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# Cheorella 細胞에서의 $^{32}$P-燐酸의 蛋白質 및 다른 窒素化合物로의 轉換

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# Incorporation of phosphate into protein and other nitrogenous compounds in *Chlorella* cells

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## Abstract

In the process of the incorporation of orthophsphate into protein and other cell constituents, the role of inorganic polyphosphate and RNA-polyphosphate complex and the correlation between them were pursued by analyzing the contents of $^{32}$P and total P in various fractions of *Chlorella* cells, which had been uniformly labeled with $^{32}$P before the inoculation in a normal "cold" medium or P-free medium during the culture. The effects of ionizing radiation and various micronutritional-element deficiencies on the phosphate incorporation into, and biosynthesis of, protein and other introgenus compounds in the cells were also observed.

When the uniformly $^{32}$P-labeled algae were grown in a normal "cold" medium the contents of $^{32}$P in the fractions of protein, DNA and RNA- polyphosphate complex increased, but those in the fraction of acid-insoluble polyphosphate decreased. On the other hand, amount of $^{32}$P in the fraction of RNA was almost unchanged in spite of rapid increase of the total P. In the growing period of $^{32}$P-labeled algae in a P-free medium, amounts of $^{32}$P in the fractions of DNA, protein and lipid increased, while those in the fractions of RNA-polyphosphate and inorganic polyphosphates decreased.

When the algal cells were irradiated with about 70,000 r of gamma-rays before the inoculation in the medium, amounts of phoshate in the fractions of DNA, RNA, nucleotides and protein decreased during the culture, compared with those of the control. However, the phosphate content in the fraction of acid-insoluble polyphosphate of the irradiated cells increased than those of the control.

In the growing period of the algae in a Mo-free medium, amounts of acid-soluble total phosphate and nucleotides of the cells increased, while the amounts of residual protein and RNA decreased compared with those of the normal cells. Amounts of alkali-labile protein and phospholipid of the cells grown in a B-free medium decreased, whereas amount of phosphate in acid-soluble fraction increased compared with the control. In general, the contents of protein and RNA in each microelement deficient cells decreased more or less, compared with those in the normal cells.

## INTRODUCTION

Suspensions of *Chlorella* Cells in the light convert orthophosphate into intracellular polyphosphate, mostly acid-insoluble(Wintermans, 1955), and the phosphorus thus incorporated into acid-insoluble polyphosphate may be further transferred, by light

-independent reactions, in part to the residual protein fraction (Nihei, 1957) and deoxyribonucleic acid (DNA) fraction (Miyachi and Tamiya, 1961). As it became recently evident by the experiments of Correll and Tolbert (1962, 1964) and Correll (1965) that there is a large amount of ribonucleic acid polyphosphate complex, in addition to acid-soluble and -insoluble poly-phosphates in the algal cells, it is desirable to clarify the correlation between these polyphosphates and their role in the metabolism.

The experiments to be described in the present paper were designed to trace the role of polyphosphates, both of inorganic and organic complex forms, and correlation between them in the process of the incorporation of inorganic orthophosphate into protein, DNA, ribonucleic acid (RNA) and phospholipid, using $^{32}$P-labeled *Chlorella* cells. The effects of environmental conditions, such as individual micronutritional-element deficiency and gamma-irradiation, on the phosphate incorporation into, and biosynthesis of, protein and other nitrogenous compounds in the cells were also observed.

## METHODS

*Chlorella ellipsoidea* cells were grown in an inorganic medium wtih provision of $CO_2$-enriched air under the light at 25°C. To obtain the uniformly $^{23}$P-labeled cells for the tracer experiments the algae were precultured in an inorganic medium containing $^{32}$P-phosphate. After a week of the culture, when the cell mass increased to more than 20 times that of the inoculum, the cells were harvested, washed twice with 0.002 M $K_2SO_4$, and then used as an inoculum in P-free or in normal "cold" medium.

### Fractionation procedures for inorganic polyphosphate and RNA-polyphosphate complex

These procedures were performed by the method of Correll and Tolbert (4) with slight modifications. The cells were extracted twice with 5% perchloric acid (PCA), and the residue was extracted successively with ethanol (twice) and ethanol-ether(3 : 1) at 60°C(3 times). The residue thus obtained was suspended in distilled water which was adjusted to pH 11.5 with KOH at room temperature for one hour to solulbize RNA-polyphosphate complex and was centrifuged and the extraction was repeated., Combined supernatant was adjusted to pH 4.5 with 10% acetic acid at 0°C (1 hour) and small amount of residue was removed. The supernatant thus obtained was adjusted to pH 3.5 with dilute HCl and then ethanol (2 vol.) was added. The precipitate(RNA-polyphosphate complex) was removed, and from the supernatant and PCA-soluble fraction both forms of polyphosphates, cold PCA-insoluble and -soluble, were collected by means of Ba-precipitation.

