THE METABOLIC ROLE OF INTRACELLULAR INORGANIC POLYPHOSPHATE DURING THE SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

by

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Introduction

The demonstration in algae and bacteria of the presence of a high molecular weight polymer of inorganic phosphate has raised questions concerning the metabolic role of the polymer and the mechanism(s) by which it is synthesized and utilized. The technique for synchronizing cultures of Chlorella pyrenoidosa[26], an alga which accumulates polyphosphate, provided an ideal tool for the investigation of the polymer during cellular growth and division. The purpose of the research described in this dissertation was to determine the relationship of inorganic polyphosphate to the total metabolism of phosphorus by C. pyrenoidosa.

The preliminary work of Schmidt and King[26] and Baker and Schmidt[1,2] suggested that polyphosphate was accumulated in Chlorella during daughter cell development for eventual utilization during nuclear and cellular division. This inference was drawn from dramatic shifts observed in the concentration of polyphosphate, expressed as percentage of total cellular phosphorus, during the growth cycle. Additional experimental evidence to support the hypothesis that the polymer was serving as a potential intracellular phosphorus donor was supplied by other investigators using a variety of microorganisms. Miyachi and Miyachi[16] and Miyachi and Tamiya[17] showed that polyphosphate fractions in C. ellipsoideae donated phosphorus for nucleic acid and phosphoprotein synthesis. Similar results were obtained by Harold[9] using Neurospora crassa. Mudd, Yoshida and Koike[18] and Sall, Mudd and Takagi[21] demonstrated reciprocal relationships between polyphosphate and ribonucleic acid phosphate in Mycobacterium chelonei and Corynebacterium diphtheriae.

Hughes, Conti and Fuller[12] observed a nucleotide dependent depolymerization of polyphosphate in Chlorobium thiosulfatophilum. Although the experimental evidence cited above showed actual polyphosphate utilization, in each instance the organism was subjected to an environmental stress (eg., phosphate deficiency, low light intensity, abnormal nucleotide concentration, use of 2, 4 dinitrophenol, etc.).

In addition to existing data which suggests an active phosphate-donor role for polyphosphate, there are recent studies which indicate that the polymer is not utilized in the absence of environmental stress. Baker and Schmidt[3] have observed that the net accumulation of polyphosphate is a linear function when expressed on a per cell basis. Periodism in the polymer concentration only appeared when the data was expressed as percent of total cellular phosphorus. Although the linear deposition of polyphosphate throughout cellular development suggested that it was not utilized during normal growth, the possibility remained that if there was significant turnover of the polymer, phosphorus originally in the polyphosphate could be incorporated into other cellular fractions. Two experimental approaches were undertaken, therefore, to determine whether polyphosphate exhibited sufficient turnover to be considered an intracellular phosphorus source during nuclear division, a period of growth when phosphorus demands for nucleic acid synthesis accelerate[11]. The transfer of synchronous, uniformly labeled (^{32}P) cells into non-radioactive culture medium immediately prior to nuclear division allowed the observation of shifts in the intracellular phosphorus components of the cells during

normal development. The shifts in endogenous phosphorus when cells were subjected to phosphate starvation could be studied by transferring normal synchronous cultures of cells into phosphorus-deficient medium immediately prior to nuclear division.

The results are divided into four parts, three of which are recent publications[1-3].

Part 1.

Intracellular Distribution of Phosphorus During Synchronous Growth of Chlorella pyrenoidosa

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Introduction

Studies of phosphorus metabolism during the synchronous growth of Chlorella were initiated by Nihei[19] who demonstrated a period of active photophosphorylation prior to cellular division in Chlorella ellipsoidea. The period of active photophosphorylation was accompanied by a rapid accumulation of cellular inorganic polyphosphate. During synchronous growth of Chlorella pyrenoidosa, Schmidt[24] observed a high rate of phosphate uptake immediately prior to nuclear division but the rate of uptake decreased rapidly at the onset of this event. Polyphosphate (metachromatic) granules accumulated in the cells immediately prior to nuclear division. Schmidt and King[26] using synchronized cells of a high temperature strain of C. pyrenoidosa, showed that although phosphate uptake was exponential, there was preferential incorporation into different phosphate compounds during different stages of cellular growth. The most striking observation was the accumulation of inorganic acid-insoluble polyphosphate prior to nuclear division and its decrease during this event. Thus, it appeared that polyphosphate might be serving as a phosphorus and/or energy source during nuclear division. Miyachi and Miyachi[16]

continued studies with C. ellipsoidea by fractionating the acid-insoluble polyphosphate into two components by differential basic extraction. The acid-soluble phosphates were also fractionated and their metabolism partially traced with radioactive phosphate.

The present paper is a continuation in the investigation of the metabolic role of inorganic polyphosphates during the synchronous growth of C. pyrenoidosa.

Methods and Materials

Organism

The alga used in these studies was the high temperature strain 7-11-05 of C. pyrenoidosa described by Sorokin and Myers[27]. The cells were synchronized by the method of Schmidt and King[26] modified by increasing the length of the light periods from 9 to 11 h.

Culture conditions

For biochemical studies, 35 l of synchronized cells were cultured in a flat Plexiglass (0.5 in thickness) chamber, having the following inside dimensions: 50 in width, 1 in thickness, and 42 in height. The culture was maintained at 38.5° by rapidly circulating water, from an Aminco 50 gal wide range constant temperature water-bath, through glass coils within the culture chamber. The temperature of the constant temperature water-bath was controlled by a thermoregulator mounted within the culture chamber. The culture medium was modified from that of Sorokin and Myers[28] by (a) doubling the levels of KNO_3 and

KH_2PO_4 to permit the exponential growth of higher cell densities, and (b) reducing the level of $MgSO_4 \cdot 7H_2O$ by 75% to eliminate clumping of cells during growth. The culture was continuously aerated with CO_2-air (3:97). A bank of twenty-two fluorescent lamps (40 W) was mounted approx. 0.5 in from each side of the culture chamber. An exponential increase in cellular dry matter and phosphorus was obtained, under the above conditions, when the initial cell density did not exceed $12 \cdot 10^6$ cells per millilitre of culture.

Biochemical methods

Cells were harvested every 2 h during the 14-h growth cycle. The cell concentration per millilitre of culture was determined using a Levy-Hausser hemacytometer. Total cellular phosphate per millilitre of culture was determined by the method of Fiske and SubbaRow[8] after washing the cells in an aqueous solution of Tris (0.125%) and $CaCl_2$ (0.01%) at pH 7.0. Approx. 2.2 ml p.c.v. of cells were washed with the same buffer and fractionated by the following procedure: (a) An initial extraction with 10% trichloroacetic acid at 0° for 10 min, followed by three similar extractions with 5% trichloroacetic acid, removed the acid-soluble phosphates. (b) Trichloroacetic acid and phospholipids were removed from the resulting residue by three extractions with 95% ethanol at 0° for 10 min followed by six extractions with ethanol-ether (3:1) for 20 min at 45°. (c) The acid-insoluble polyphosphates and nucleic acids were extracted from the lipid-free residue with 1 N KOH for 60 min at 37° followed by two washes of the residue with 1 N KOH.

The phosphate compounds of the cold trichloroacetic acid extract were separated using 0.5 g Norit-A columns (1.5 cm x 1.2 cm). Prior to addition of the extracts to the columns, the Norit-A was soaked in 10% aqueous EDTA (pH 6.5); then, washed three times with deionized water. After determining the total phosphate, orthophosphate and Δ-8 min labile phosphate (amount of orthophosphate released in 8 min in 1 N H_2SO_4 at 100°) levels in aliquots of the cold trichloroacetic acid extract, the remaining extract was added to the Norit-A column and the non-adsorbable phosphate quantitatively eluted with cold deionized water. The orthophosphate level (after correction for volume changes) of the column eluate equaled the level in the untreated extract indicating that no hydrolysis of acid labile phosphates occurred. Acid-soluble inorganic polyphosphate was determined as Δ-8 min labile phosphate in the column eluate. The difference between the total phosphate level of the column eluate and of the untreated trichloroacetic acid extract was designated the Norit-A adsorbable phosphate. The non-adsorbed organic phosphate was determined by subtracting the sum of the orthophosphate and Δ-8 min labile phosphate levels from the total phosphate level of the column eluate.

The acid-insoluble polyphosphate and the nucleic acids of the 1 N KOH extract were separated on Norit-A columns identical to those previously described. The 1 N KOH extracts were adjusted to approx. pH 6 before addition to the columns. The nucleic acids were adsorbed to the Norit-A and the acid-insoluble polyphosphate was eluted with deionized water.

Results and Discussion

Polyphosphate extraction and quantitation

Schmidt and King[26] attempted to extract acid-insoluble polyphosphate quantitatively from the high temperature strain of C. pyrenoidosa with a 5-min extraction at 95° with 5% trichloroacetic acid, after the cells had been previously extracted with cold 5% trichloroacetic acid, 70% ethanol, and a solution of ethanol-ether. This hot trichloroacetic acid extraction of polyphosphate had been used previously by Wiame[29] and by Mudd et al.[18]. Wiame[29] and Chayen et al.[6] showed that natural or synthetic polyphosphates were 30% hydrolyzed to orthophosphate under the above conditions; therefore, all polyphosphate levels were corrected for 30% hydrolysis after extraction. Many investigators[18,19,21,23,30] precipitated the polyphosphate from the trichloroacetic acid extract in the cold with barium at approx. pH 4.2. The orthophosphate released from the barium precipitate after a 7-min hydrolysis at 100° with 1 N HCl or H_2SO_4 was assumed to be polyphosphate-phosphate.

Schmidt[25] ran an orthophosphate analysis along with a 7-min labile phosphate analysis directly on the hot trichloroacetic acid extract of C. pyrenoidosa. It was assumed that during the 7-min labile phosphate analysis that the hydrolysis of the phosphate anhydride bonds of polyphosphate was complete, while the hydrolysis of the sugar-phosphate ester linkages of the nucleic acids and the amino acid-phosphate ester bonds in phosphoproteins was assumed to be negligible. The level of 7-min labile phosphate in the hot trichloroacetic acid extract, when corrected for 30%

hydrolysis, equaled the combined levels of orthophosphate and 7-min labile phosphate (uncorrected for hydrolysis) in the extract. Therefore, all of the orthophosphate in the hot trichloroacetic acid extract could seemingly be accounted for as arising from the 7-min labile phosphate (polyphosphate) during the hot trichloroacetic acid extraction. However, recent investigations in this laboratory (Fig. 1) have shown that only approx. 50% of the cellular polyphosphate is removed from C. pyrenoidosa by a 5-min hot trichloroacetic acid extraction. Longer extraction times were undesirable because of considerable release of orthophosphate into the extract from other cellular components, probably nucleic acids and phosphoproteins. The apparent hydrolysis of nucleic acids or phosphoproteins, during extended extraction times in 5% trichloroacetic acid at 95°, necessitated a study of the possible instability of nucleic acids in the trichloroacetic acid extract to the 7-min labile phosphate analysis as used by Schmidt[25] and Schmidt and King[26]. Herrmann[11] of this laboratory, demonstrated 4% hydrolysis of RNA and DNA in 1 N H_2SO_4 at 100° in 7 min. Thus, the validity of the analysis for 7-min labile phosphate in the presence of nucleic acids seems questionable.

A search for a method in which polyphosphate could be quantitatively extracted from the cells without undergoing hydrolysis to orthophosphate led to the use of the Schmidt and Thannhauser[23] extraction procedure. An extraction-time study was made (Fig. 2B) to determine the minimum time required to quantitatively extract nucleic acid and polyphosphate from the cells. After 30 min the total phosphate of the 1 N KOH extract ceased to increase significantly. Herrmann[11] observed a similar trend for

Fig. 1. Extraction of phosphorus compounds from a lipid-free cell residue of C. pyrenoidosa with 5% trichloroacetic acid (TCA) at 95°.

