degree of phosphate starvation of algae affect the maximum uptake of phosphorus by these micro-organisms in the manner illustrated in figure 58. As the ratio P_s/P_c decreases from 1.0 to 0.3, the maximum per cent PO_4 by dry weight of algae increases from 9.5 to 21.0. When the degree of phosphate starvation increases further however, a decrease in the maximum phosphorus uptake by algae is observed. Purely on this basis, the optimum starvation point (O.S.P.) may be defined as that value of the ratio P_s/P_c (0.3 in this case) which activates the algae most for the maximum uptake of phosphate from the substrate.

The interesting features of the 'lag period' curve in figure 58 should be noted. Below the point of optimum phosphate starvation ($p_s/p_c = 0.3$), the shape of the 'lag period'

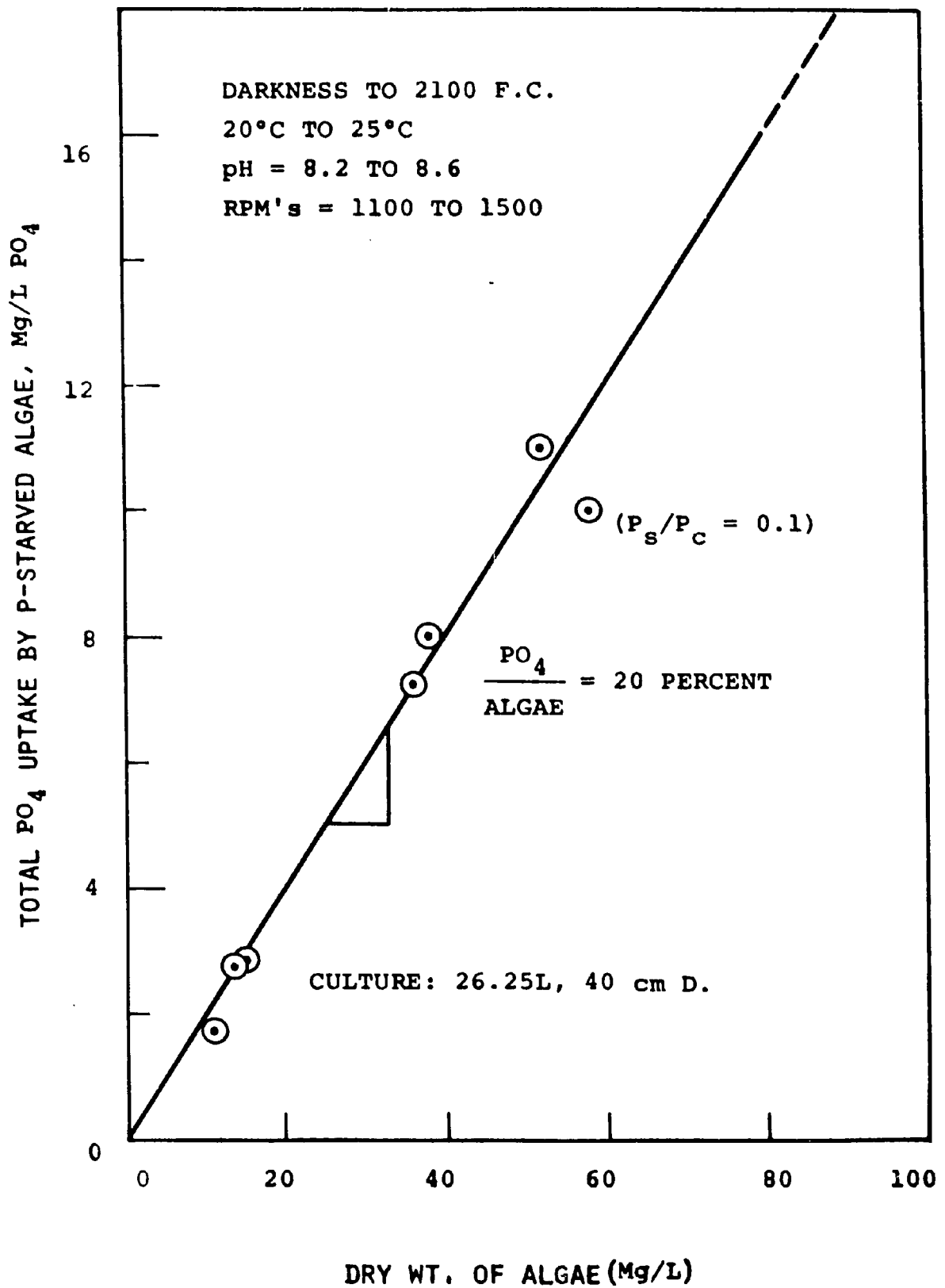


Figure 57. Characteristic High PO₄ Uptake by P-Starved *Chlorella* and *Scenedesmus*

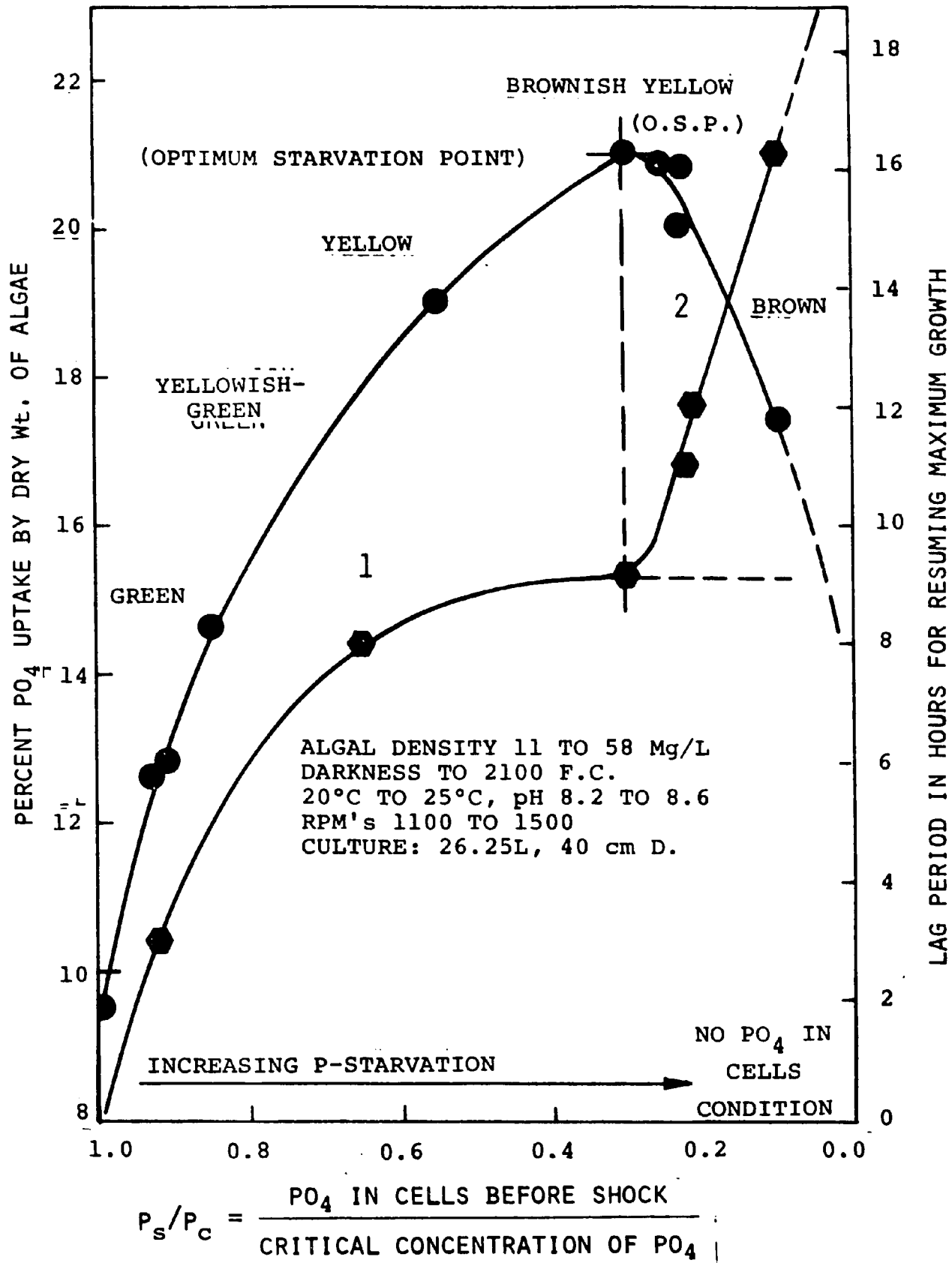


Figure 58. High PO_4 Uptake by P-Starved *Chlorella* and *Scenedesmus* as a Function of the Degree of P-Starvation Expressed as P_s/P_c

curve resembles the familiar biochemical oxygen demand curve. Above this point, the curve takes off as a straight line on a steep incline. The fact that this curve has a break exactly at O.S.P., strengthens the belief in O.S.P. and to some extent, explains the nature of the operating mechanisms.