### Analysis of DNA, RNA, Protein and other cell constituents

According to the method of Schmidt-Thannhauser (1945), acid-insoluble nonlipid and nonpolyphosphate samples of the cells were treated with 1N KOH at 37°C for 18 hours to hydrolyze RNA, and were subsequently neutralized with 5% PCA. PCA was further added to coprecipitate DNA and protein with potassium perchlorate formed by the neutralization. The supernatant was separated by centrifugation (RNA-fraction) and the residue was further extracted with 5% PCA in boiling bath for 15 minutes to remove DNA fraction from the residual protein fraction.

Amounts of phosphate in each fractions of the algal cells were determined spectrophotometrically after hydrolysis of P-compounds to orthophosphate followed by treatment with amonium molybdate, or by measuring their radioactivity taken into account the specific activity of $^{32}$P in the starting culture medium. Form the acid-soluble fraction of the cells, amounts of ortho-P and nucleotidic labile-P were determined by the methods of Belenblum and Chain (1938) and Crane and Lipmann (1953), respectively. Amounts of DNA and RNA were also expressed in relative optical density at 260 millimicron. From the acid-hydrolysates of the RNA fraction as residual protein fraction, amino acid quantity was also measured by the ninhydrin reaction (Troll and Cannan, 1953) for the amounts of alkali-labile and-stable protein, as it was found that a large

amount of protein was present in the RNA fraction of the cells separated from the above procedure (Aoki and Hase, 1964).

## RESULTS AND DISCUSSION

### Incorporation of intracellular $^{32}P$ in P-free medium

When the algal cells grown in the normal medium were inoculated in the P-free medium under the light, they could grow to some extents showing about 4-fold increase in the packed cell volume within 70 hours using the intracellular P-reserve. The uniformly $^{32}P$-labeled algal cells were further incubated in the P-free medium under the light, and the changes in $^{32}P$-content in various fractions of the cell materials were analyzed during the culture. The results obtained are reproduced in Fig. 1.

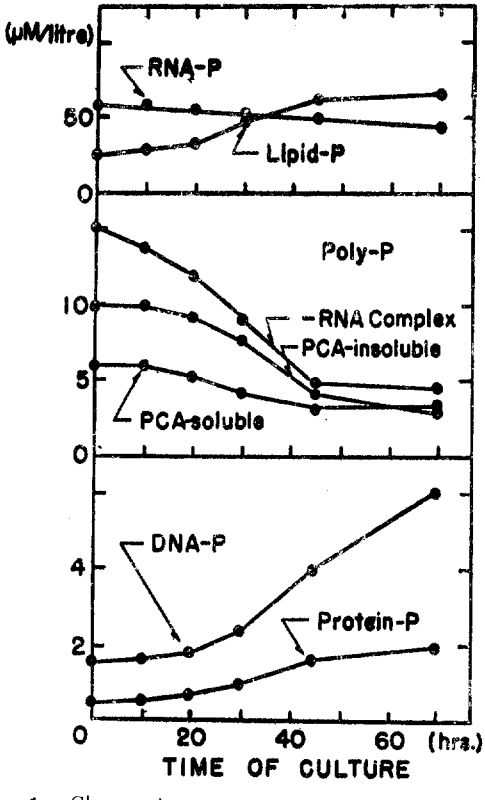


**Fig. 1.** Changes in amounts of $^{32}P$-phosphate in various fractions of the uniformly $^{32}P$-labeled *Chlorella* cells during the culture in a P-free medium.

In the culture period of the labeled algae in the P-free medium amounts of $^{32}P$ in the fractions of DNA, protein and lipid increased significantly showing the indispensable function of these substances as cell constituents, while amounts of $^{32}P$ in the fraction of RNA-polyphosphate complex decreased markedly as well as in the fractions of cold PCA-soluble and -insoluble polyphosphates. On the other hand, amounts of $^{32}P$ in the fraction of RNA remained almost unchanged, showing rather decreasing tendency. This indicated that under the condition of P-deficiency the synthesis of phosphoprotein in the cells could proceed without accompanying new RNA synthesis.