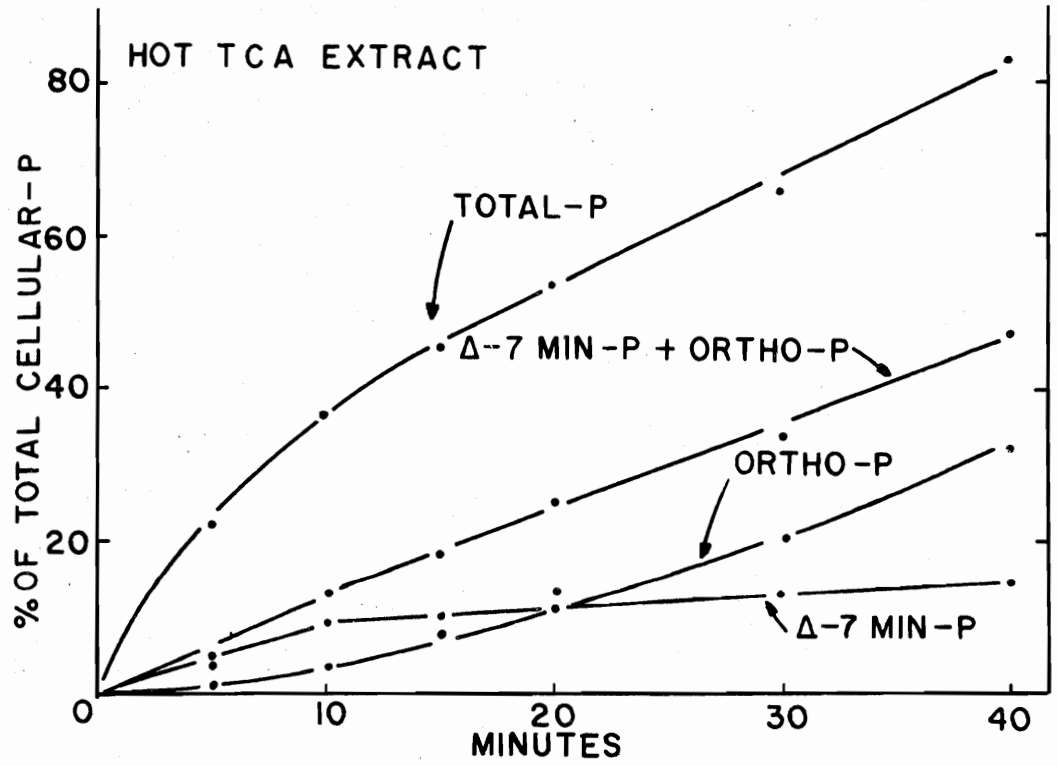
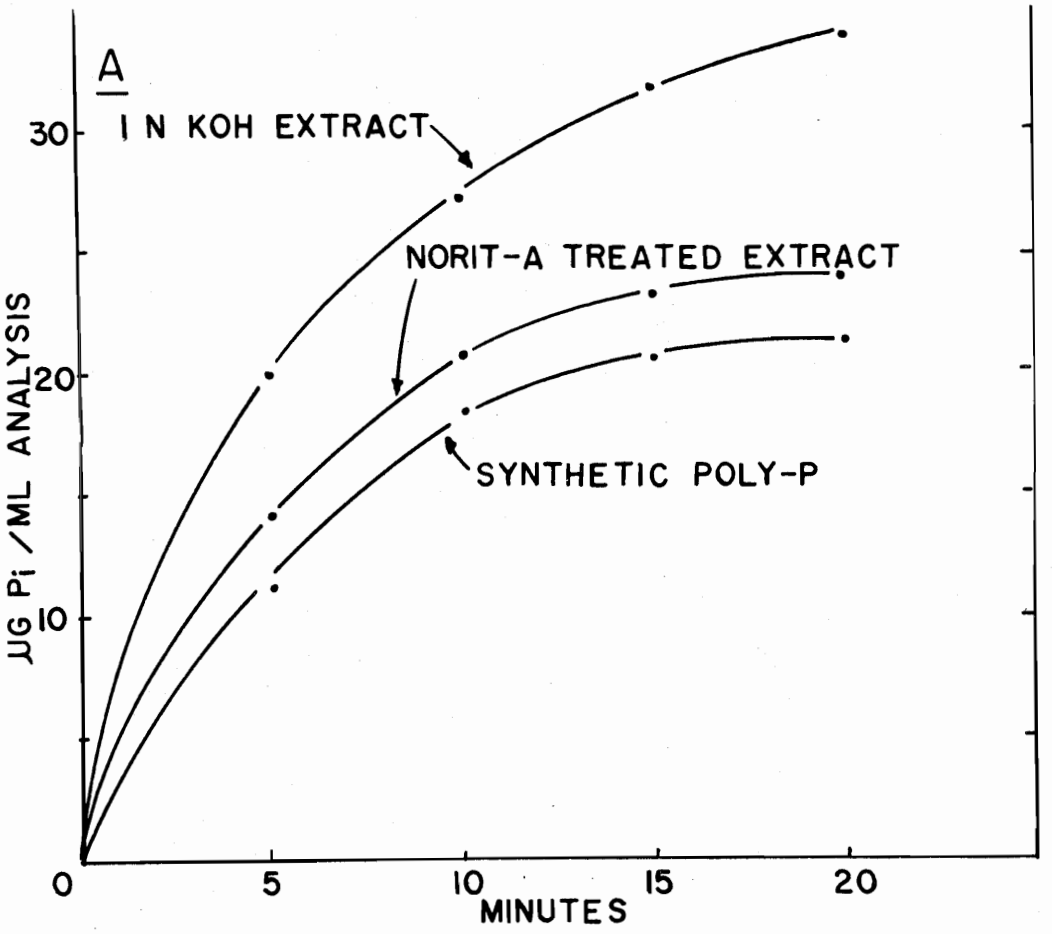
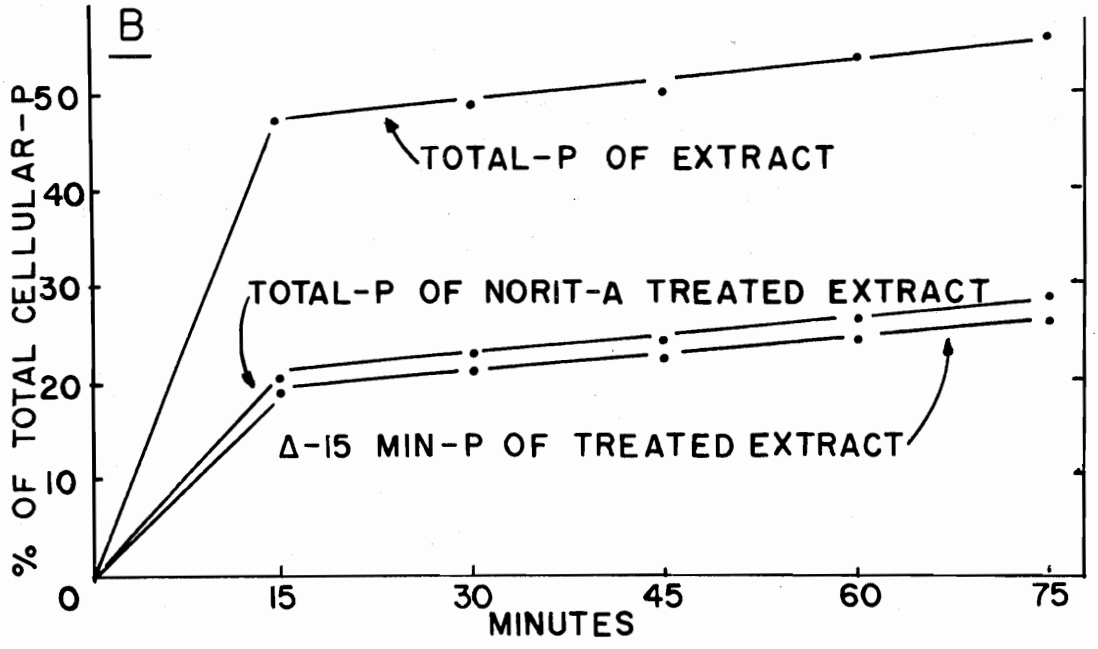

HOT TCA EXTRACT
% OF TOTAL CELLULAR-P
80
60
40
20
0
TOTAL-P
Δ-7 MIN-P + ORTHO-P
ORTHO-P
Δ-7 MIN-P
10
20
30
40
MINUTES

Fig. 2A. Comparison of the hydrolysis (in 1 N H_2SO_4 at 100°) of the phosphorus compounds in a 1 N KOH extract from a lipid-free cell residue of C. pyrenoidosa, before and after treatment with Norit-A, with a synthetic inorganic high-molecular-weight polyphosphate. (B) Extraction of phosphorus compounds from a lipid-free cell residue of C. pyrenoidosa with 1 N KOH at 37°.


A
1 N KOH EXTRACT
NORIT-A TREATED EXTRACT
SYNTHETIC POLY-P
JUG Pi /ML ANALYSIS
30
20
10
0
5
10
15
20
MINUTES


B
TOTAL-P OF EXTRACT
TOTAL-P OF NORIT-A TREATED EXTRACT
Δ-15 MIN-P OF TREATED EXTRACT
% OF TOTAL CELLULAR-P
50
40
30
20
10
0
15
30
45
60
75
MINUTES

RNA-ribose and ultraviolet absorption at 260 mμ for the extract. Extraction periods longer than 60 min or with 2 N KOH, as used by Miyachi and Tamiya[17], resulted in considerable solubilization of the protein fraction of the cells. The protein gave the extract an extremely high viscosity and a dark amber color which interferred with subsequent polyphosphate analysis. Therefore, polyphosphate extractions were restricted to 60 min. At the end of the 60-min extraction period, no orthophosphate could be detected in the extract.

Before attempting to develop a quantitative analysis for the polyphosphate, extracted with 1 N KOH from C. pyrenoidosa, some of the chemical properties of a high-molecular-weight synthetic polyphosphate synthesized by the method of Pfanstiel and Iler[20] as modified by Kornberg[14] were investigated. The hydrolysis of the synthetic polyphosphate to orthophosphate in 1 N H_2SO_4 at 100° is shown in Fig. 2A. The hydrolysis of the polyphosphate was not complete in 7 min as previously reported[30]. Approx. a 15-min hydrolysis period was required for the complete conversion of the polyphosphate to orthophosphate.

When the (neutralized) 1 N KOH extract was hydrolyzed in 1 N H_2SO_4 at 100°, orthophosphate continued to be released into the extract even after 15 min (Fig. 2A). This indicated that compounds other than high-molecular-weight polyphosphates were being hydrolyzed. It seemed probable that these compounds were nucleic acids. Therefore, the 1 N KOH extract was adjusted to approx. pH 6 and passed through a column of Norit-A to remove the nucleic acids. The polyphosphates in the extract were assumed not to be adsorbed by the Norit-A because synthetic polyphosphates

were not adsorbed under the same conditions. 95% of the total phosphate in the Norit-A column eluate could be hydrolyzed to orthophosphate in 15 min. The hydrolysis curve was similar to that obtained for the high-molecular-weight synthetic polyphosphate (Fig. 2A). No RNA[11] or protein[11] could be detected in the Norit-A column eluate; therefore, it appears that the main phosphate component of the eluate is a high-molecular-weight inorganic polyphosphate.