It would appear that P-starvation up to the O.S.P., does not significantly compel the microorganisms to break down their nucleic acids for extracting phosphorus from these genetically crucial compounds. Since most of the genetic material remains intact, a supply of external phosphorus to these cells would restore their internal phosphorus compounds rather quickly and they could start reproducing without a major lag.

On the other hand, the cells which are overly starved (P_s/P_c below 0.3) could have started breaking down some of their genetic molecules and extracting the phosphorus for bare survival. It is logical to assume that the microorganisms would do this only as a last resort and definitely not, until they are forced to. In the process of this fight for the survival of the fittest, some cells would die off and still others would become biologically inactive for a long period of time. Addition of phosphorus to the medium with a dead or inactive cell would not help. Hence, the lag period does increase rather rapidly when the algae are overly starved. The same reasoning would hold when explaining the decrease of maximum phosphorus uptake by these cells.

Based on the routinely observed changes in the color of algae due to phosphorus starvation, appropriate notations

are made in figure 58. Admittedly, the visual observation of color is a qualitative measure of P-starvation of the green algae but the simplicity of this method is its greatest strength.

E. PURE CULTURE STUDIES

Some studies on P-uptake by P-starved algae were conducted using the batch culture apparatus and pure *Chlorella* cultures. The results obtained during two such experiments are illustrated in figures 59 and 60. Environmental conditions are as indicated on these figures. The ratio P_s/P_c carries the same meaning as in the continuous culture study reported above and the dry weight of algae refers to the initial value, i.e. at time zero.

In the case of figure 59, 590 mg P-starved algae extracted 132 mg phosphate from the medium within the first 6 hours which represents about 22% PO_4 by dry weight of algae. Out of the maximum phosphorus extracted 97.5% was absorbed by the algae during the first 3 hours. As can be seen in figure 59, the algal cells retained their phosphate even though the concentration of this nutrient in the solution outside the cells varied from 40 to 36 mg/l, compared with the concentration inside the cell which varied from 132 to 135 mg/l during the same period.

Internal saturation of cells with phosphate is apparent too since the algae did not absorb any significant amount of phosphorus from time 16 hours to 30 hours in figure 59. This

