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# INTRACELLULAR DISTRIBUTION OF PHOSPHORUS DURING SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

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## 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.

## INTRODUCTION

Studies of phosphorus metabolism during the synchronous growth of Chlorella were initiated by NIHEI[1] 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[2] 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[3], 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[4] continued studies with *C. ellipsoidea* by

Abbreviation: p.c.v., packed cell volume.

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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[5]. The cells were synchronized by the method of SCHMIDT AND KING[3] 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[6] 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[7] 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 × 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 $\Delta$-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 $\Delta$-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 $\Delta$-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[3] 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[8] and by MUDD *et al.*[9]. WIAME[8] and CHAYEN *et al.*[10] 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[1,9,11–13] 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[20] 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[20] and SCHMIDT AND KING[3]. HERRMANN[14] of this laboratory, demonstrated 4% hydrolysis of RNA and DNA in 1N $H_2SO_4$ at 100° in 7 min. Thus, the validity of the analysis for 7-min labile phosphate in the presence of nucleic acids seems questionable.

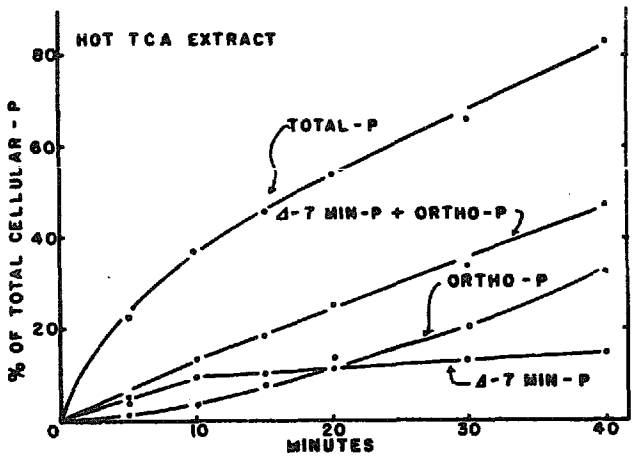


Fig. 1. Extraction of phosphorus compounds from a lipid-free cell residue of *C. pyrenoidosa* with 5% trichloroacetic acid (TCA) at 95°.

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[15] 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 1N KOH extract ceased to increase significantly. HERRMANN[14] observed a similar trend for RNA-ribose and ultraviolet absorption at 260 mμ for the extract. Extraction periods longer than 60 min or with 2N KOH, as used by MIYACHI AND TAMIYA[16], 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 1N KOH from *C. pyrenoidosa*, some of the chemical properties of a high-molecular-weight synthetic polyphosphate, synthesized by the method of PFANSTIEL AND ILER[17] as modified by KORNBERG[18] were investigated. The hydrolysis of the synthetic polyphosphate to orthophosphate in 1N $H_2SO_4$ at 100° is shown in Fig. 2A. The hydrolysis of the polyphosphate was not complete in 7 min as previously reported[13]. 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