Harold[9] extracted nucleic acids and polyphosphates from Neurospora crassa with 0.5 N perchloric acid at 70°. The nucleic acids were removed from the acid extract with Norit-A. The polyphosphate was considered to be the acid labile phosphate remaining in the extract. It would seem, however, that this hot acid extraction would have the undesirable characteristics of hydrolyzing polyphosphate and phosphoproteins to orthophosphate.

Cellular growth

Before metabolic shifts observed during the synchronous growth of algae can be interpreted as truly reflecting the metabolism of a cell during normal development, it is imperative to show that neither nutrient concentration nor light intensity have become limiting to the cells during the course of synchronous growth.

It was reported earlier by this laboratory[24,26], when nutrient concentration and light intensity are in excess of the growth requirements for the completion of one synchronous growth cycle, synchronized cells exhibit linear logarithmic increases in dry weight, total cellular phosphorus,

and total cellular sulfate for the entire growth cycle. If light intensity or nutrient concentration did become limiting, a negative deviation from each of the linear logarithmic growth curves was observed.

In the experiments reported in this paper, linear logarithmic increases in total cellular phosphorus (Fig. 3) and cellular dry weight were observed throughout each synchronous growth cycle. The cell count data in Fig. 3 are representative of the high degree of synchrony that was obtained in these experiments.

Intracellular distribution of phosphate

During synchronous growth, the intracellular pools of acid-insoluble polyphosphate, cold trichloroacetic acid soluble phosphates, and phospholipids exhibited trends similar to those observed by Schmidt and King[26] (Fig. 4). However, the levels of intracellular polyphosphate, at any stage of synchronous growth, were higher than previously reported because of improved techniques of extraction and analysis. It should be emphasized that the level of polyphosphate phosphorus (as percentage of total cellular phosphorus) began to decrease 4h before the initiation of nuclear division and reached its lowest level of the growth cycle at the end of nuclear division. The pool of acid-soluble phosphates and phospholipids showed an inverse trend. Whether or not the acid-insoluble polyphosphate served as a phosphate donor for these two phosphate pools will have to be ascertained using radioactive phosphate as a tracer.

Cold trichloroacetic acid soluble phosphate

To determine which phosphate component(s) were contributing to the rapid increase in the total phosphate of the 0° trichloroacetic acid

Fig. 3. Relationship between cell number and total cellular phosphorus during synchronous growth of C. pyrenoidosa.

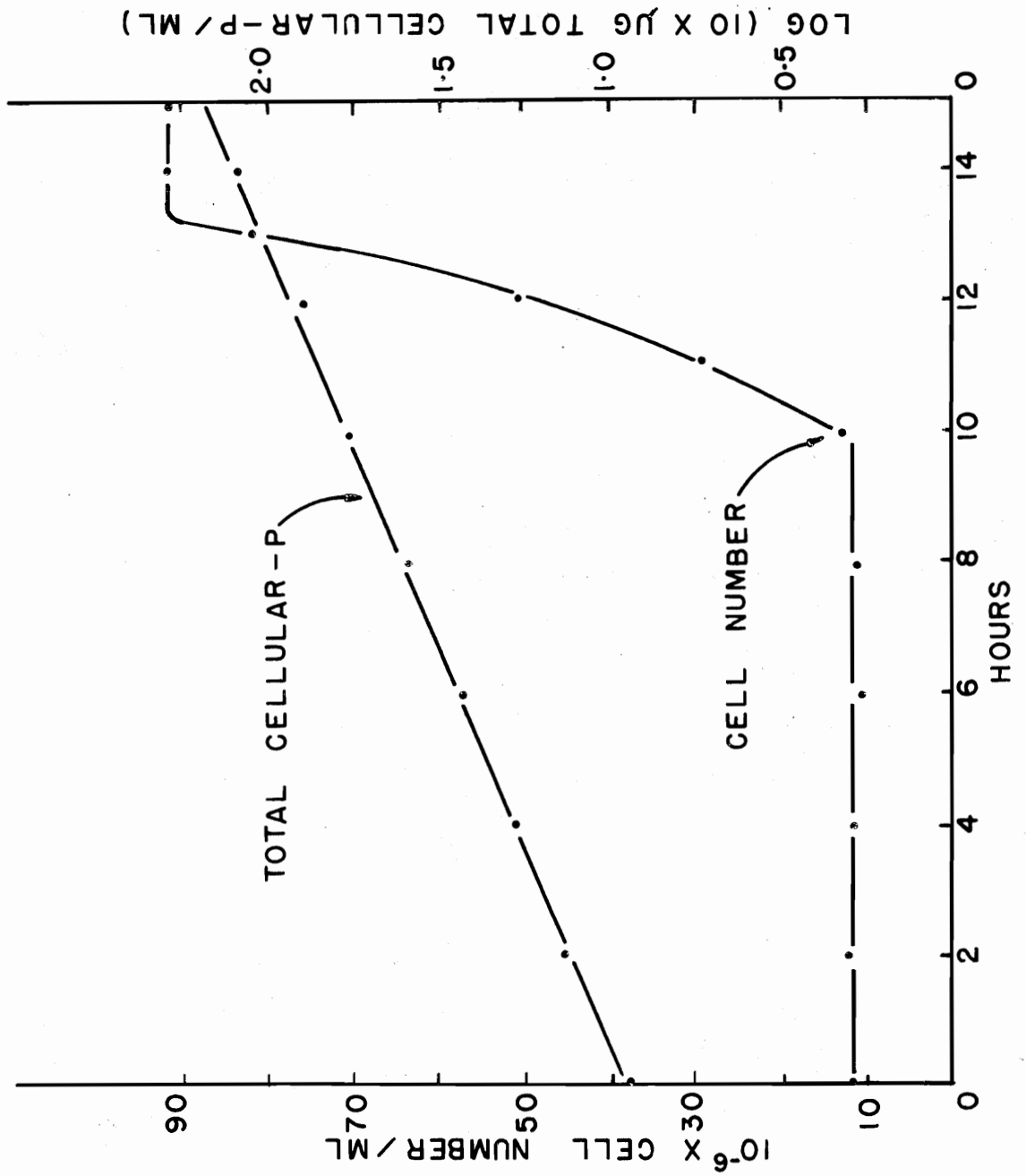
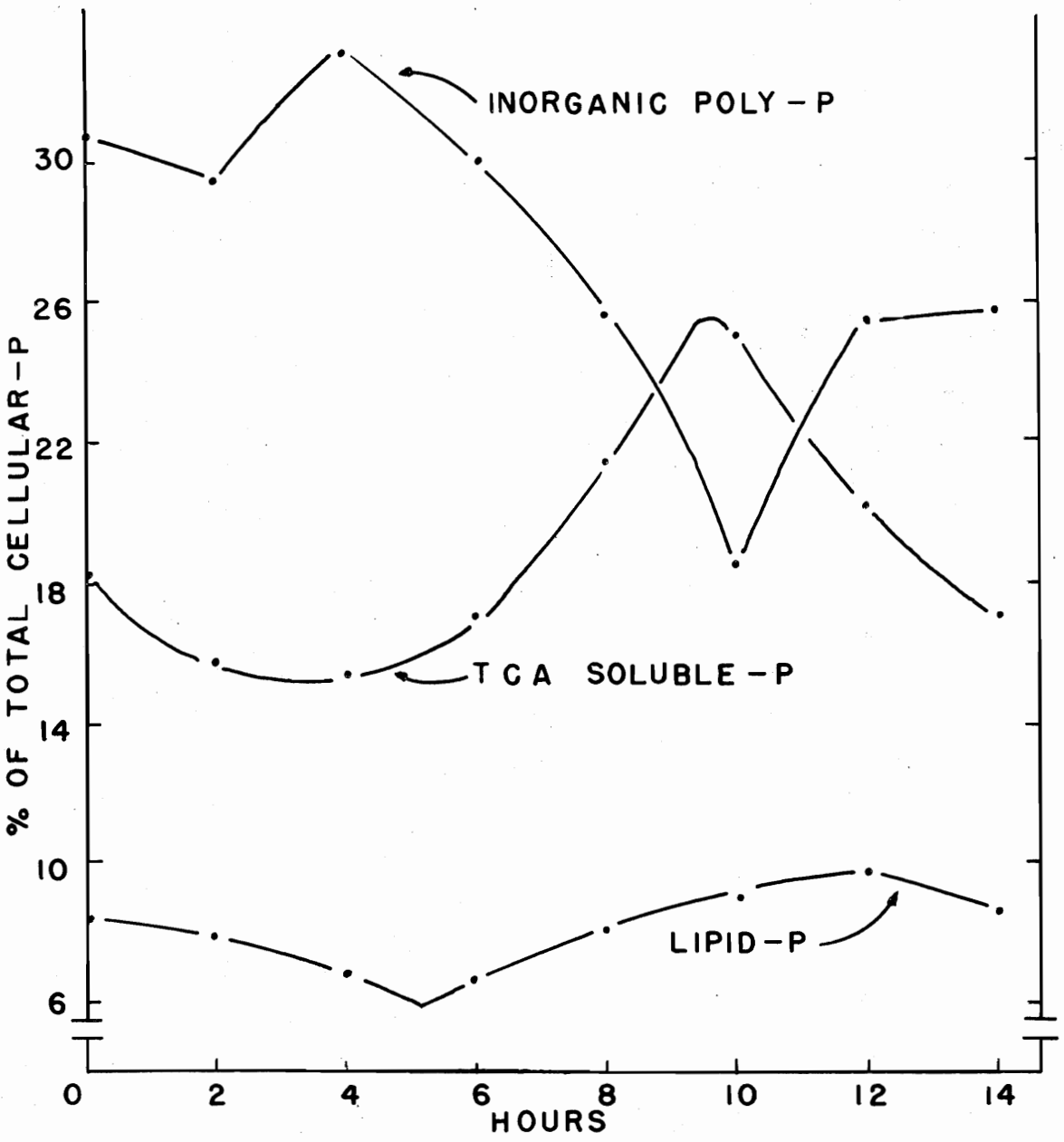

LOG (10 X µG TOTAL CELLULAR-P/ML)
2·0
1·5
1·0
0·5
0
10^{-6} X CELL NUMBER/ML
90
70
50
30
10
0
2
4
6
8
10
12
14
HOURS
TOTAL CELLULAR-P
CELL NUMBER

Fig. 4. Intracellular distribution of phosphorus during synchronous growth of C. pyrenoidosa. These data represent averages of two experiments. TCA, trichloroacetic acid.