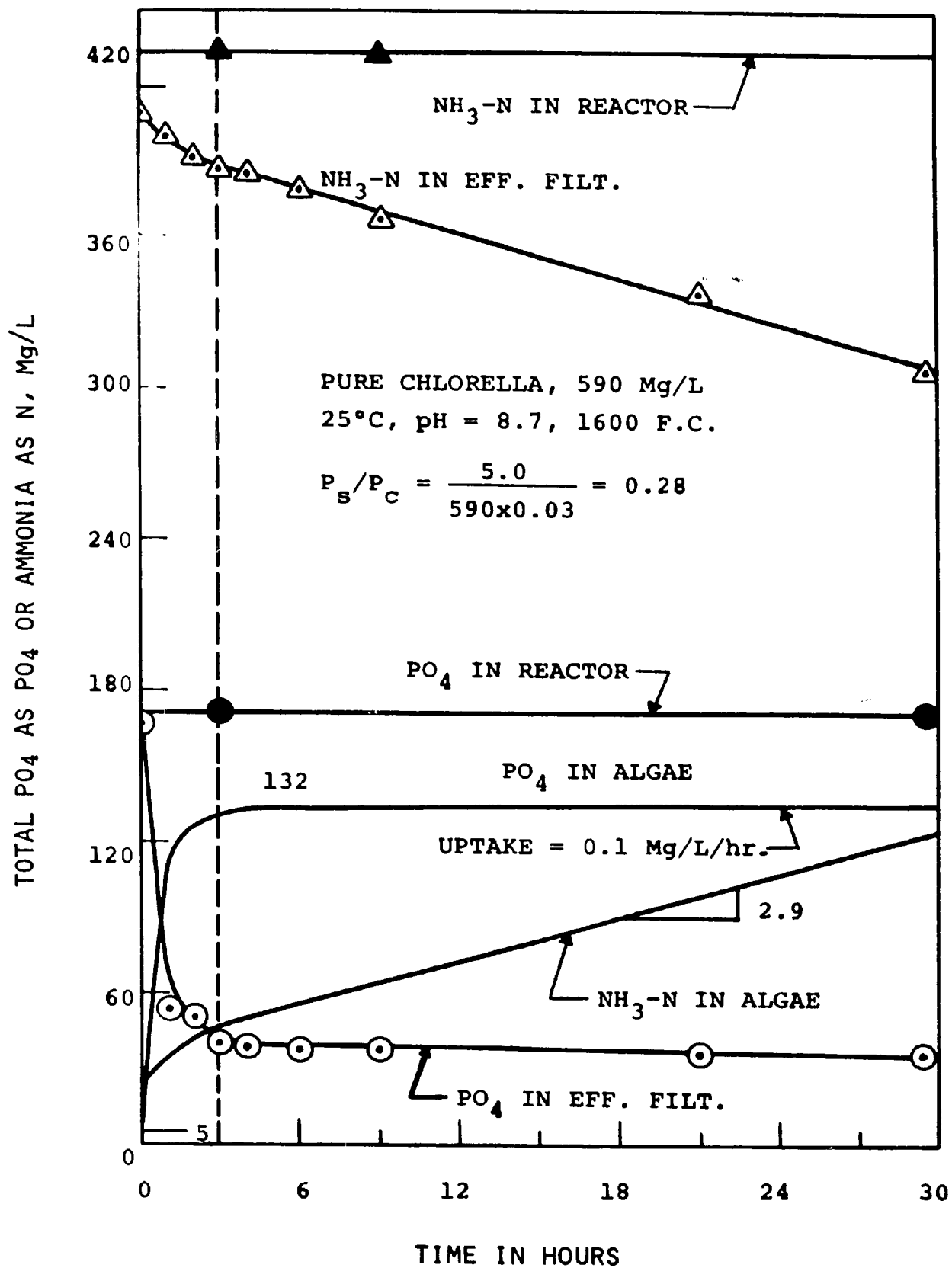


Figure 59. High P-Uptake by P-Starved Chlorella During a Batch Experiment

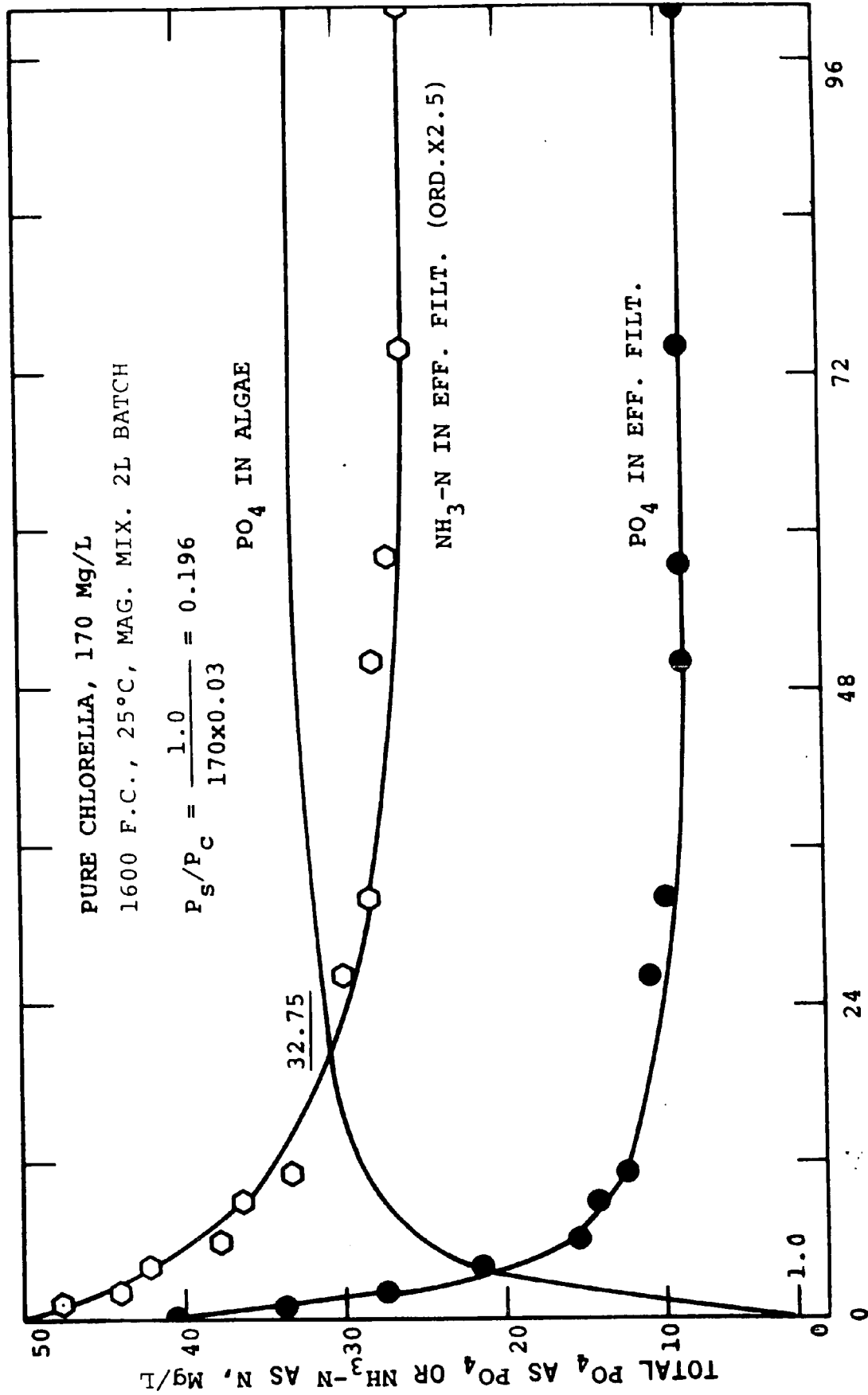


Figure 60. High P-Uptake but No Subsequent Growth of P-Starved Chlorella in a Batch Experiment

would strengthen further the concept of optimum starvation since by definition, that is the point of maximum possible phosphorus uptake by the P-starved algal culture.

Another important characteristic of P-starved algae to be noted in figure 59, is the pattern of the uptake of $\text{NH}_3\text{-N}$. Relatively little nitrogen was absorbed during the first three hours when most of the phosphate absorption took place. Thereafter, 29 mg of $\text{NH}_3\text{-N}$ were being picked up by the algae for each mg of PO_4 from the medium per 10 hours. No significant mass growth of the culture was observed during this period. Hence, this constant rate of nitrogen extraction from the substrate could not be attributed to the growth of microorganisms measured in terms of increase in the dry weight density of the culture.

One may speculate however, that the excessive fats and the carbohydrates accumulated by the cells during phosphorus starvation were being broken down and proteins synthesized within the same cells, using the internal carbon skeletons and the external nitrogen at a constant rate. If this is true, the constant and relatively high rate of $\text{NH}_3\text{-N}$ uptake from the medium by the cells can be understood quite easily on this basis.

In the case of figure 60, 170 mg algae did not absorb its maximum amount of PO_4 (32.75 mg or 19.6% by dry weight of algae) until after a time lapse of almost 2 days. However, the culture was still most active to the pick of phosphorus during the first 6 hours since it absorbed about 78% of the maximum during this period. There was no additional absorption

of phosphates by the algae during the last two days of this experiment.

NH₃-N absorption by the algae is not shown in figure 60 but a relative idea can be obtained by comparing the 'PO₄ in effluent filtrate' and the 'NH₃-N in effluent filtrate' curves. It should be noted that NH₃-N curve is scaled down 2.5 times. Although some NH₃-N was being absorbed by the algae without an accompanying absorption of phosphate, it cannot be said that the pattern is similar to the one reported above in the case of figure 59.

It may be summarized that the pure culture batch study results, although not as consistent as the continuous culture results, generally support and extend the constant density culture results as far as the phosphorus uptake by the P-starved algae is concerned.

F. LITERATURE COMPARISONS

Round (128) has discussed briefly some experimental findings on the P-uptake by the P-starved algal cells. According to him, phosphorus depleted cells can absorb phosphates in the dark whereas replete cells can do so only in the light. He also reported that phosphate deficiency in algae results in the accumulation of large amounts of fat. Alkholy (129) stated that P-starved cells of Chlorella pyrenoidosa absorb phosphorus before they start growing. He observed the maximum and minimum phosphorus limits in this microorganism to be 1.5×10^{-6} and 0.1×10^{-6} microgram per cell, respectively.

Kuhl (130) reviewed some literature available on this aspect and stated that in P-deficient cells of Ankistrodesmus braunii, chlorophyll synthesis was apparently enhanced in the first stages of P-deficiency, but a marked chlorosis appeared in the later stages. Development of chlorosis due to phosphorus deficiency has also been reported in Hydrodictyon meticulatum. Other symptoms of P-starvation included the accumulation of fat, starch and cell-wall substances. Accumulation of fat due to phosphorus deficiency has also been observed in Chlorella vulgaris.

The experimental findings reported in this chapter are in general agreement with these references. However, one of the earlier investigators had reported to the contrary. According to Ketchum (131), uptake of phosphate by P-deficient cells of Nitzschia closterium was always significantly greater in the light than in the dark. He also stated that the P-deficient cells of Chlorella pyrenoidosa recovered more rapidly when illuminated than when kept in darkness. The results obtained during this investigation would not support the conclusions drawn by Ketchum.

Fogg (8) has illustrated the phenomenon of nitrogen starvation using the microorganisms Monodus subterraneus as an example. When growing exponentially, this alga has a protein content of almost 70% by dry weight, high content of chlorophyll and nucleic acids, low content of fat and carbohydrates, and high photosynthetic and respiratory activity. Nitrogen - deficient cells on the other hand, have a protein content of less than 10%, low contents of chlorophyll and

nucleic acids, high contents of reserve polysaccharide and fat, and extremely low photosynthetic and respiratory activity.

This author believes that the above noted characteristics of nitrogen starvation are very similar to the ones observed for phosphorus starvation of algal cells in this study. From this standpoint, nitrogen and phosphorus deficiencies in algal cells would appear to produce very similar effects on microorganisms.

CHAPTER X

DISCUSSION AND CONCLUSIONS

The experimental observations, made using the constant density continuous uni-algal-culture techniques reported in this thesis, have established three phosphate regimes in algal growth as illustrated for a specific case in Figure 61. For an algal cell density of 50 mg/L and for the culturing conditions indicated, three distinct regions of PO_4 —cell interaction are defined quantitatively in this figure.

In this case, the rate of growth of the algae appears to depend on the concentration of PO_4 in the substrate only from 0 to 1.5 mg/L PO_4 , i.e. 0 to 3% PO_4 (as PO_4) by dry weight of algal cells. This range of phosphate concentrations is marked 'Zone I' in Fig. 61 and is called the growth dependent zone. Since the growth rate of algae is seen to decline at phosphate concentrations below 1.5 mg/L and is independent of this nutrient above the level of 3% PO_4 by dry weight of cells, this concentration has been designated as the critical concentration (CCP). It should be emphasized that below the critical level, algal mass growth is strictly proportional to the phosphate concentration present, while above the critical level, growth is constant and independent of the phosphate concentration.