During the culture period of the algal cells in the P-free medium there was no practical release of $^{32}P$ into the surrounding medium. Therefore, the results obtained may be interpreted as indicating that during the cucture period of the algae in a P-free medium the $^{32}P$ in polyphosphates including RNA-polyphosphate complex has been transferred to other cell constituents such as protein, DNA and lipid directly or via some substance, presumably adenosine triphosphate.

### Incorporation of $^{32}P$ and total P in normal "cold" medium

The uniformly $^{32}$ P-labeled *Chlorella* cells were inoculated in the normal "cold" medium under the light and the changes in the amounts of total P and $^{32}P$ in various fractions of the cell materials were pursued during the culture. After inoculation of the $^{32}P$-labeled algae in the normal "cold" medium, the cell mass has increased about 7-fold compared with the inoculum within 50 hours.

As represented in Fig. 2 amounts of $^{32}P$ in the fractions of protein, DNA and RNA-polyphosphate complex increased considerably as well as in the fractions of lipid and cold PCA-soluble polyphosphate, although the increase of $^{32}P$ in the latter two fractions was very slight compared with those in the former three fractions. In the fractions of DNA, protein and RNA-polyphosphate the course of increase of $^{32}P$ approached very nearly to that of the total P for a significant period, whereas in the fraction of lipid the phosphate derived from the surrounding medium increased by far in excess

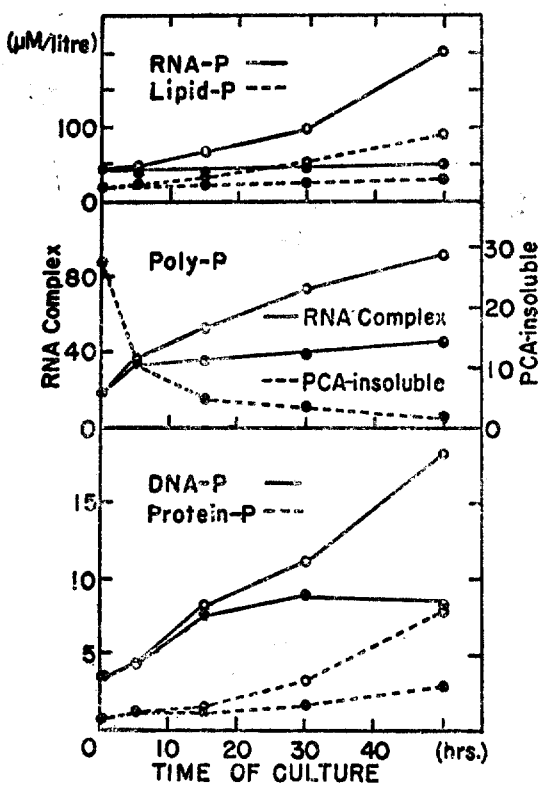


**Fig. 2.** Changes in amounts of $^{32}$P-phosphate and total phosphate in various fractions of the uniformly $^{32}$P-labeled *Chlorela* cells during the culture in the normal "cold" meldium. Solid circle, $^{32}$P; open circle, total P.

compared with the $^{32}$P derived from the intracellular P-source. On the other hand, amounts of $^{32}$P in the fraction of PCA-insoluble polyphosphate decreased rapidly and markedly, whereas in the fraction of RNA it was almost unchanged in spite of rapid increase of total phosphate.

These results indicated that during the growing period of the labeled algae in the normal "cold" medium the $^{32}$P in the PCA-insoluble polyphosphate has been transferred to phosphoprotein, DNA and RNA-polyphosphate complex. The data also concurred with the interpretation of Miyachi and Tamiya (1961) that the phosphorus used in the synthesis of DNA and protein was primarily taken from intracellular polyphosphate and the phosphorus used in the synthesis of RNA was, for the most part, taken from the extracellular P-source. It is considered that the PCA-insoluble polyphosphate is principal acceptor of phosphorus from exogenous phosphate through photochemical reaction of the algal cells and from this substance the phosphorus is transferred to the cell constituents, such as DNA and protein, or other P-reservoir in the cell.