INORGANIC POLY - P
TCA SOLUBLE - P
LIPID - P
% OF TOTAL CELLULAR - P
30
26
22
18
14
10
6
0
2
4
6
8
10
12
14
HOURS

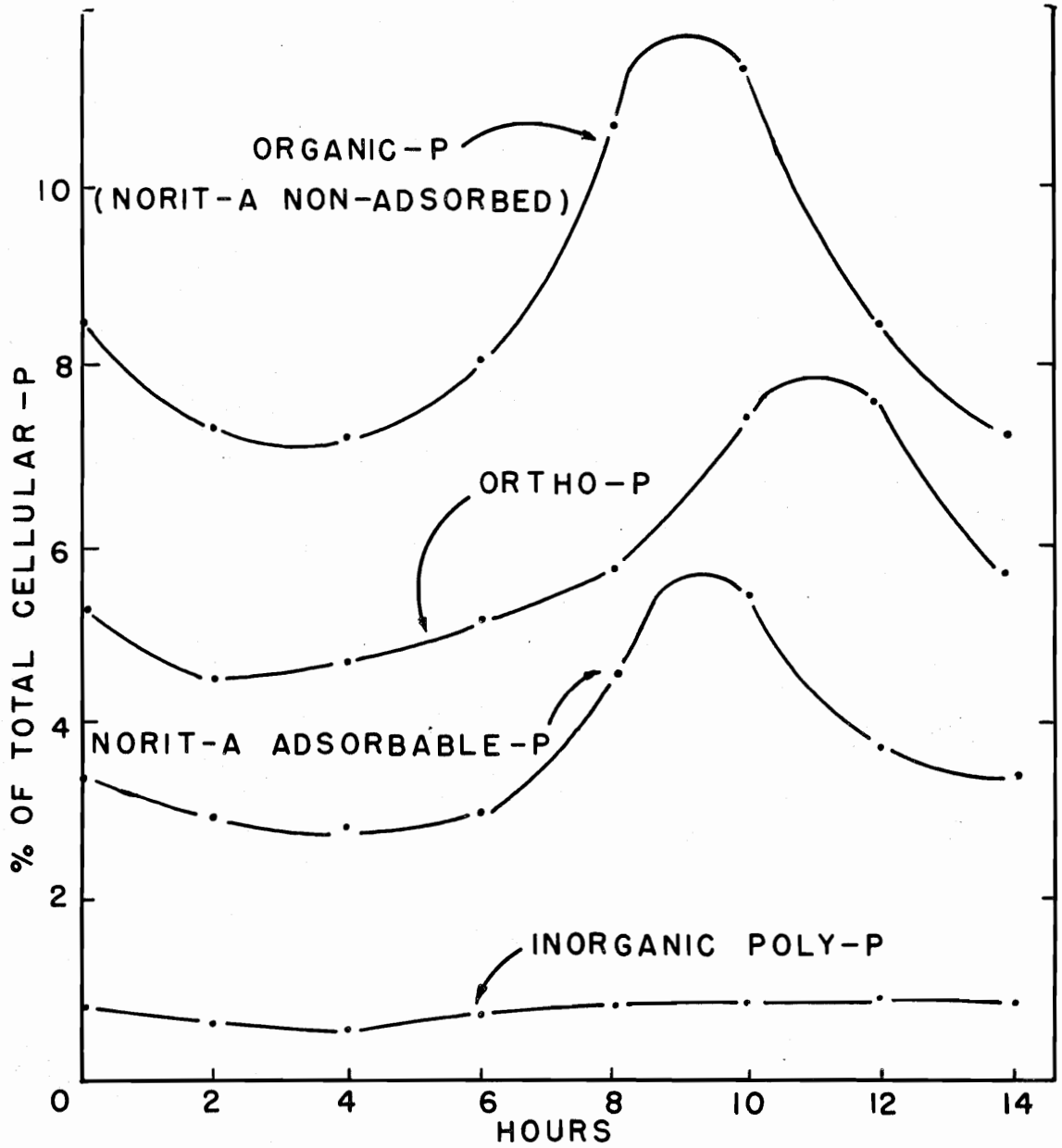
extract of the cells during nuclear division, the extract was separated into four phosphate fractions: orthophosphate, acid-soluble inorganic polyphosphate, Norit-A adsorbable phosphate, and organic phosphates not adsorbable on Norit-A.

When the phosphate fractions were expressed as percent of total cellular phosphate, all fractions increased during nuclear division except the acid-soluble polyphosphates (Fig. 5). The pool of acid-soluble polyphosphates decreased slightly during the early stages of growth, but returned to its initial level and remained essentially constant during the latter stages of cellular development.

Although orthophosphate, Norit-A adsorbable phosphate, and Norit-A non-adsorbable organic phosphate levels increased during nuclear division, only the Norit-A adsorbable phosphates and the Norit-A non-adsorbable organic phosphates reached their peak values during nuclear division. Orthophosphate reached its maximum value at the end of nuclear division. Thus, the phosphate components which contributed most significantly to the rise in total phosphate of the trichloroacetic acid extract were, in decreasing order of contribution, the organic phosphates not adsorbable on Norit-A, orthophosphate, and Norit-A adsorbable phosphates.

Miyachi and Miyachi[16], likewise, demonstrated periodism in the incorporation of phosphate into the trichloroacetic acid soluble fraction of synchronized cells of C. ellipsoidea during cellular development. However, their data suggested periodism was almost quantitatively due to changes in amounts of acid-soluble polyphosphate and nucleotide labile phosphate fractions. No periodism in the levels of orthophosphate and non-nucleotide

Fig. 5. Phosphorus components of the cold trichloroacetic acid extract from cells of C. pyrenoidosa during synchronous growth. These data represent averages of two experiments.


ORGANIC-P
(NORIT-A NON-ADSORBED)
ORTHO-P
NORIT-A ADSORBABLE-P
INORGANIC POLY-P
% OF TOTAL CELLULAR-P
HOURS
0
2
4
6
8
10
12
14

phosphate was reported. The phospholipids of C. ellipsoidea, likewise, did not fluctuate in concentration during synchronous growth as was observed in C. pyrenoidosa. Thus, there appears to be a striking difference in the phosphorus metabolism of these two organisms.

Until the trends for the synthesis of RNA and DNA are elucidated, and the phosphate components of various cellular extracts are identified and their metabolism traced with radioactive phosphate, speculation regarding the significance of the observed shifts in phosphorus metabolism in C. pyrenoidosa will be premature.

Summary

During the synchronous growth of Chlorella pyrenoidosa, strain 7-11-05, shifts in the levels of several cellular phosphate components were observed. Inorganic acid-insoluble polyphosphate reached its peak concentration early in cellular development, then decreased reaching a minimum value at the end of nuclear division. The phospholipids and the pool of acid-soluble phosphates exhibited an inverse trend by increasing dramatically during nuclear division. The pool of acid-soluble phosphate was fractionated into orthophosphate, Norit-A adsorbable phosphate, organic phosphates non-adsorbable on Norit-A, and acid-soluble polyphosphates. All acid-soluble phosphate components, except the polyphosphates, increased during nuclear division. The acid-soluble polyphosphate level remained essentially constant throughout cellular development.

Part 2.

Further Studies on the Intracellular Distribution of Phosphorus During Synchronous Growth of *Chlorella pyrenoidosa*

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Introduction

The rapidly increasing volume of literature concerning the synchronous growth of microorganisms (as reviewed by Burns[4], Scherbaum[22], Zeuthen[31], and Campbell[5]) is indicative of the realization by many investigators that the synchronization technique is useful for mapping the biochemical and biophysical events associated with the stepwise growth and division of a single cell during its normal life history. However, at least two criteria must be satisfied before the metabolic shifts observed during synchronous growth can be said to truly reflect the "normal" behavior of a cell during its development. First, it should be demonstrated that periodism in any biochemical or biophysical event is recurrent in successive generations of synchronous growth without repeated exposure of the cells to the synchronization treatment. If the metabolism of synchronized cells is only followed in the first growth cycle following the synchronization treatment, it is possible that the observed metabolic shifts do not reflect the normal metabolic behavior of the cells, but merely a convalescence from an environmental stress or shock. Second, although culture conditions (nutrient concentration, pH,

light intensity, etc.) may be changing during synchronous growth, it is essential to show that the limits within which these change do not at any time during synchronous growth change the growth rate of the cells. For example, if the essential nutrients are not in excess of the growth requirements of the cells for the completion of one synchronous growth cycle, the depletion of these nutrients would limit the growth rate of the cells and cause metabolic shifts which may be falsely assumed to be typical of normal cellular development. Too frequently, either one or both of the above criteria are overlooked by investigators employing synchronized cells as biochemical tools. Therefore, it is impossible to speculate on the interrelationships between metabolic events recorded for the spectrum of organisms under investigation.

In the present paper, an attempt is made to evaluate the effect of the synchronization treatment (intermittent illumination) on the intracellular distribution of phosphorus during synchronous growth of Chlorella pyrenoidosa. The experimental approach involves following the intracellular distribution of phosphorus through several generations of synchronous growth to determine whether the periodism in phosphate metabolism observed in the first cycle[1], immediately after the synchronization treatment, would be recurrent through all synchronous growth cycles, thereafter. Evidence is presented which shows to what extent periodism in phosphorus metabolism is induced by intermittent illumination.

Methods and Materials

Organization and culture conditions

The alga used in these studies was the high temperature strain 7-11-05 of C. pyrenoidosa described by Sorokin and Myers[27]. The cells were synchronized by the intermittent illumination method of Schmidt and King[26] as modified by Baker and Schmidt[1]. The cultural conditions, growth chamber, and culture medium were identical with those described by Baker and Schmidt[1].

After the cells had completed one synchronous growth cycle (14 h) following the synchronization treatment, the cells were diluted with fresh culture medium, pre-equilibrated at 38.5° with CO_2-air (3:97), to the original concentration existing prior to cellular division. This dilution allowed the cells to complete a second and part of a third generation of logarithmic growth, in cellular dry weight and total cellular phosphorus, during which the studies reported in this paper were made.

In an experiment to determine the effect of storage at -15° on the intracellular distribution of phosphorus in fresh frozen cells, asynchronous cells were cultured for 48 h in continuous light prior to harvest.

Biochemical methods

To determine the intracellular distribution of phosphorus during the second cycle of synchronous growth, cells were harvested at one or two hourly intervals from the initiation of cell division (daughter cell release) in the first synchronous growth cycle until the completion of cell division at the end of the second cycle. Cell number per milliliter of culture was determined using a Levy-Hausser hemacytometer. Total cellular phosphorus

was determined by the method of Fiske and Subbarow[8] after the cells were washed as previously described[1]. Approximately 1.0 ml packed cell volume of cells was washed, frozen, and stored at -15° until extraction with (a) cold trichloroacetic acid (b) cold 95% ethanol (c) ethanol-ether at 45° (d) KOH at 37°. The phosphorus components in each extract were separated and analyzed by the procedures of Baker and Schmidt[1]. Total nucleic acid phosphorus was estimated as the difference between the total phosphorus of the KOH extract and its polyphosphate content.

The asynchronous cells which were used to study the effect of prolonged storage at -15° on the intracellular distribution of phosphorus were washed and resuspended in buffer. Aliquots of equal size were pipetted from this suspension into centrifuge tubes, centrifuged to pellets, and stored at -15°. At intervals over a period of one month, pellets of cells were removed from storage, immediately extracted, and the intracellular phosphorus components were determined[1].