Phosphorus concentrations from 1.5 to 5.0 mg/L PO_4 constitute 'Zone II' in Fig. 61. This zone is referred to as the zone of 'Storage' or of 'Luxury Uptake' of phosphorus.

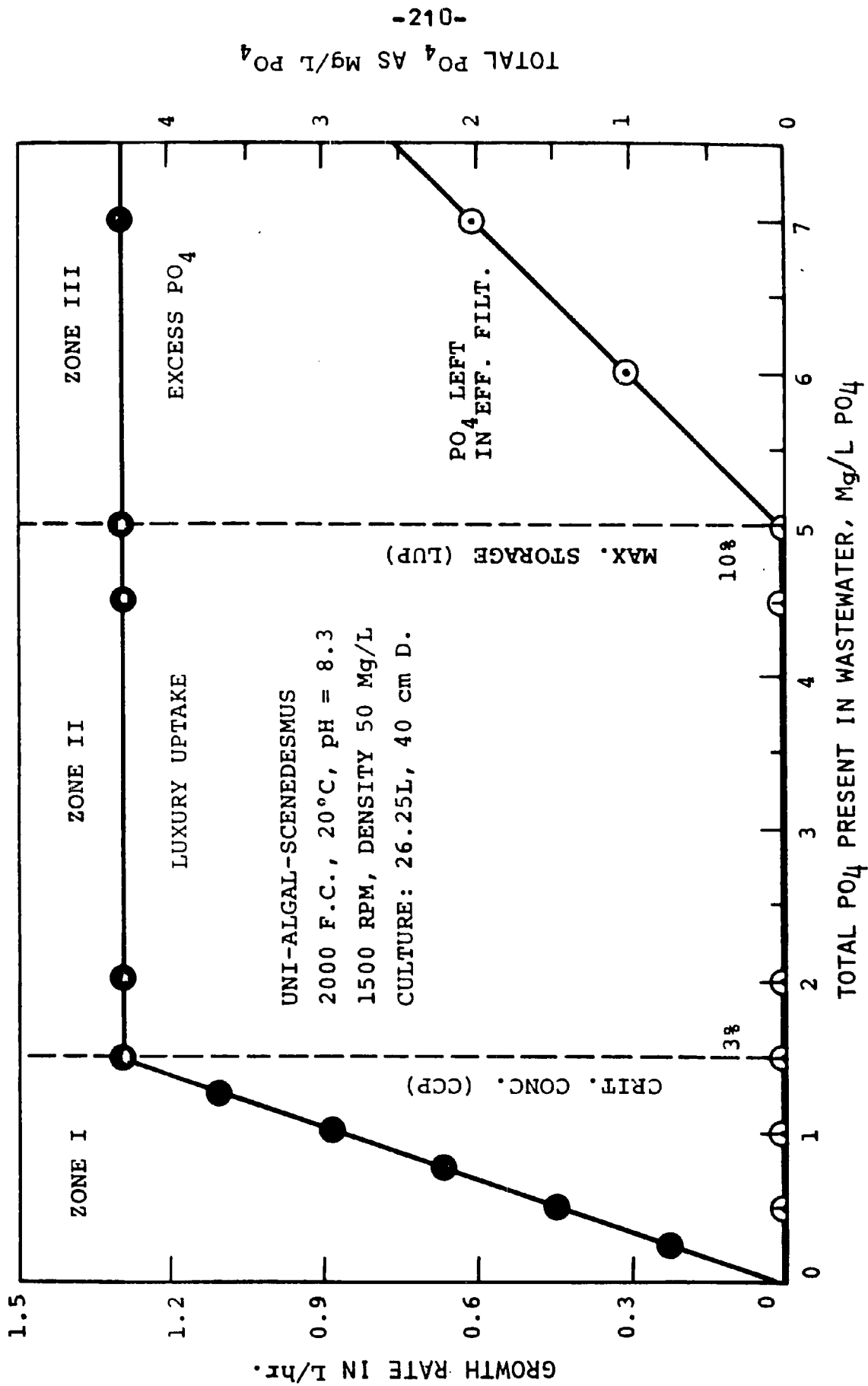


Figure 61. Three Regions of PO₄ Supply for Algal Growth

Whatever concentration of PO_4 is fed to the culture up to the level of 10% PO_4 by dry weight of cells, no phosphate residual appears in the effluent filtrate (the culture fluid which passes through a good quality 1.2 micron pore size filter). This additional and variable absorption of phosphorus by algae has no effect on the maximum steady state growth rate of algae. On this basis, the 'luxury uptake' can be defined in general as, "that phosphate incorporated into cells above the critical level". In the case of Fig. 61, it would appear that all phosphate in the algal cell mass exceeding 3.6% as PO_4 or 1% as P, on a dry weight basis, would define the magnitude of "luxury uptake".

Concentrations of phosphorus above 5.0 mg/L (at least up to 24 mg/L) have no effect either on the rate of growth of algae or on the magnitude of luxury uptake of phosphate by algae. For this reason, 'Zone III' in Fig. 61 is designated as the 'Excess PO_4 ' zone or the zone of saturation. It is clear from the ' PO_4 left in effluent filtrate' line in this figure that algae do not absorb any portion of the phosphate added to the culture in excess of the level of 10% PO_4 by weight of the cells. Therefore, this level constitutes the upper limit of the cellular potential for the storage of phosphorus under the experimental conditions used.

Actual values of both the critical concentration of PO_4 (CCP) and the luxury uptake of phosphorus (LUP) are dependent on the environmental conditions under which the algal cells are grown. The effects of Light Intensity, Cell Density, Diurnal cycling, Temperature, and Mixing on CCP and LUP were

investigated in this study. Summary discussions (along with summary graphs) concerning the effects of each of these parameters on the values of CCP, the growth rate, and LUP are presented in chapter VIII, where these factors are discussed individually in detail. Only the most general and important conclusions will be summarized here concerning each environmental parameter studied.

The pertinent conclusions derived from the P-uptake study of the P-starved cells (reported on in chapter IX) will be listed below after listing the general conclusions regarding the effect of various environmental factors. For the sake of clarity, all the important conclusions concerning the different factors are grouped below under appropriate headings.

SECTION I

A. LIGHT INTENSITY EFFECTS

An algal cell density of 50 mg/L was maintained throughout, while the light intensity at the face of the culture tank was varied from 400 to 2250 foot candles. The study of the effects of light intensity on the constant density algal culture has made the following conclusions possible:

1. Within the range of the values of light intensity studied, the higher the intensity of light available to the culture, the higher the rate of growth of the algae.
2. The value of CCP is an inverse function of light intensity, i.e. the lower the value of luminous energy,

the higher the value of the critical concentration of phosphate will be.

3. When the growth rate of algae in L/hr was plotted against the values of light intensity measured at the front face of the culture tank, the conventional curve, showing three regions of dependence of the growth rate on light intensity, resulted.
4. A new method, utilizing a term designated as effective average light intensity (EALI), has been developed to express the average luminous energy available to the culture for net photosynthesis.
5. The growth rate of algae versus 'EALI' do not plot as a curve (3 above) but yield a straight line which has a slope of 0.056 L/hr/10 foot candles.
6. The CCP versus 'EALI' also plot as a straight line, with a slope of -0.05 milligram PO_4 /L/10 foot candles, up to an EALI value of 200 F.C. Beyond this point, the magnitude of dependence of CCP on luminous energy appears to decrease.
7. A relative increase in algal cell numbers with a corresponding decrease in the cell size, thus holding the biomass constant, is observed under continuous but low light conditions.