It may be remarked that so-called polyphosphate "D" of Miyachi et al., collected from RNA fraction treated with 2 N KOH, must be a portion of RNA-polyphosphate complex comparing the results of the present study with the behavior of poly-P "D" in their experiment (Kanai et al., 1965, Miyachi et as., 1964). When the PCA-insoluble nonlipid sample of the algal cells was extracted with cold KOH at pH 9, the supernatant obtained, so-called poly-P "B" fraction (Miyachi and Tamiya 1961), contained a large amount of stable phosphate compound (up to about 40% of all) which has a absorption peak at 260 mμ, in addition to labile polyphosphate (Lee, 1964 a)Therefore, it may also be remarked that some portion of RNA-polyphosphate complex could be incorporated into poly-P "B" fraction in their experiment.

It was shown experimentally by Meyerhof et al. (1953) and Yoshida (1955) that the energy of P-O-P bonds in the polyphosphate molecule is of the same magnitude as that of the energy-rich phosphate bond. The RNA-polyphosphate complex, therefore, must be a final reservoir of both phosphate and energy, which accumulates so-called energy-rich phosphate bond from PCA-insoluble polyphosphate in the cell under the condition of excess phosphate and light. It appears that the RNA-polyphosphate complex may be the main component of the metachromatic volutin granule in the *Chlorella* cells (Badour, 1959, Kim 1966) grown in the presence of excess phosphate.

**Effects of ionizing radiation**

*Chlorella ellipsoidea* cells were irradiated with gamma-rays from cobalt-60 before the incubation in the normal medium, and the sensitivity of the cells to gamma-rays was determined by measuring the photosynthetic and respiratory activities. The photosynthetic activity was found to be more sensitive to radiation than the respiratory activity of the cells

(Lee, 1964 b). About 70,000 r were irradiated to the algal cell suspension, in which the cell mass occupied 0.38 millilitre in packed volume per millilitre suspention, for three hours. It was observed that irradiated algal cells exhibited subsequently about 9% reduction in photosynthetic activity and 5% inhibition of glucose respiration.

The growth of the irradiated algal cells was considerably retarded showing about 3-fold increase in packed cell volume within 45 hours in contrast with 5-fold increase in the nonirradiated control cells. As represented in Fig. 3 amounts of phosphate

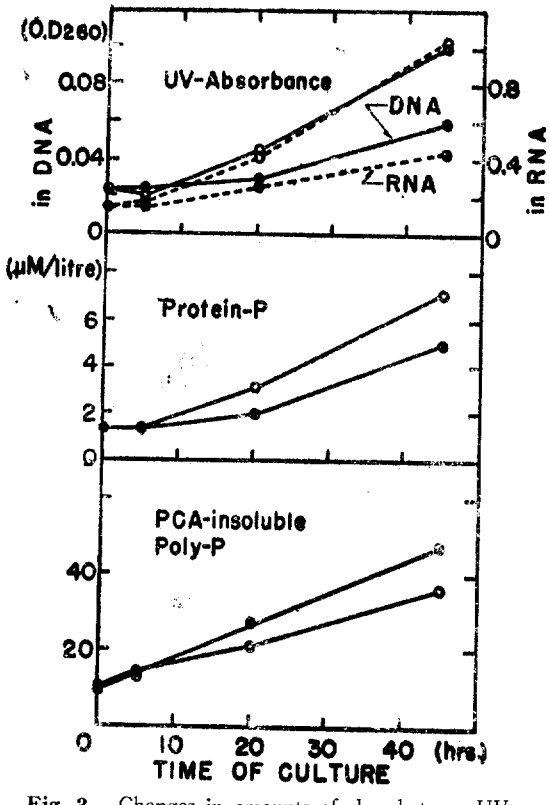


**Fig. 3.** Changes in amounts of phosphates or UV-absorbing materials in various fractions of the gamma-irradiated *Chlorella* cells during the culture. Gamm-irradiation was held before the inoculation in the medium. Solid circle, irradiated; open circle, control.

and UV-absorbing substances of the irradiated cells decreaded in the fractions of DNA, RNA, nucleotides and residual protein compared with those of the nonirradiated normal cells during the culture. However, the content of phosphate in the PCA-insoluble fraction of the irradiated cells increased more than that of the control in spite of growth retardation effect of ionizing radiation.

The results may, therefore, be interpreted to indicate that the preliminary irradiation of gamma-rays to the algal cells inhibited the incorporation of phosphorus from PCA-insoluble polyphosphate into DNA and nucleotides in the cells, and that the biosynthetic activities of phosphoprotein and RNA decreased owing to either this indirect effect on DNA synthesis or the direct effect of gamma-ray itself, and the growth of the irradiated cells was consequently retarded.