Results and Discussion

Hydrolysis of intracellular phosphorus compound(s) in frozen algae

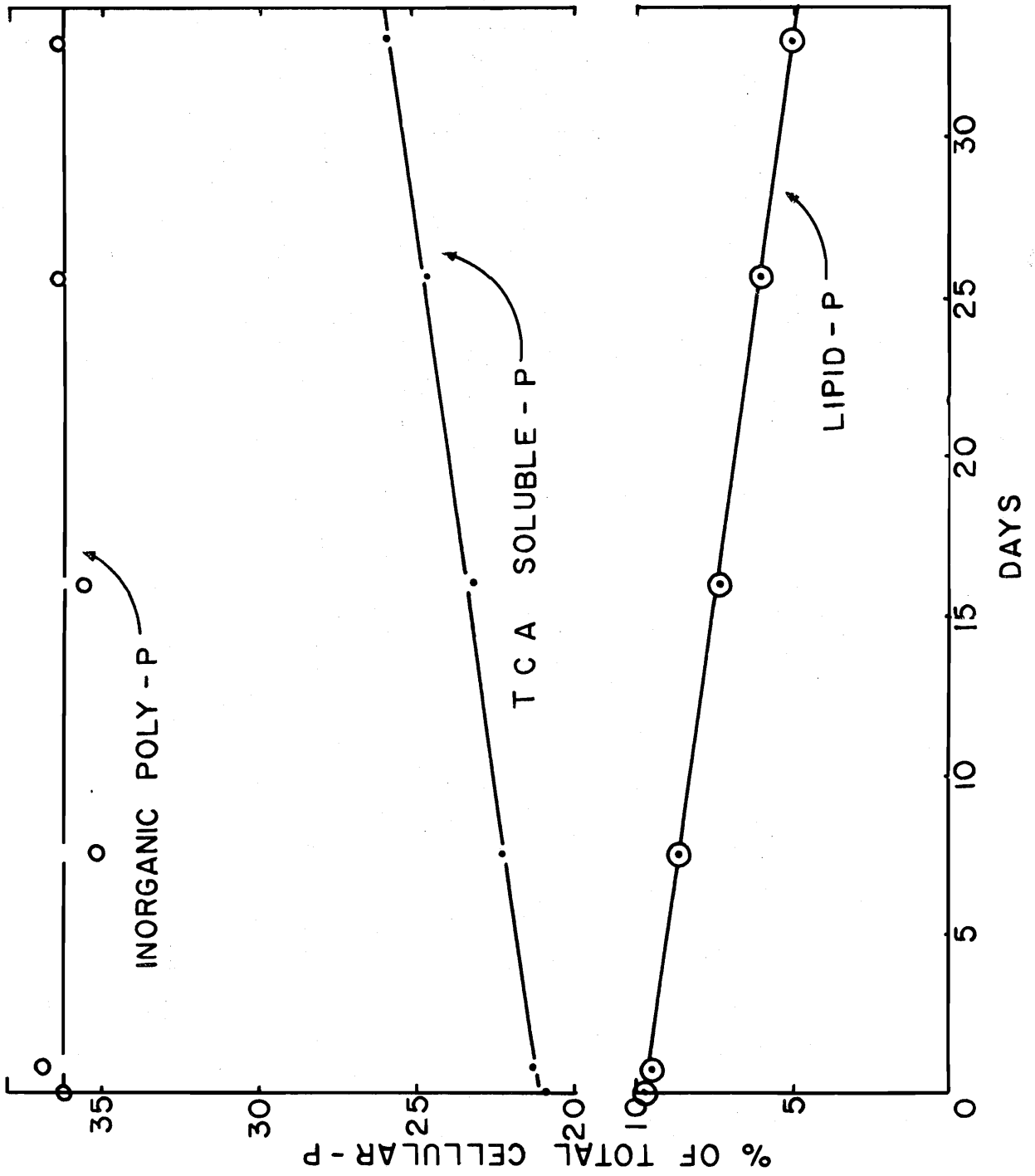
In replicate experiments, in which the intracellular distribution of phosphorus was followed during the first cycle of synchronous growth of *C. pyrenoidosa*[1], the periodism observed in the distribution of all phosphorus components during cellular development was very reproducible. However, the absolute quantity of phosphorus (expressed as percent of total cellular phosphorus) in certain cellular extracts varied between replicate experiments. It appeared that more phosphorus was extractable

with cold trichloroacetic acid in those experiments in which the cells had been frozen and stored for long periods (1-3 months) at -15° prior to extraction and analysis. The increased extraction of acid-soluble phosphorus appeared to be at the expense of phospholipid phosphorus. Therefore, a study was made, during a one-month period, to determine the effect of -15° storage on the intracellular distribution of phosphorus in fresh frozen algae (Fig. 1).

The level of inorganic acid-insoluble polyphosphate remained unchanged throughout the one-month storage period. However, the phospholipid phosphorus level decreased linearly (5%) during this same period. The loss of phosphorus from the phospholipid fraction could be quantitatively accounted for in the linear increase in the cold trichloroacetic acid-extractable phosphorus. The increase in the acid-soluble phosphorus could be attributed to increases in orthophosphate and Norit-A non-adsorbable organic phosphorus.

Thus, it appears that prolonged storage of fresh frozen cells at -15° results in the chemical or enzymic hydrolysis of the phospholipid fraction to orthophosphate and some organic phosphorus component(s) soluble in cold trichloroacetic acid. Therefore, in order to obtain a more truly quantitative picture of the phosphorus pool sizes during cellular development, all studies on synchronous cultures reported in this and further research papers from this laboratory will be made on cells stored no longer than one week at -15°.

Fig. 1. The effects of freezing and storage at -15° upon the distribution of intracellular phosphorus in asynchronous C. pyrenoidosa.


% OF TOTAL CELLULAR-P
INORGANIC POLY-P
TCA SOLUBLE-P
LIPID-P
DAYS
0
5
10
15
20
25
30
35

Cellular growth

It was reported earlier from this laboratory[1,13,24-26] that when nutrient concentration and light intensity are in excess of the growth requirements for the completion of one synchronous growth cycle, synchronized cells of C. pyrenoidosa exhibit linear logarithmic increases in dry weight, total cellular phosphorus, and total cellular sulfur for the entire growth cycle. If light intensity or nutrient concentration did become limiting, a negative deviation from each of the linear logarithmic growth curves was observed.

In the experiments reported in this paper, linear logarithmic increases in total cellular phosphorus (Fig. 2) and cellular dry weight were observed during two consecutive synchronous growth cycles in continuous light. The cells were diluted at the end of the first cycle to prevent low light intensity or nutrient concentration from limiting the growth rate of the cells in the second cycle. It should be noted that the rate of increase (slope) of total cellular phosphorus was virtually the same in both cycles. The cell count data shown in Fig. 2 are indicative of the high degree of synchrony maintained through both synchronous growth cycles.

Intracellular distribution of phosphorus during synchronous growth

The intracellular levels (expressed as percent of total cellular phosphorus) of acid-insoluble inorganic polyphosphate, cold trichloroacetic acid-soluble phosphates, and phospholipids during part of the first, the entire second, and part of the third cycles of synchronous growth are shown in Fig. 3A.

Fig. 2. Relationship between cell number and total cellular phosphorus during successive synchronous growth cycles of C. pyrenoidosa.

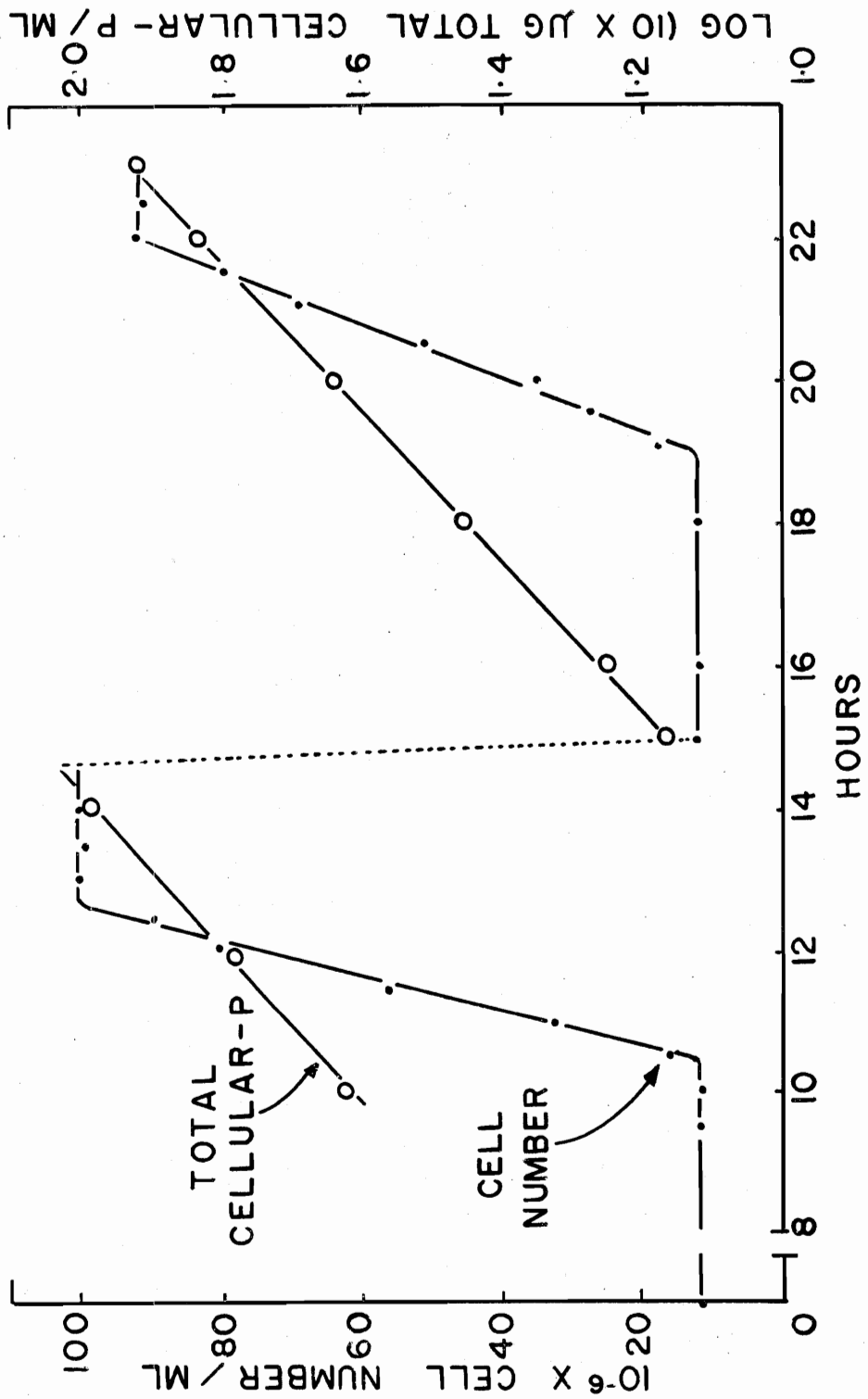
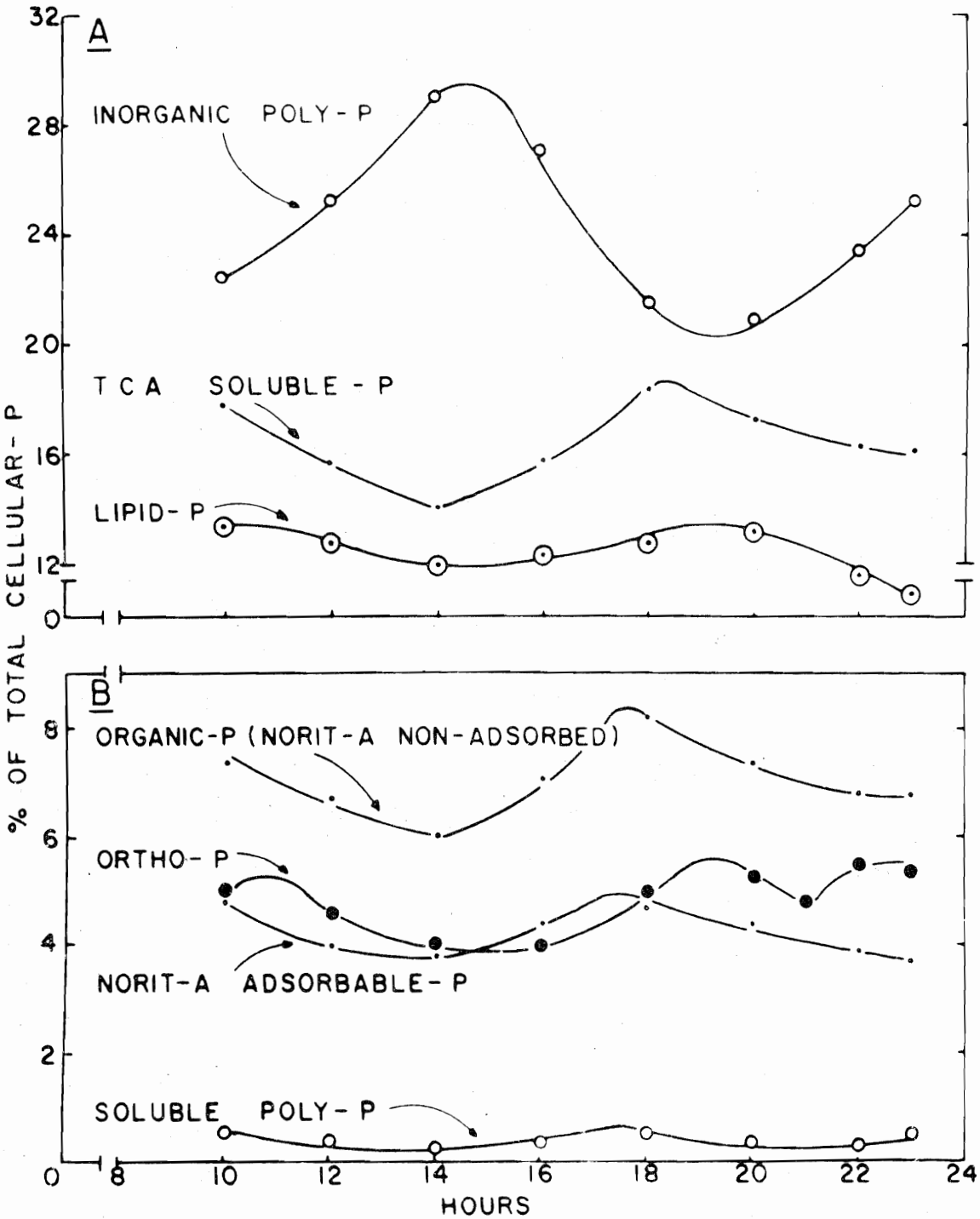