B. CELL DENSITY EFFECTS

The lower the value of algal cell density in a turbidostat, the higher the value of EALI is found at a fixed supply of luminous energy to the culture. A light intensity value of

2000 F.C., measured at the front face of the culture, was held constant throughout this study. The cell density was varied from 8 mg/L to 100 mg/L dry weight of algae. The major conclusions derived from this study are as follows:

1. Relatively high growth rates of the algae (indicating theoretical generation times in the order of 7 to 8 hours) are observed when the cell density is in the range of 8 to 10 mg/L.
2. The fact that the luxury uptake of phosphorus is a 'normal' behavior of the algal cells has been established very clearly by the experimental observations made at low cell densities (below 15 mg/L dry weight of algae).
3. The magnitudes of the luxury uptake of phosphorus and of CCP, as percents by weight, have been found to be higher at lower cell densities.
4. The concept of 'unavailable' phosphate at cell densities lower than 40 mg/L algae has been developed and partially elucidated.
5. Growth and phosphorus uptake patterns of the green algae *Chlorella* and *Scenedesmus* in a river water feed and in the synthetic substrate have been found to be very similar, if not identical at algal cell densities ranging from 8 to 40 mg/L.
6. Regular patterns of growth of and P-uptake by algae at cell densities approximating 100 mg/L algae have been found. The percent by weight values for LUP and CCP are found to be lower at these higher cell densities.

7. Using the experimental data, an algal cell yield (biomass produced/unit time) curve has been generated which appears to indicate an optimum cell yield point at a microorganism density of 40 mg/L.
8. The experimental rates of growth of the algae at various cell densities have been predicted successfully by employing the EALI method.
9. A modified form of the EALI method (to suit the natural systems) whereby it might be possible to predict the light intensity-algal photosynthesis relationships, may find practical applications.

C. DIURNAL CYCLE EFFECTS

On the basis of 12 hr. day and 12 hr. night cycles, the following conclusions have been reached:

1. No significant phosphorus uptake by or leakage from algal cells takes place during the 12 hr. dark period following the continuous lighting period during steady state growth conditions.
2. The mass of algal growth in the dark after a 12 hour period of continuous lighting, declines to a low level.
3. During this period, a dramatic increase in cell counts but not in cell mass is observed.
4. The overall efficiency of phosphorus removal by algal cultures would diminish, below the steady state level, when used in a natural light-dark environment. As a first approximation, an efficiency factor of 64% is indicated by these results for green algae

similar to *Scenedesmus* and *Chlorella*.

D. TEMPERATURE EFFECTS

Based on the experimental observations reported in this thesis, the following major conclusions concerning temperature may be cited:

1. The higher the temperature (between 10 to 35°C), the lower the value of CCP observed and this relation is that of a straight line with a slope of (-) 0.047 mg PO_4 /L/°C at a culture density of 50 mg/L and with other conditions as noted on Fig. 42.
2. Temperatures up to 25°C enhance the rate of growth of the green algae studied, while temperatures above this point have an adverse effect on growth. The locus of points that have resulted from observing the growth rates at different temperatures would appear to have a slope of 0.068 L/hr/°C for the specific conditions noted in figure 42.
3. In addition to observing some 'thermal death' in the green algal culture at 30°C, traces of phosphate in the effluent filtrate were observed.

E. MIXING EFFECTS

The following 'tentative' conclusions may be drawn from the limited amount of study that was conducted on mixing effects on the growth of algae:

1. Within the range of the degrees of mixing imparted the culture by the variation in the speed

of a specific mixer impeller from 600 to 1500 RPM, the rate of growth of the algae certainly depended on the degree of mixing.

2. This direct dependence of the growth rate of algae on the value of RPM used has been approximated by a straight line having a slope of 0.059 L/hr/100 RPM under the conditions noted in figure 48.
3. The same pattern is observed within algal cell density values of 34 and 100 mg/L. However, the value for the slope of this line is a function of the algal cell density.
4. Atleast at 100 mg/L algal cell density, CCP appears to be independent of the degree of turbulence (Fig.47).

SECTION II

PHOSPHATE UPTAKE BY P-STARVED ALGAE

The following pertinent conclusions may be listed under this heading:

1. The maximum uptake of phosphorus by phosphate-starved algae is a function of the 'degree' of P-starvation which has been defined by the ratio P_s/P_c .
2. Within the limits of this study, the rate as well as the equilibrium amount of P-uptake by the P-starved cells appears to be independent of the environmental conditions. This of course, does not include the concentration of PO_4 in the medium which is responsible for changing the P-uptake pattern of the P-starved algae.

3. Under 'optimum ' starvation conditions ($P_s/P_c=0.3$), the algal cells can extract atleast 20% PO_4 on the basis of their dry weight from the culture medium. This percentage decreases as the degree of P-starvation goes above the optimum starvation point(Over starvation) or declines below this point (inadequate starvation).

APPENDIX I

RELATIVE AMOUNTS OF DIFFERENT LIPIDS

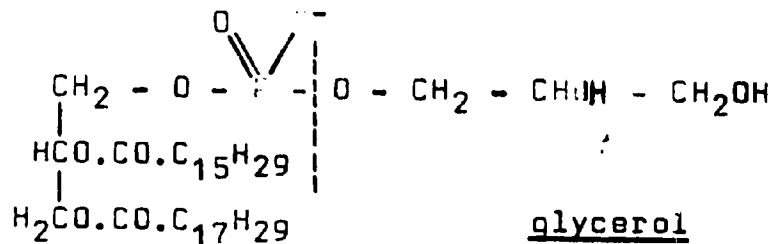
IN SPINACH CHLOROPLASTS (39)

Number of Lipid Molecules per 100 Molecules of Chlorophyll	% by Weight of Total Lipids
<u>100 chlorophyll:</u>	<u>20.8</u>
70 chlorophyll a	
30 chlorophyll b	
<u>21.6 carotenoids:</u>	<u>2.8</u>
<u>22.1 quinone and related compounds:</u>	<u>3.3</u>
15.7 plastoquinones A,B,C, and D	
6.4 vitamin K and other compounds	
<u>50.6 phospholipids:</u>	<u>9.1</u>
23.0 phosphatidyl glycerol	
18.0 phosphatidyl choline	
5.7 phosphatidyl inositol	
2.9 phosphatidyl ethanolamine	
1.0 phosphatidic acid	
<u>234 glycolipids:</u>	<u>44.3</u>
150 monogalactosyl diglyceride	26.8
63 digalactosyl diglyceride	13.4
21 sulpholipid	4.1
<u>24.6 sterols:</u>	<u>2.2</u>
<u>2.9 prenols:</u>	<u>0.5</u>
<u>Unidentified lipids:</u>	<u>17.0</u>

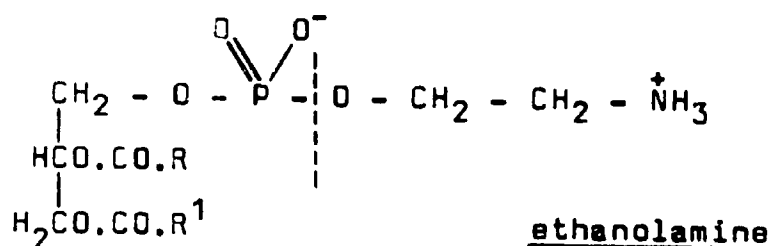
APPENDIX II

STRUCTURE OF CHLOROPLAST PHOSPHOLIPIDS (39)

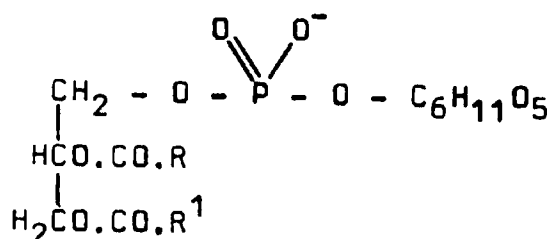
A. Phosphatidyl glycerol: ($C_{40} H_{70} O_{10} P$)



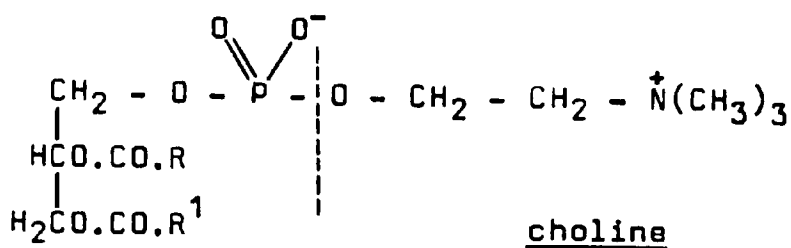
B. Phosphatidyl ethanolamine (Cephalin):