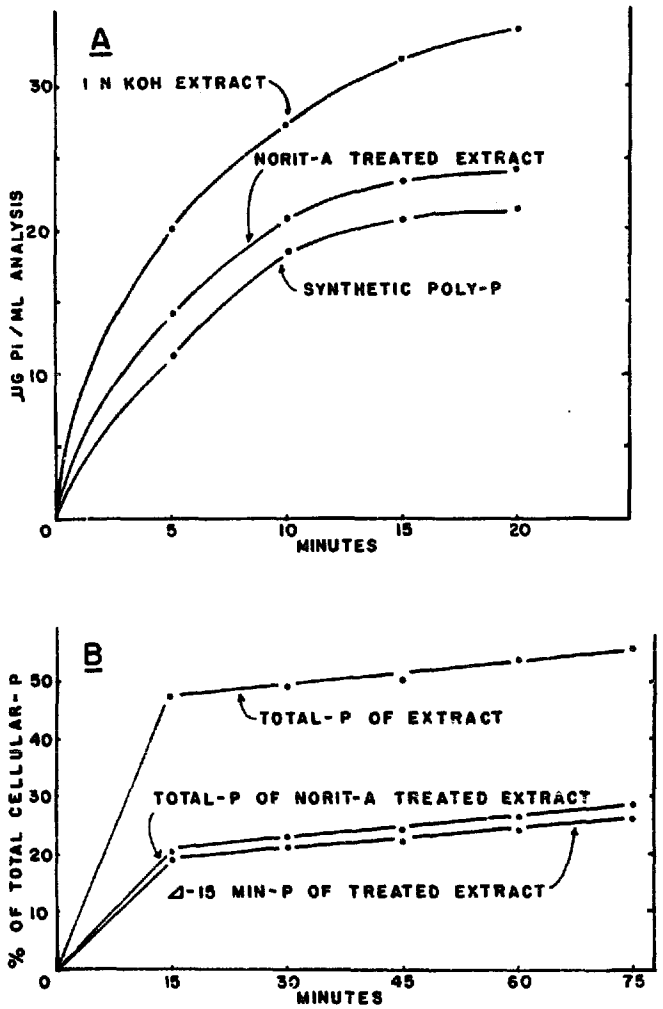


Fig. 2A. Comparison of the hydrolysis (in 1 N $H_2SO_4$ at 100°) of the phosphorus compounds in a 1 N KOH extract from a lipid-free cell residue of *C. pyrenoidosa*, before and after treatment with Norit-A, with a synthetic inorganic high-molecular-weight polyphosphate. (B) Extraction of phosphorus compounds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

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[14] or protein[14] 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[19] extracted nucleic acids and polyphosphates from *Neurospora crassa* with 0.5 N perchloric acid at 70°. The nucleic acids were removed from the acid ex-

tract 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[2,3], 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[3] (Fig. 4). However, the levels of intracellular polyphosphate, at any stage of synchronous growth, were higher than

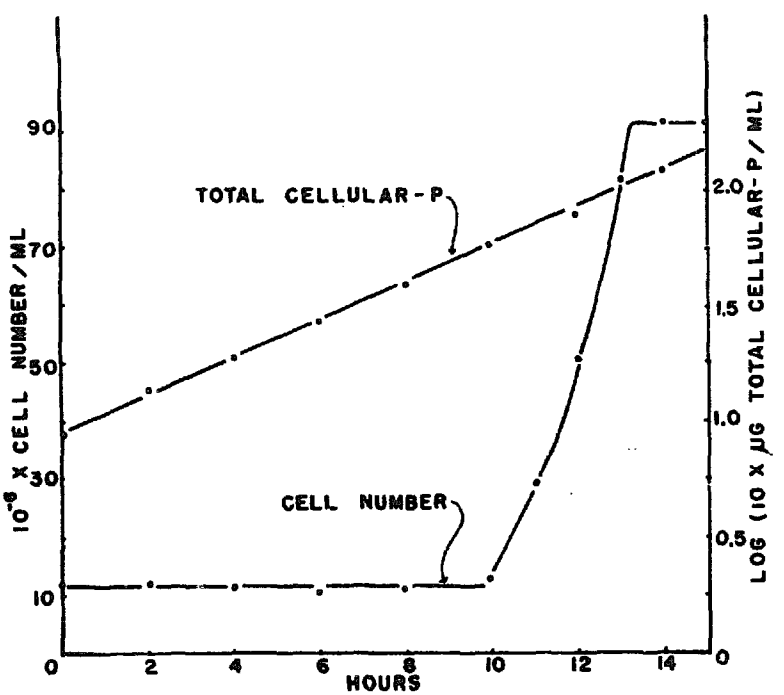


Fig. 3. Relationship between cell number and total cellular phosphorus during synchronous growth of *C. pyrenoidosa*.

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.

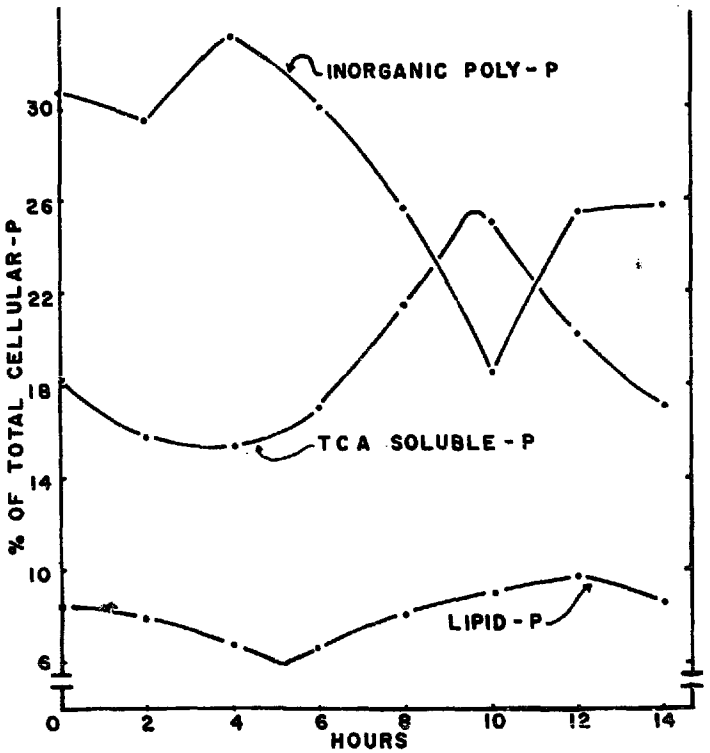


Fig. 4. Intracellular distribution of phosphorus during synchronous growth of *C. pyrenoidosa*. These data represent averages of two experiments. TCA, trichloroacetic acid.