LOG (10 X µG TOTAL CELLULAR- P/ML)
2.0
1.8
1.6
1.4
1.2
1.0
10-6 X CELL NUMBER/ML
100
80
60
40
20
0
TOTAL CELLULAR-P
CELL NUMBER
8
10
12
14
16
18
20
22
HOURS

Fig. 3. (A) Intracellular distribution of phosphorus during synchronous growth of C. pyrenoidosa. (B) Phosphorus components of the cold trichloroacetic acid extract from cells of C. pyrenoidosa during synchronous growth. Nuclear division began at 7.75 h and cellular division was initiated at 10.25 h or cellular development in the first growth cycle; these events occurred at 17.5 h and 19.0 h, respectively, in the second growth cycle.


A
INORGANIC POLY-P
TCA SOLUBLE-P
LIPID-P
B
ORGANIC-P (NORIT-A NON-ADSORBED)
ORTHO-P
NORIT-A ADSORBABLE-P
SOLUBLE POLY-P
% OF TOTAL CELLULAR-P
HOURS
0
12
16
20
24
28
32
2
4
6
8
10
12
14
16
18
20
22
24

Acid-insoluble inorganic polyphosphate increased steadily in concentration, from the initiation of cell division (daughter cell release) in the first synchronous growth cycle, until reaching a maximum value during the first half of the second cycle. After reaching this maximum, the level of the polyphosphate decreased steadily during nuclear division (nuclear division begins approx. 2.25-2.5 h prior to cell division[25]) reaching its lowest concentration at the beginning of cell division in the second cycle. The same periodism in the levels of this phosphate polymer appear to continue into the third cycle of synchronous growth.

However, the pattern of acid-insoluble polyphosphate accumulation, during the first cycle of synchronous growth, as reported earlier by this laboratory[1], was somewhat different from that reported above for the second and third cycles of synchronous growth. In the first cycle, the level of polyphosphate decreased significantly during the first 2 h of growth, but increased to its highest level of the cycle by the end of the fourth hour of growth. (The initial decrease in the level of acid-insoluble polyphosphate, during early cellular development, was recently confirmed by sampling at hourly intervals during the first 5 h of synchronous growth.) After the fourth hour, however, the periodism in polyphosphate accumulation was identical to that observed in the second and third cycles of synchronous growth. It appears, therefore, that (a) the synchronization treatment (intermittent illumination) only significantly alters polyphosphate metabolism during the first 4 h of synchronous growth immediately following this treatment (b) the recurrent decrease in polyphosphate level immediately prior to and during nuclear division is a normal characteristic of cellular development in this organism.

Preliminary results, from studies on nucleic acid metabolism during synchronous growth, have revealed that the accumulations of total nucleic acid phosphorus and acid-insoluble polyphosphate phosphorus (percent of total cellular phosphorus) exhibited an inverse relationship to each other. For example, the peculiar shifts in polyphosphate level, observed during the first 4 h of synchronous growth in the first but not in the second or third cycles, were followed by almost equal but opposite shifts in the level of total nucleic acid phosphorus during the same time interval. Thus, it appears that the synchronization procedure induces abnormal, non-recurrent periodism in both nucleic acid and polyphosphate phosphorus metabolism during early growth in the first synchronous growth cycle.

The periodism in levels of cold trichloroacetic acid-soluble phosphates and phospholipids during the second growth cycle (Fig. 3A) was very similar to that reported for the first cycle of synchronous growth. However, at the beginning of the third growth cycle (21-23 h), several deviations from this periodism were observed. During early growth in the third growth cycle, the phospholipid level decreased to a lower value than observed during early growth in the preceding growth cycles. Concomitantly, the trichloroacetic acid-soluble phosphorus level increased instead of decreasing as observed during a similar stage of cellular development in the first and second growth cycles. The increase in trichloroacetic acid-soluble phosphorus was attributable to increases in orthophosphate and organic phosphorus (Fig. 3B).

The apparent difference in the metabolism of the trichloroacetic acid-soluble phosphates and phospholipids at the beginning of the third

growth cycle is difficult to explain. However, it is possible that (a) the periodism, observed during the first two growth cycles, for these two cellular fractions, was induced by the synchronization treatment and that only by the beginning of the third cycle was the "normal" pattern of the metabolism of these cellular components becoming apparent, or (b) the metabolism of the daughter cells beginning the third growth cycle was altered by the accumulation of metabolic waste products in the culture medium from the previous two cycles, in spite of the dilution (8-fold) of the cells with fresh medium at the end of the first cycle.

The second explanation has a weakness in that these metabolic waste products would have to be specific inhibitor(s) of only the processes associated with the metabolism of one or more of the trichloroacetic acid-soluble and phospholipid components, since phosphate uptake, respiration rate[7], and the levels of the other cellular phosphate components do not deviate from the trends observed in the second cycle. However, the high viscosity and foaming of the culture medium at the beginning of the third growth cycle do indicate an accumulation of extracellular substances. Further experimentation will be necessary to clarify this issue.

Summary

After synchronization by intermittent illumination, the intracellular distribution of phosphorus in Chlorella pyrenoidosa was followed for several successive synchronous growth cycles in continuous light. The synchronization procedure induced non-recurrent periodism in the

concentrations of acid-insoluble inorganic polyphosphate and total nucleic acid phosphorus during early stages of growth in the first growth cycle. Periodism in the levels of acid-soluble phosphates and phospholipids was similar throughout the first and second synchronous growth cycles, but deviated from established trends at the beginning of the third cycle. The deviations in the periodism of these two cellular fractions are discussed in reference to the synchronization procedure and to the accumulation of metabolic waste products from the first two growth cycles. A study was also made of the effects of freezing and storage of cells at -15° on the intracellular distribution of phosphorus.

Part 3.

Polyphosphate Metabolism During Nuclear Division in Synchronously Growing Chlorella

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Recent investigations in this[1,2,26] and other[10,16-18,21] laboratories have attempted to elucidate the metabolic role of inorganic acid-insoluble polyphosphate, $(P_i)_x$, in organisms that accumulate this phosphate polymer during cellular growth. The intracellular level of $(P_i)_x$ in synchronously growing Chlorella pyrenoidosa was previously reported[1,2,26] to fluctuate dramatically from 35 percent of the total cellular phosphorus during early growth to 18 percent by the end of the period of nuclear division. Furthermore, a reciprocal relationship was observed between the levels of $(P_i)_x$ and those of nucleic acid-[2,11], acid-soluble-[1,2,26], and phospholipid-[1,2,26] phosphates during synchronous growth of this organism. These studies suggested that $(P_i)_x$ may be serving, during normal cellular development, as an intracellular phosphorus and/or energy source for the synthesis of nucleic acids and other phosphate compounds during nuclear division, a period when phosphorus and/or energy demands of the cell accelerate. The present study indicates, however, that $(P_i)_x$ does not serve as a phosphorylating agent during normal cellular growth and division.

The alga used in this study was the high temperature strain 7-11-05 of C. pyrenoidosa. The cells were synchronized by the intermittent

illumination method of Schmidt and King[26] as modified by Baker and Schmidt[1]. During synchronization, the cells were cultured on a medium[1] containing ^{32}P-phosphate (1.8 mC/g P). By the end of the third dark period of the synchronization procedure, the cells were uniformly labeled. After synchronization, the cells were harvested by centrifugation, resuspended in fresh radioactive medium to a cell density of 10^8 cells per ml, and transferred to a flat Plexiglass growth chamber[24] of 1/4" inside thickness with a capacity of 900 ml. Under these conditions, an exponential increase in total cellular phosphorus was observed for the duration of the experiment (10 h).

After 7 h of synchronous growth (immediately prior to the initiation of nuclear division), the cells in the entire culture were separated from the radioactive culture medium by rapid filtration onto Millipore filters followed by several washes with non-radioactive culture medium. The cells were resuspended to their original cell density in non-radioactive culture medium, returned to the growth chamber, and equilibrated[2] for a short time before turning on the lights. One-half hour elapsed from the moment of harvest until the cells were returned to conditions optimum for their growth.

Samples of 100 ml of culture were taken at 50 min intervals from 7.5 to 10 h of synchronous growth (the period of nuclear division[2,7]). The cells were separated immediately from the culture medium by filtration, washed, and the intracellular phosphate components extracted, separated, and determined as previously reported[1,2]. Radioactivity of the phosphate fractions was determined using a Model 500 B Tri-Carb Liquid Scintillation Spectrometer.

Although the intracellular level of $(P_i)_x$ appeared to shift dramatically during synchronous growth of C. pyrenoidosa when expressed as percent of total cellular phosphorus (Fig. 1), no periodism was observed when the concentration of this phosphate polymer was expressed on a per cell basis. The $(P_i)_x$ level, expressed on a per cell basis, increased linearly throughout cellular development (Fig. 1).

Because the uptake of orthophosphate, P_i, is an exponential function during cellular development while $(P_i)_x$ deposition is a linear function, a greater percentage of the P_i absorbed during early growth is utilized for $(P_i)_x$ synthesis than in the latter stages of cellular development. On a percentage basis, therefore, the $(P_i)_x$ level would appear to increase during the initial phases and decrease during the latter stages of cellular development; thus, explaining the apparent paradox between the two methods of expressing the concentration of $(P_i)_x$.

The linear increase in $(P_i)_x$ throughout cellular development suggested that the polymer was not an intracellular phosphorylating source. The possibility remained, however, that the turnover rate of $(P_i)_x$ could vary during the growth cycle. The present study was conducted, therefore, to determine whether $(P_i)_x$ accumulated prior to nuclear division would exhibit significant turnover during this event when the demands for phosphorus and energy increase abruptly.

Fig. 2 shows the intracellular distribution of phosphorus in the nucleic acid, acid-soluble, lipid, and $(P_i)_x$ fractions during the period of nuclear division. The changes in total radioactivity of these phosphate fractions are indicative of the fate of endogenous phosphorus

Fig. 1. Polyphosphate accumulation during synchronous growth of C. pyrenoidosa expressed as percent of total cellular phosphorus (●---●) and as µg polyphosphate phosphorus per cell (▲---▲). Nuclear division began at 7.5 h and cell division at 10 h.