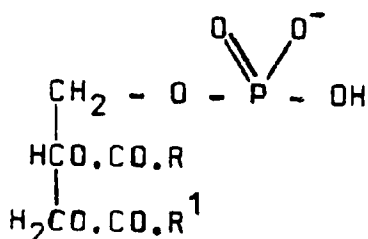
C. Phosphatidyl inositol:



D. Phosphatidyl choline (Lecithin):



E. Phosphatidic acid:



APPENDIX III

INTRACELLULAR DISTRIBUTION OF CERTAIN ENZYMES IN AUTOTROPHICALLY GROWN CELLS OF EUGLENA GRACILIS (39)

Enzyme Name and Probable Function	Location With Respect To Chloroplast
<u>Photosynthetic Electron Transport Enzymes:</u>	
1. Cytochrome b_6	Inside
2. Cytochrome - 55^2	Inside
3. Ferredoxin	Inside
4. $NADPH_2$ cytochrome c reductase	Inside & Outside
5. $NADPH_2$ diaphorase	-do-
6. Transhydrogenase	-do-
7. Adenylate Kinase	-do-
<u>Enzymes of Photosynthetic CO_2 Fixation Cycle:</u>	
8. Carboxydismutase	Inside
9. 3-phosphoglycerate Kinase	Inside & Outside
10. $NADP$ -glyceraldehyde- PO_4 dehydrogenase	Inside
11. Triose phosphate isomerase	Inside & Outside
12. Fructose diphosphate aldolase	-do-
13. Alkaline fructosediphosphatase	Inside
14. Transketolase	Inside & Outside
15. Phosphopentoisomerase	-do-
16. Ribulokinase	Inside

APPENDIX III (Contd.)

Enzyme Name and Probable Function	Location With Respect To Chloroplast
<u>Other Enzymes:</u> 17. Phosphohexoisomerase 18. Phosphofructokinase 19. Phosphoglycerate mutase 20. Enolase 21. Pyruvate Kinase 22. Phosphoglucomutase 23. Neutral fructosediphosphatase 24. UDPG pyrophosphorylase 25. Transaldolase 26. Glucose-6-PO ₄ dehydrogenase 27. 6-Phosphogluconate dehydrogenase 28. Isocitrate dehydrogenase 29. Malate dehydrogenase 30. Malic Enzyme 31. Glutathione reductase 32. NADH ₂ - cytochrome c reductase 33. NADH ₂ - glyoxylate reductase 34. NADPH ₂ - glyoxylate reductase 35. Ribonuclease 36. Pyrophosphatase	Outside -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- -do- Inside & Outside

APPENDIX IV

PHOSPHORUS COMPOUNDS IN THE CHLOROPLAST AND VOLUTIN

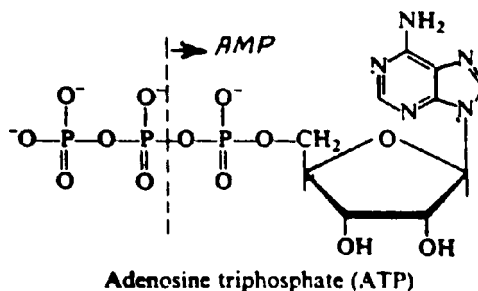
GRANULE FRACTIONS ISOLATED FROM CHLORELLA (41)

Phosphorus Compound	Chloroplast per 100 mg		Volutin Granule micro mole P per 100 mg
	PURE	IMPURE	
<u>Acid Soluble Fraction:</u>			
Inorganic P	2.8	2.9	11.5
15 minute - P,			
Nucleotidic	1.7	1.3	22.0
Non-nucleotidic (Polyphosphate "A")	0.3	1.1	93.2
Total P	6.6	8.3	----
<u>Lipid - P:</u>	6.0	6.2	----
<u>Polyphosphate "B" Fraction:</u> (pH 9 - Extract)			
15 minute - P	2.5	1.1	2.5
Total P	5.8	3.4	2.9
<u>Polyphosphate "C" Fraction:</u>			
15 minute - P	0.9	0.4	0.9
Total P	1.5	0.8	----
<u>RNA - Fraction:</u>			
Total P	5.1	8.1	11.6
<u>DNA - Fraction:</u>			
Total P	0.2	0.4	0.0
<u>Protein Fraction:</u>			
Total P	1.0	0.6	0.0
Total P in all Fractions:	26.2	27.8	
Note: $26.2 \mu\text{M-P} = 0.81 \text{ mg P/100 mg chloroplasts} = 0.81\% \text{ by wt.}$			

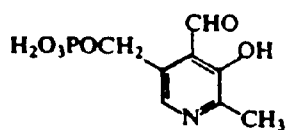
APPENDIX V

STRUCTURE OF SOME VITAL PHOSPHORUS CONTAINING COMPOUNDS (40)(42)

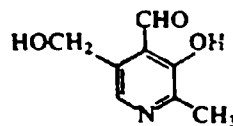
- A. Adenosine Triphosphate (ATP), Adenosine Monophosphate (AMP), and Adenosine Diphosphate (ADP) - Purine Nucleotides:



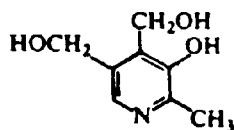
- B. Pyridoxal Phosphate (Crucial Coenzyme in Transformations of Amino Acids):



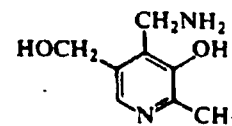
Pyridoxal phosphate



Pyridoxal

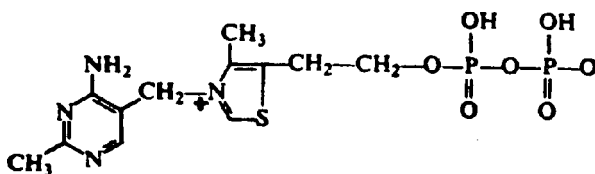


Pyridoxol



Pyridoxamine

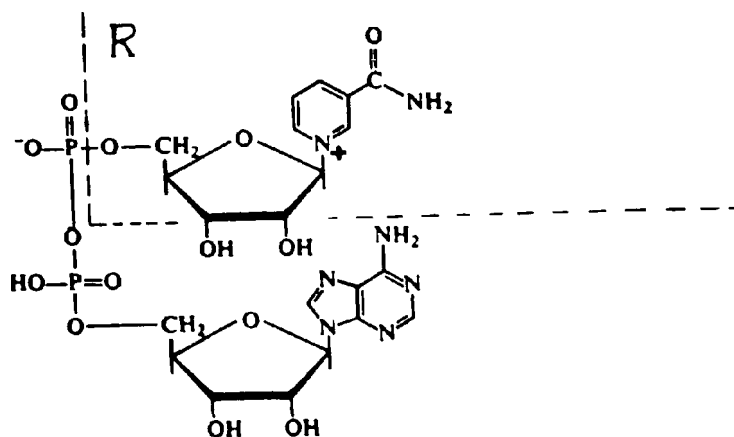
- C. Thiamine Pyrophosphate (A Coenzyme of Universal Occurrence in Living Systems, also Very Important as a Plant Hormone):



Thiamine pyrophosphate

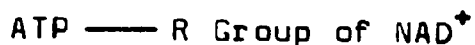
APPENDIX V (Contd.)