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 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 per cent 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.

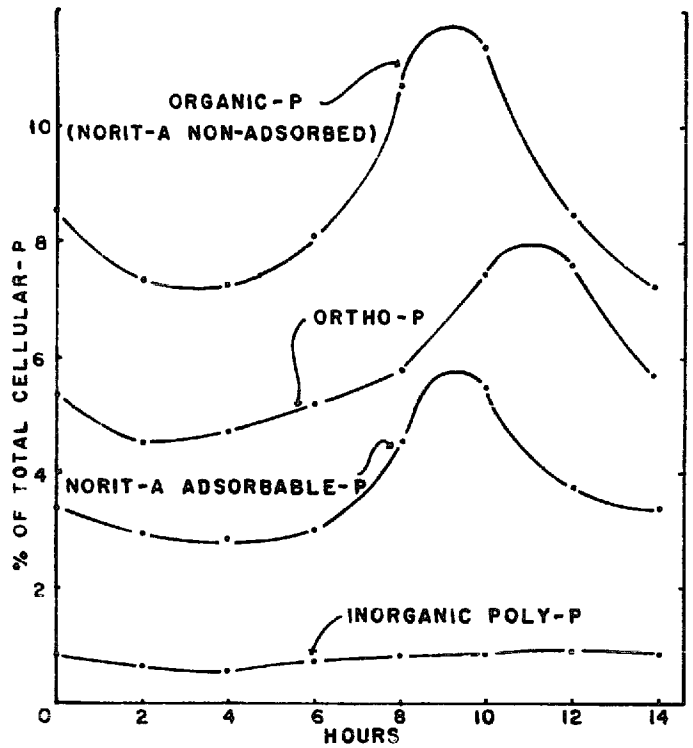


Fig. 5. Phosphorus components of the cold trichloroacetic acid extract from cells of *C. pyrenoidosa* during synchronous growth. These data represent averages of two experiments.

MIYACHI AND MIYACHI[4], 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 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.

ACKNOWLEDGEMENT

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## REFERENCES


[1] T. NIHEI, *J. Biochem. Tokyo*, 42 (1955) 245.
[2] R. R. SCHMIDT, *J. Exptl. Cell Res.*, 23 (1961) 209.
[3] R. R. SCHMIDT AND K. W. KING, *Biochim. Biophys. Acta*, 47 (1961) 391.
[4] S. MIYACHI AND S. MIYACHI, *Plant Cell Physiol. Tokyo*, 2 (1961) 415.
[5] C. SOROKIN AND J. MYERS, *Science*, 117 (1953) 330.
[6] C. SOROKIN AND J. MYERS, *J. Gen. Physiol.* 40 (1957) 579.
[7] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[8] J. M. WIAME, *Biochim. Biophys. Acta*, 1 (1947) 234.
[9] S. MUDD, A. YOSHIDA AND M. KOIKE, *J. Bacteriol.*, 75 (1958) 224.
[10] R. CHAYEN, S. CHAYEN AND E. R. ROBERTS, *Biochim. Biophys. Acta*, 16 (1955) 117.
[11] T. SALL, S. MUDD AND A. TAKAGI, *J. Bacteriol.*, 76 (1958) 640.
[12] G. SCHMIDT, L. HECHT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 166 (1946) 775.
[13] J. M. WIAME, *J. Biol. Chem.*, 178 (1949) 919.
[14] E. C. HERRMANN, unpublished data.
[15] G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[16] S. MIYACHI AND H. TAMIYA, *Plant Cell Physiol. Tokyo*, 2 (1961) 405.
[17] R. PFANSTIEL AND R. K. ILER, *J. Am. Chem. Soc.*, 74 (1952) 6059.
[18] S. R. KORNBERG, *Biochim. Biophys. Acta*, 26 (1957) 294.
[19] F. M. HAROLD, *J. Bacteriol.*, 83 (1962) 1047.
[20] R. R. SCHMIDT, *Dissertation*, Virginia Polytechnic Institute, Blacksburg, Va. (U.S.A.), 1961.