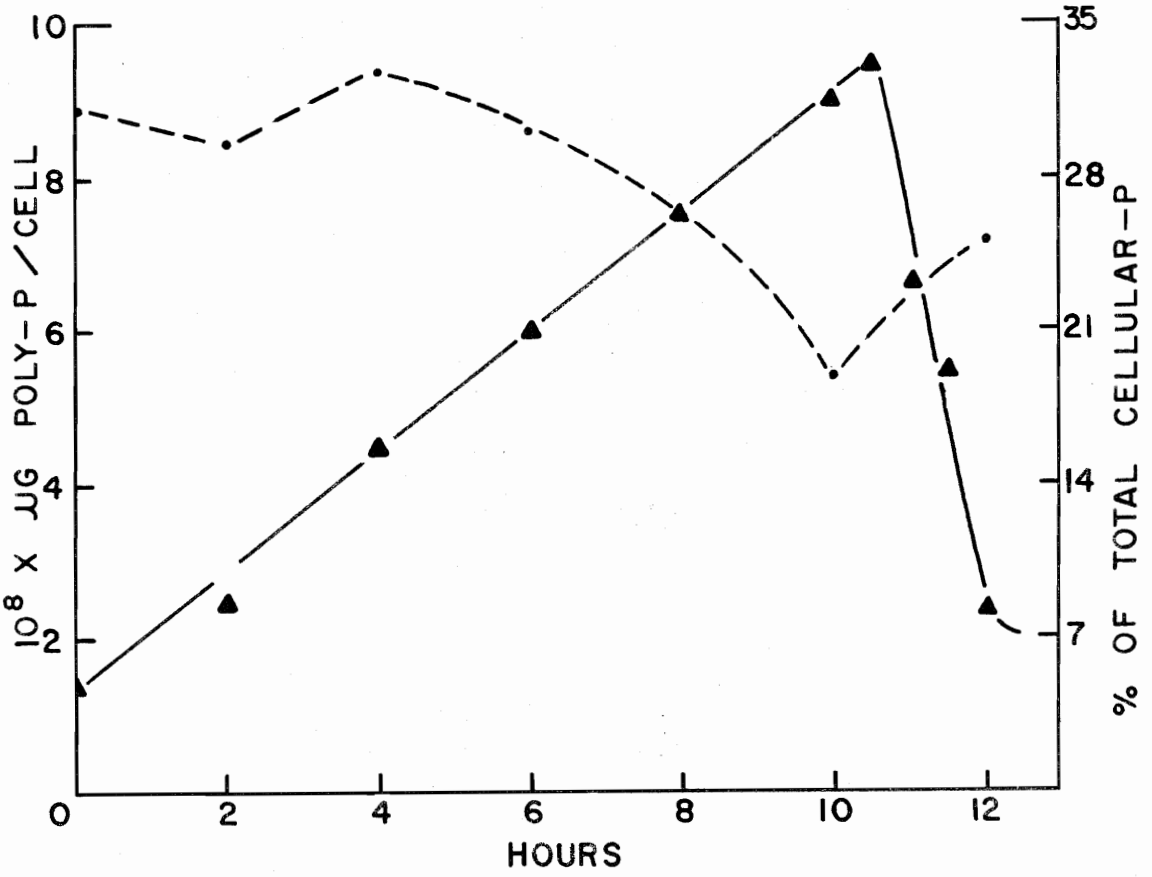
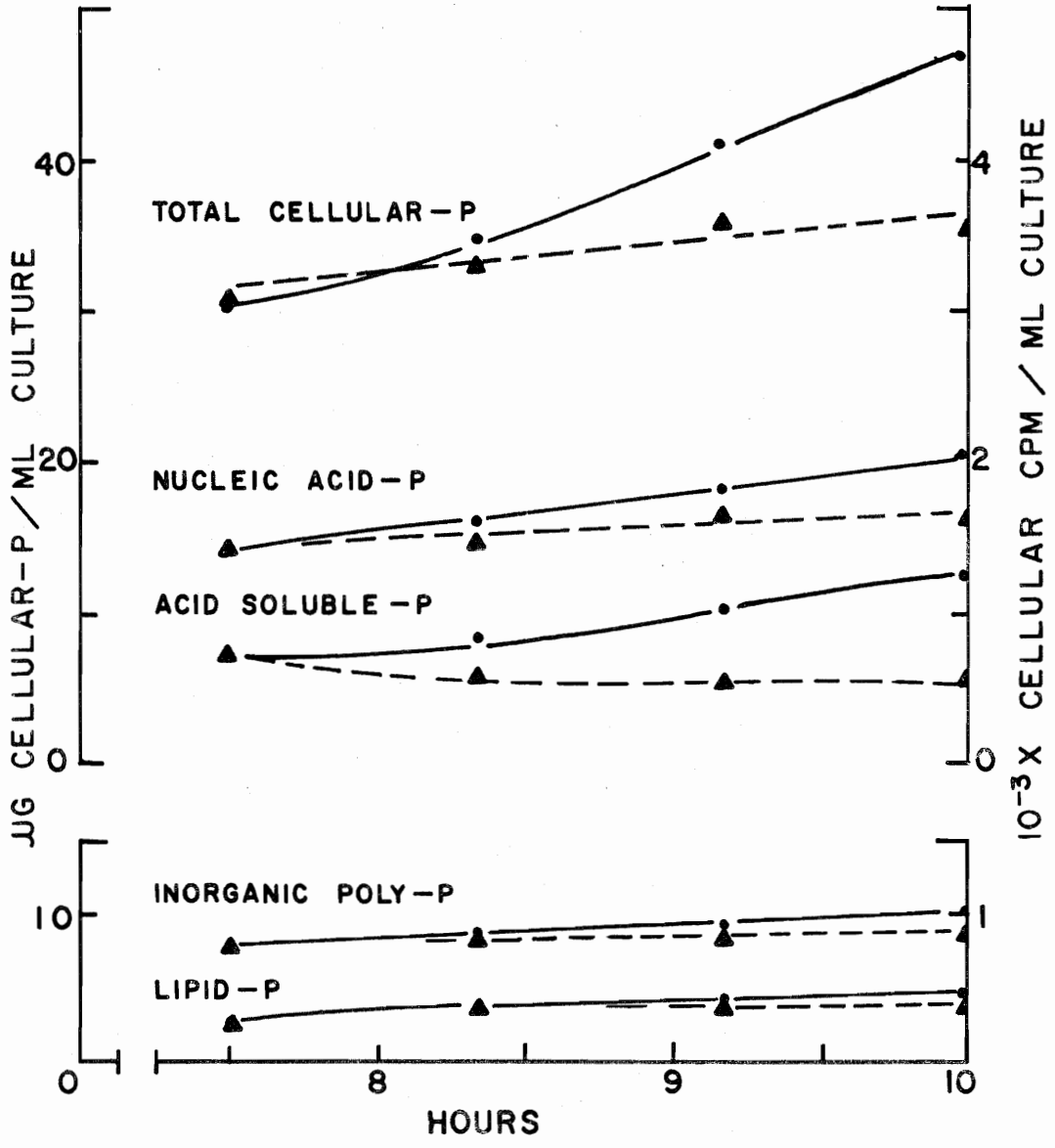

10
8
6
4
2
0
10^8 X µG POLY-P / CELL
35
28
21
14
7
% OF TOTAL CELLULAR-P
2
4
6
8
10
12
HOURS

Fig. 2. The intracellular distribution of phosphorus (●——●) and radioactivity (▲---▲) when uniformly labeled (^{32}P) cells of C. pyrenoidosa were placed on a non-radioactive culture medium during nuclear division.


TOTAL CELLULAR – P
NUCLEIC ACID – P
ACID SOLUBLE – P
INORGANIC POLY – P
LIPID – P
40
20
0
10
0
4
2
0
1
8
9
10
HOURS
µG CELLULAR-P / ML CULTURE
10⁻³ X CELLULAR CPM / ML CULTURE

accumulated prior to nuclear division. Since there is no loss of radioactivity from the $(P_i)_x$ fraction, but actually an increase accompanying the linear deposition, there would appear to be negligible turnover of this phosphate polymer during normal cellular development. Apparently, the exponential uptake of P_i from the culture medium was sufficient to meet the phosphorus requirements of the dividing cell.

The pool of acid-soluble phosphates (P_i, sugar phosphates, nucleotides, etc.) exhibited significant turnover with apparent loss of radioactivity to other cellular phosphate components. The rapid increase in nucleic acid phosphorus with only slight increase in radioactivity suggests that P_i from the culture medium is the main source of phosphorus for this cellular fraction.

Studies with a variety of microorganisms[10,16-18,21] have shown that when cells are placed under an environmental stress, i.e., P_i starvation, low light intensity, etc., $(P_i)_x$ can be utilized (probably indirectly) as a phosphorylating source for nucleic acid and phosphoprotein synthesis. It would appear from the present study, however, that $(P_i)_x$ is not utilized in the absence of an environmental stress but is only accumulated at a constant rate during cellular growth to be distributed between the daughter cells at cell division, increasing their chances of survival should an environmental stress be encountered.

Part 4.

Induced Utilization of Polyphosphate During Nuclear Division in Synchronously Growing Chlorella

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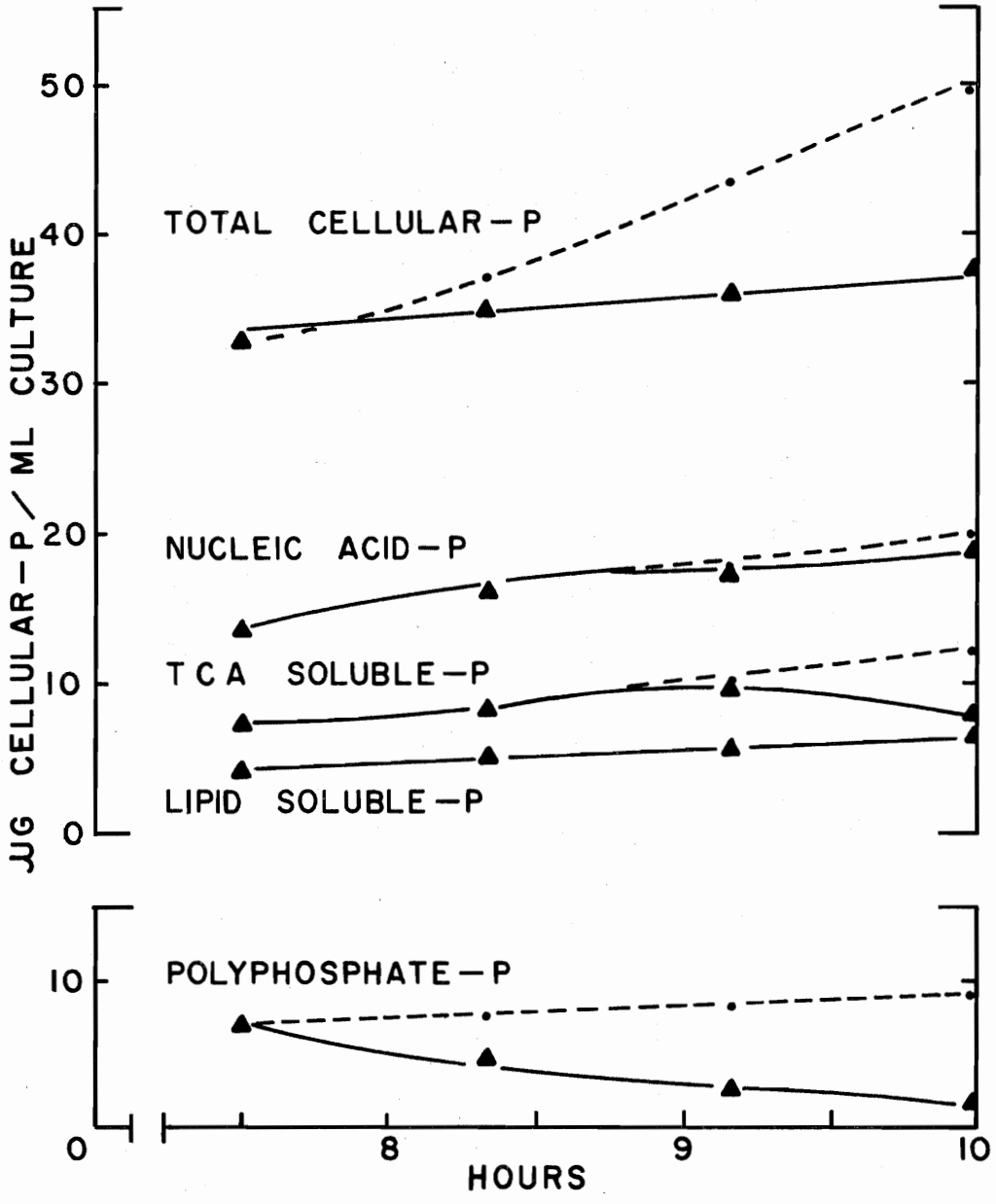
Current investigators[10,16-18,21] have demonstrated the utilization of inorganic polyphosphate, $(P_i)_x$, for nucleic acid and phosphoprotein synthesis in a number of microorganisms subjected to environmental stresses; i.e., phosphate starvation, low light intensity, abnormally high nucleotide concentrations, addition of oxidative phosphorylation uncoupling agents, etc. S. Miyachi[15] and F. M. Harold[9] have proposed mechanisms whereby the $(P_i)_x$ of Chlorella ellipsoidea and Neurospora crassa could enter into the general phosphorus metabolic pathways of the respective organisms. However, in the absence of an environmental stress, there has been no evidence to indicate any significant role for $(P_i)_x$ other than intracellular phosphate storage. This laboratory[3] has recently shown that during the period of nuclear division in synchronously growing C. pyrenoidosa, cultured in a medium containing ample phosphorus for normal cellular development, there was accumulation but no utilization of $(P_i)_x$. The present study was conducted to determine if $(P_i)_x$ could be utilized as an intracellular phosphorus source during nuclear division in Chlorella if the cells were subjected to phosphate starvation.