- D. Nicotinamide Adenine Dinucleotide. (NAD^+ , A Coenzyme Involved in a Large Number of Oxidation-Reduction Reactions Catalyzed by Dehydrogenases):

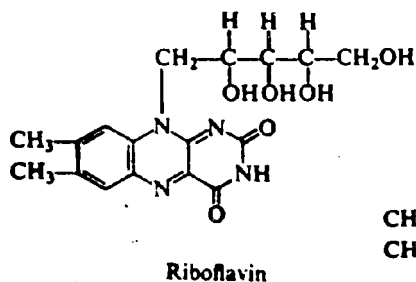


Nicotinamide adenine dinucleotide (NAD^+)

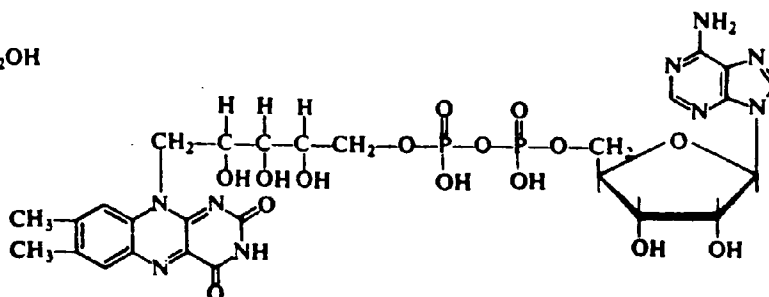
- E. Nicotinamide Adenine Dinucleotide Phosphate (NADP^+ , Function Similar to NAD^+):



- F. Flavin Adenine Dinucleotide (FAD , A Flavin Coenzyme Extremely Important in Catalysis of many Dehydrogenation Reactions)

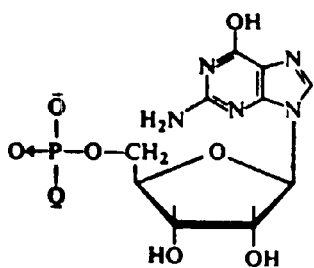


Riboflavin

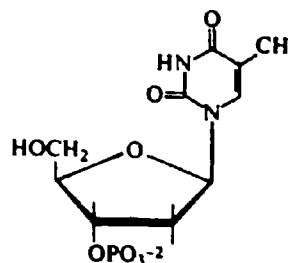


Flavin adenine dinucleotide (FAD)

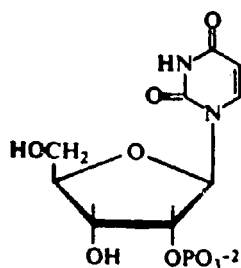
G. Nucleotides (True Monomeric Units of the Nucleic Acids):



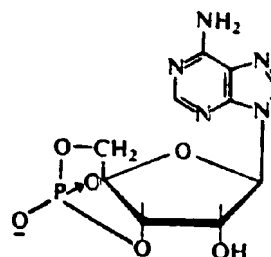
Guanosine-5'-phosphate
(9- β -D-Ribofuranosylguanine-5'-phosphate)
a 5'-ribonucleotide



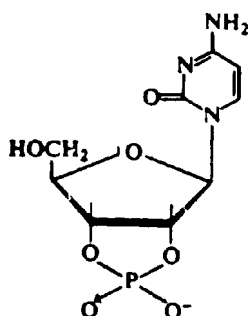
Thymidine-3'-phosphate
(1- β -D-Deoxyribofuranosylthymine-3'-phosphate)
a 3'-deoxyribonucleotide



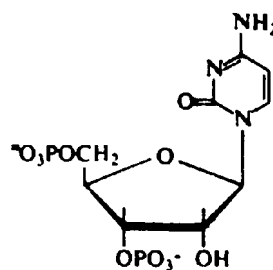
Uridine-2'-phosphate
(1- β -D-Ribofuranosyluracil-2'-phosphate)
a 2'-ribonucleotide



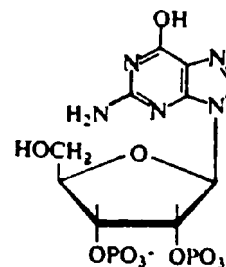
Adenosine-3'-5' cyclic phosphate



Cytidine-2'-3'-cyclic phosphate

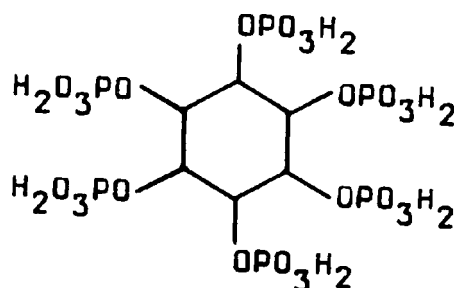


Cytidine-3'-5'-diphosphate



Guanosine-2'-3'-diphosphate

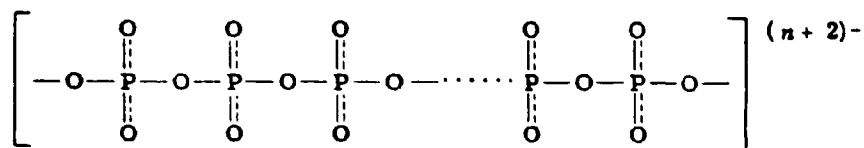
H. Phytic Acid (Usually Present in Plants as Calcium or Magnesium Salt Known as Phytin):



APPENDIX V (Contd.)

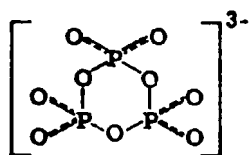
- I. Condensed Phosphates (High Energy Phosphates Serving Microorganisms Internally as P and Energy Source):

1. Linear Polyphosphates (Salts of Anions of $(P_nO_{3n+1})^{(n+2)-}$ formula)

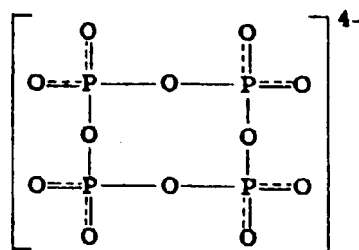


Polyphosphate

2. Cyclic Polyphosphates (Salts of Anions of $(P_nO_{3n})^{n-}$ Formula)



Trimetaphosphate

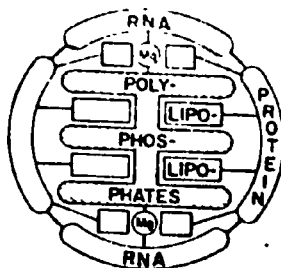


Tetrametaphosphate

Tetrametaphosphate can have boat and chain forms. Metaphosphates may have infinite chains rather than rings, e.g. $(KPO_3)^{2-}$ (43).

APPENDIX VI

DIAGRAM INDICATING POSSIBLE COMPOSITION OF A BACTERIAL POLYPHOSPHATE (VOLUTIN) GRANULE (49).



APPENDIX VII

PROCEDURE FOR TOTAL PHOSPHATE ANALYSIS

REAGENTS:

1. Sulfuric acid solution: add 625 ml of concentrated H_2SO_4 slowly while stirring to about 1.2 liters of distilled water. Cool and dilute to 2 liters.
2. Ammonium molybdate solution: dissolve 50 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 350 ml of distilled water. Add 155 ml of concentrated H_2SO_4 slowly while stirring to 800 ml of distilled water. Cool. Add the molybdate solution to the acid solution and dilute to 2 liters.
3. Stannous chloride solution: dissolve 5 g of fresh $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 200 ml of glycerol.
4. Potassium persulfate: use "Baker Analyzed" reagent grade $\text{K}_2\text{S}_2\text{O}_8$ crystals (or equivalent) in 1 lb. lot.

STEPS FOLLOWED:

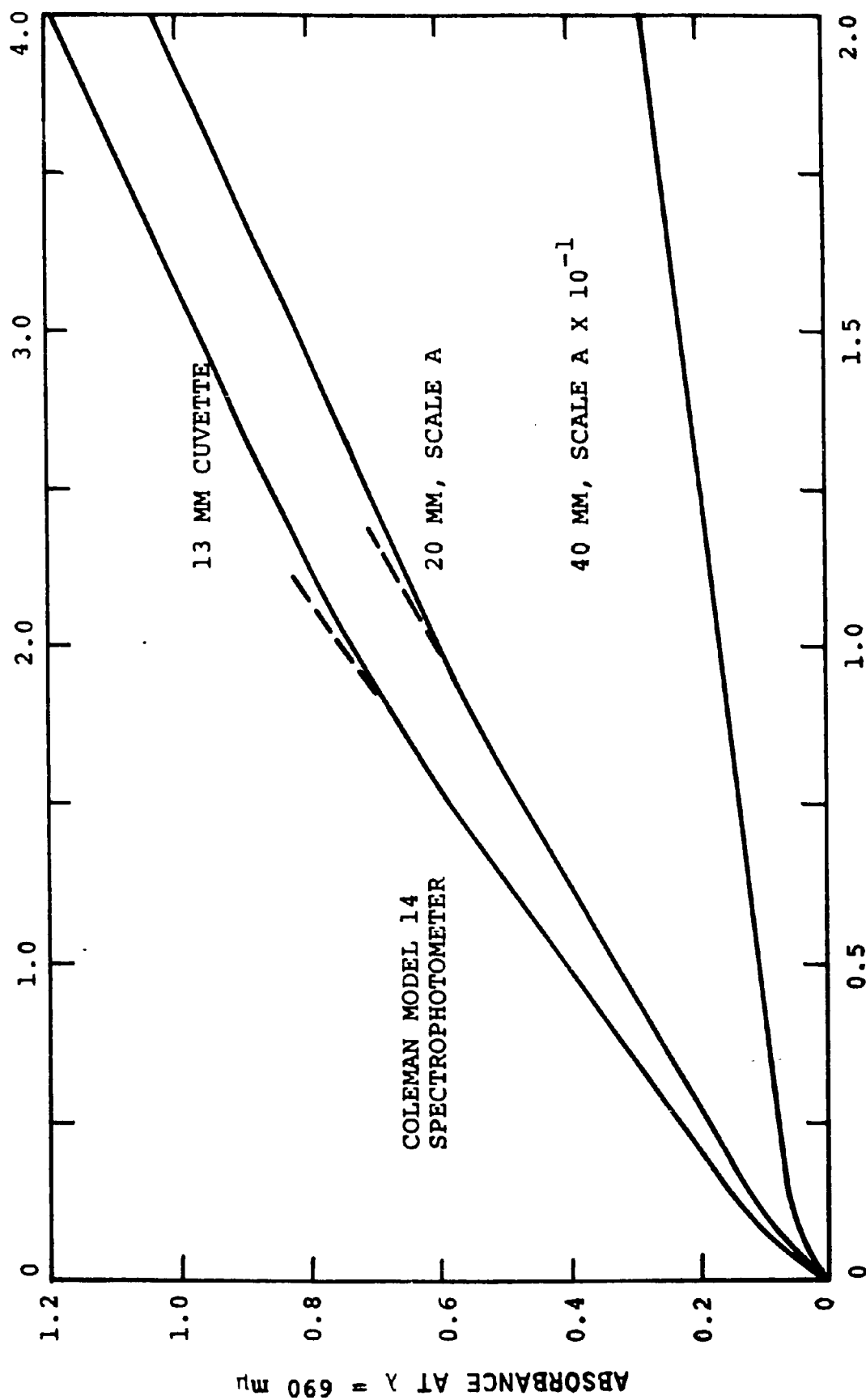
1. Add 2 ml of H_2SO_4 solution to 100 ml of diluted sample in a 250-ml Erlenmeyer flask.
2. Add 0.85 ± 0.05 g of $\text{K}_2\text{S}_2\text{O}_8$ using a specially prepared plexiglass measuring spoon.
3. Boil gently on a hot plate until about half the sample is evaporated (usually 35 to 45 minutes, depending upon the sample constituents and the location of the flask on the hot plate).
4. Cool to room temperature, transfer the sample to a 100

APPENDIX VII. (Contd.)

- ml - volumetric, and dilute to the mark with distilled water.
5. Transfer 50 ml to a clean 250 ml - Erlenmeyer flask.
 6. Add 2 ml of ammonium molybdate solution and mix.
 7. Add 3 drops from an eye dropper of stannous chloride solution and mix.
 8. Allow 10 minutes for color development.
 9. During this 'wait' period, prepare the reference blank from the remaining 50 ml sample (step 4) in exactly the same manner except using strong-acid solution in place of the molybdate solution.
 10. Fill the appropriate cuvette having a 13,20, or 40 mm. light path, depending upon the estimated phosphate concentration in the sample and determine the optical density at 690 millimicron wavelength.

APPENDIX VIII

B. PHOSPHATE SCALE FOR 13 MM LIGHT PATH



A. TOTAL PHOSPHATE AS MILLIGRAM PER LITER PO₄

Appendix VIII. Standard Curves for Total Phosphate Determination

APPENDIX IX

ELEMENTARY ANALYSES OF DOMESTIC SEWAGE (110)

Element	CALIFORNIA SEWAGE SOURCES			
	San Francisco	Richmond	Concord	Contra Costa
C	91	176	66	101
H	14	---	77	---
N	25	50	22	36
P	8	11	13	6
K	15	14	13	---
Mg	9	22	58	---
Ca	24	17	40	---

APPENDIX X

COMPOSITION OF SYNTHETIC FEED

Reagent Added	Quantity (mg/l)	Sewage Effluent (106) (107)
1. Na HCO ₃	250	190
NH ₄ Cl	75	105
K ₂ HPO ₄	0.18 to 14	32
Mg SO ₄ .7H ₂ O	450	
2. Fe SO ₄ .7H ₂ O + KOH + EDTA → AERATED	5 + 15 + 26	
3. Mn SO ₄ .H ₂ O	1.55	
Zn SO ₄ .7H ₂ O	0.22	
H ₃ BO ₃	2.86	
Mo O ₃	0.02	
Cu SO ₄ .5H ₂ O	0.08	
4. pH comparison:	pH = 7.5 ± 0.3	pH = 7.7 ± 0.5

APPENDIX XI

MACRONUTRIENTS PROVIDED BY THE CULTURE MEDIUM

NUTRIENT ELEMENT	COMPOUND ADDED	AS Mg/L	MOLES	BATCH, CRIT. CONC. (5)
CARBON	NaHCO_3	250	3×10^{-3}	2.5×10^{-2}
NITROGEN	NH_4Cl	75	1.4×10^{-3}	2.5×10^{-2}
PHOSPHORUS	K_2HPO_4	0.18	1×10^{-6}	1.8×10^{-4}
		14	8×10^{-5}	
SULFUR	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	450	1.8×10^{-3}	
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22	7.6×10^{-7}	
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08	3.2×10^{-7}	
	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.55	9.2×10^{-6}	
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	24.9	9.0×10^{-5}	
	Total Sulfur =		1.9×10^{-3}	2.0×10^{-4}
POTASSIUM	K_2HPO_4	0.18	2×10^{-6}	
		14	16×10^{-5}	
	KOH	15	2.7×10^{-4}	
	Range =		2.7×10^{-4} to 4.5×10^{-4}	4.3×10^{-4}
MAGNESIUM	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	450	1.8×10^{-3}	2.0×10^{-3}
SODIUM	NaHCO_3	250	3×10^{-3}	

APPENDIX XII

MICROELEMENTS PROVIDED BY THE CULTURE MEDIUM

NUTRIENT ELEMENT	COMPOUND ADDED	A mg/L		CRIT. CONCENT. (IN BATCH) VALUE REFER.
IRON	Fe^{+3} -EDTA	5.1	9.9×10^{-5}	1.3×10^{-5} (5) 2.6×10^{-8} (81)
MANGANESE	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.55	9.0×10^{-6}	1.0×10^{-7} (5)
CHLORINE	NH_4Cl	75.0	1.4×10^{-3}	3.4×10^{-2} (5)*
ZINC	$\text{ZnSO}_4 \cdot \text{H}_2\text{O}$	0.22	7.5×10^{-7}	7.7×10^{-7} (5) 1.0×10^{-7} (82) 1.0×10^{-6} (87)
BORON	H_3BO_3	2.86	4.6×10^{-5}	9.0×10^{-6} (5)
MOLYBDENUM	Mo_3O_3	0.02	1.4×10^{-7}	Not (89) Required (5)
COPPER	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08	3.2×10^{-7}	1.0×10^{-7} (68)
CALCIUM	Present in Ann Arbor Tap Water.			Replaceable (68) by Strontium
VANADIUM	Traces in tap water?			Accelerates (94) Photosynthesis under high light
COBALT	Traces in tap water?			Stimulated the growth of <i>Scenedesmus</i> (96)

*Critical concentration reported is for a blue-green alga.

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