The alga used in this study was the high temperature strain 7-11-05 of C. pyrenoidosa. The cells were synchronized by the intermittent illumination method of Schmidt and King[26] as modified by Baker and Schmidt[1]. After synchronization, the cells were harvested by centrifugation,

resuspended in fresh culture medium[2] to a cell density of 10^8 cells per ml, and transferred to a flat Plexiglass growth chamber[3]. After 7 h of synchronous growth (immediately prior to the initiation of nuclear division), the cells of the entire culture were separated from the culture medium by rapid filtration onto Millipore filters. The cells were washed and resuspended in phosphate-free culture medium (normal medium containing 1.25 g/l tris hydroxymethyl aminomethane in place of KH_2PO_4) to their original cell density, returned to the growth chamber, and equilibrated[2] for a short period of time before turning on the lights. One-half hour elapsed from the moment of harvest until illumination was restored. Samples of 100 ml of culture were taken at 50 min intervals from 7.5 to 10 h of synchronous growth (the period of nuclear division[7,24]). The cells were separated immediately from the culture medium by filtration, washed, and the intracellular phosphate components extracted, separated, and determined as previously reported[1,2].

Fig. 1 shows the intracellular concentration of phosphorus in the nucleic acid, acid-soluble, lipid, and $(P_i)_x$ fractions during the period of nuclear division in normal and phosphate-deficient cultures of Chlorella. The $(P_i)_x$ is the only phosphorus fraction which began immediately to drop in concentration under conditions of phosphate starvation. After approximately 50% of the $(P_i)_x$ was utilized (at 8.8 h), the acid-soluble phosphates (ortho, nucleotidic, sugar phosphates, etc.) dropped in concentration to the level observed at the onset of the experiment. The nucleic acid and phospholipid fractions increased during phosphate deficiency essentially at the same rate as normal cultures for

Fig. 1. Intracellular distribution of phosphorus in C. pyrenoidosa during nuclear division in normal (●---●) and phosphorus-free (▲---▲) culture medium.


50
40
30
20
10
0
TOTAL CELLULAR – P
NUCLEIC ACID – P
T C A SOLUBLE – P
LIPID SOLUBLE – P
JUG CELLULAR – P / ML CULTURE
10
0
POLYPHOSPHATE – P
8
9
10
HOURS

the duration of the experiment. The loss of phosphorus from the $(P_i)_x$ fraction can be quantitatively accounted for in the increases of phosphorus in the nucleic acid and phospholipid fractions.

The synthesis of $(P_i)_x$ has been demonstrated by Kornberg[14] to proceed according to the following reaction: $x\ ATP \xrightleftharpoons{kinase} (P_i)_x + x\ ADP$. The utilization of $(P_i)_x$ could conceivably occur by the reversal of the kinase reaction and/or by the action of phosphatases. Harold[9] has indicated that the hydrolysis of $(P_i)_x$ by phosphatases, ultimately to orthophosphate, P_i, is the major route of polymer breakdown in Neurospora. The evidence presented to support the hypothesis included the demonstration that $(P_i)_x$ will not synthesize ATP, or exchange phosphate to the terminal group of ATP, when oxidative phosphorylation and fermentation are inhibited; thus, the operation of the reversal of the polyphosphate kinase under these conditions is excluded. It was further observed that in pulse incorporation of $^{32}P_i$ into mycelium, insoluble $(P_i)_x$ became labeled before soluble, lower molecular weight $(P_i)_x$. This would suggest that the lower molecule weight $(P_i)_x$ was synthesized by the hydrolytic cleavage of insoluble $(P_i)_x$ and not from direct synthesis via ATP.

Although there is insufficient evidence at the present time to state the actual mechanism of $(P_i)_x$ utilization in C. pyrenoidosa under phosphate deficiency, several significant conclusions can be made concerning the enzyme(s) governing polymer breakdown. Since there is immediate utilization of $(P_i)_x$ when Chlorella cells are placed in phosphate-free medium (Fig. 1), a constitutive enzyme is probably responsible for depolymerization. An inducible system would have required a lag

period during which time protein synthesis could occur. Therefore, phosphate starvation appears to have activated preexisting enzyme(s) either by exposure of the enzymes to endogenous activators or by the removal of endogenous inhibitors. One possibility is that intracellular P_i is inhibiting the potentially active hydrolytic enzyme(s) and that during phosphate starvation intracellular P_i falls below the critical level for this inhibition. A second possibility is the production of enzyme activators through the disturbance of reaction equilibria during phosphate starvation.

It appears that $(P_i)_x$ has no functional role as an energy or phosphorus source during normal growth in the high temperature strain of C. pyrenoidosa. However, should an environmental stress result in an alteration of phosphorus metabolism, $(P_i)_x$ could be utilized (through the action of phosphatases and/or the reversal of the polyphosphate kinase) as an immediate source of phosphorus.

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In the preparation of a thesis which will terminate formal graduate work, a student is reminded of the many persons who have contributed towards his academic and personal advancement during graduate study. The author wishes to express particular thanks to Dr. R. R. "Bob" Schmidt, friend and major professor, and Dr. R. W. Engel, head of the Department and counselor, for their continuous help and consideration.

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Acknowledgement is due Milton S. Hershey (1857-1945). The success of his chocolate empire is surpassed only by the success with which his memory builds men.

To my wife and daughter go sincere thanks for understanding and endurance.

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Vita

Albert LeRoy Baker was born October 9, 1939 in Hagerstown, Maryland, son of Albert Leroy and Eileen Palmer Baker. He was graduated from the Milton Hershey High School, Hershey, Pennsylvania, in June, 1957. In September, 1957 he entered Bridgewater College, Bridgewater, Virginia, and received a Bachelor of Arts degree, cum laude, in chemistry in May, 1961. In September, 1961 he entered the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia to begin graduate study.

He is a member of the Lambda Society (Bridgewater College), Phi Lambda Upsilon and Phi Sigma society.

In December, 1960 he was married to the former Wanda Carol Harmon. A daughter, Carol Jo Lee, was born in January, 1962.

A. LeRoy Baker

The Metabolic Role of Intracellular Inorganic Polyphosphate During the Synchronous Growth of *Chlorella pyrenoidosa*

Abstract

In order to determine the significance of inorganic acid-insoluble polyphosphate in relation to the general phosphorus metabolism of *Chlorella pyrenoidosa*, six complementary studies were conducted. Methods were developed for the extraction, separation and estimation of the phosphorus components of Chlorella. A revised procedure for inorganic polyphosphate determination, involving extraction with base and purification with Norit-A, is presented.

Shifts in the intracellular distribution of phosphorus during the synchronous growth cycle of *C. pyrenoidosa* were observed. Inorganic acid-insoluble polyphosphate reached its peak concentration early in cellular development, then decreased reaching a minimum value at the end of nuclear division. The phospholipids and the pool of acid-soluble phosphates exhibited an inverse trend by increasing dramatically during nuclear division. Organic phosphates non-adsorbable on Norit-A, ortho-phosphate and Norit-A adsorbable phosphates, components of the acid-soluble pool, increased during nuclear division. The acid-soluble polyphosphate level remained essentially constant throughout cellular development.

To determine the extent to which the synchronization treatment of intermittent illumination induced periodism in the intracellular distribution of phosphorus in *C. pyrenoidosa*, the levels of the phosphorus components were followed for successive synchronous growth cycles in

continuous light. It was observed that non-recurrent periodism in the concentrations of polyphosphate and nucleic acid phosphorus was induced during early stages of growth in the first growth cycle. Periodism in the levels of acid-soluble phosphates and phospholipids was similar throughout the first and second synchronous growth cycles, but deviated from established trends at the beginning of the third cycle. The deviations in the periodism of these two cellular fractions are discussed in relation to the synchronization procedure and to the accumulation of metabolic waste products from the first two growth cycles. A study was also made of the effects of freezing and storage of Chlorella on the intracellular distribution of phosphorus.

Uniformly labeled (^{32}P) synchronous cultures of C. pyrenoidosa were placed in non-radioactive culture medium during the period of nuclear division, and the redistribution of endogenous phosphorus accumulated prior to this period was determined. It was observed that polyphosphate did not exhibit significant turnover, suggesting the polymer was not a phosphorus source during normal cellular development. The pool of acid-soluble phosphates (ortho, nucleotidic, sugar phosphates, etc.) turned over rapidly demonstrating loss of radioactivity to other cellular phosphate components. The accumulation of nucleic acid phosphorus was accompanied by only slight increases in radioactivity, suggesting that orthophosphate from the culture medium is the main source of phosphorus for this cellular fraction.

A final study was conducted to determine if polyphosphate could be utilized as an intracellular phosphorus source during nuclear division

in Chlorella if the cells were subjected to phosphate starvation. It was observed that the polymer provided phosphorus for nucleic acid and phospholipid synthesis under the environmental stress imposed. Conclusions were drawn concerning the induced utilization of polyphosphate in C. pyrenoidosa subjected to phosphate deficiency.

Considering the research presented in this thesis and contributions of other workers in the field of polyphosphate metabolism, the function of the polymer in Chlorella was proposed. It appeared that polyphosphate had no functional role as an energy or phosphorus source during normal growth. However, the polymer could be utilized when an environmental stress results in an intracellular orthophosphate deficiency. The induced utilization of polyphosphate during stress periods could contribute significantly to the mechanisms of survival in C. pyrenoidosa.