**Effects of various micronutritional-element Deficiencies**

*Chlorella ellipsoidea* cells were grown in Fe-, Cu-, Mn-, Zn-, MO- or B-deficient media and the effects of this micronutritional-element deficiency on the incorporation of phosphate from the external and intracellular P-sources into major cell constituents, such as protein, nucleic acids and phospholipid were pursued. The algal cells grown in each micronutritional element-deficient medium for 11 days through second subculture were analyzed for the contents of phosphate, amino acids and UV-absorbing materials in various fractions of the cells. Growth rate of the algal cells in each micro-element deficient medium decreased more or less. The cell mass, however, increased about 80-fold in packed volume through second successive culture for 11 days, even in the case of Fe-deficient medium in which algal cells exhibited the most severe growth retardation of all.

When the algal cells were grown in a Mo-free medium amounts of total phosphate and nucleotides in acid-soluble fraction of the cells increased, whereas amounts of residual protein and RNA decreased compared with those of the normal cells. The results obtained may be considered to indicate that the incorporation of phosphate into protein from the early photosynthetic products, which are soluble in cold PCA, and the biosynthesis of RNA from the acid-soluble nucleotides were inhibited by Mo-deficiency.

The growth of the algal cells in Fe-free medium was retarded with the severe cholorosis, and the

photochemical oxygen output and amount of phosphate in PCA-soluble fraction of the cells decreased remarkably. Such decreases are more severe even though the low content of chlorophyll in the cells owing to the Fe-deficiency is considered. Therefore, it is deduced that Fe takes part in the active uptake of phosphate from external medium and in the photosynthetic process itself, possibly by its participation in the photophosphorylaion coupled with electron transport.

When the algae were grown in a B-free medium amounts of alkali-labile protein and phospholipid of the cells decreased, while the amount of phosphate in acid-soluble fraction increased considerably compared with those of the normal cells in spite of growth retardation effect of B-deficiency. It is therefoie, considered that the biosynthesis of protein and phospholipid from the acid-soluble phosphate compounds is inhibited by B-deficiency.

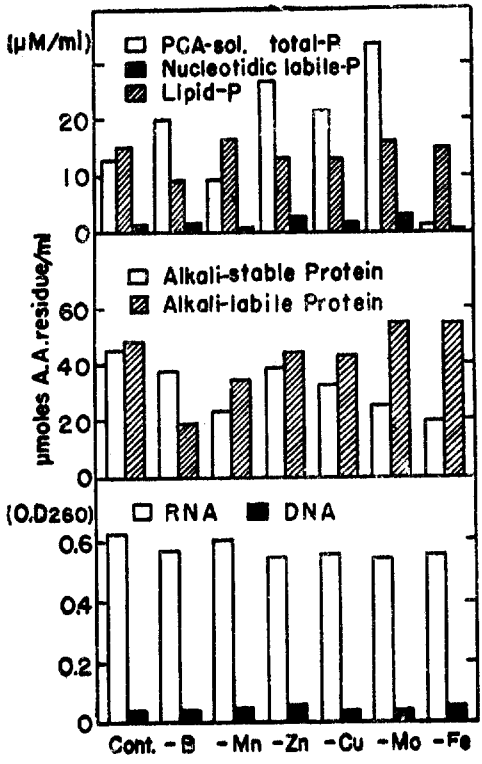


**Fig. 4.** Amounts of phosphates, amino acids and UV-absorbing materials in various fractions of the *Chlorella* cells raised in each micronutritional-element deficient medium for 11 days through second subculture.

As represented in Fig. 4 the analysis of chemical composition of the cells grown in each microelement deficient medium exhibited to be somewhat different from that of the normally raised cells. Amounts of phosphate in lipid fraction of Mn-deficient cells and in acid-soluble fractions of Fe- and Mn-deficient cells decreased, but increased considerably in acid-soluble fractions of Mo-, Cu-, Zn-, and B-deficient cells. In general, amounts of protein and RNA in each microelement deficient cells decreased more or less, compared with those of the normal cells.

## CONCLUSIONS

The phosphorus incorporated into acid-insoluble polyphosphate in the cells from the external medium has been furher transferred in part to DNA, protein and RNA-polyphosphate complex as well as acid-soluble polyphosphate, under the conditions of excess phsophate and light. On the other hand, the phosphorus used in the synthesis of RNA and phospholipid was, for the most part, taken from the external medium. Under the condition of P-deficiency, however, the phosphorus in RNA-polyphosphate complex and acid-soluble polyphosphate as well as acid-insoluble polyphosphate, has been transferred to protein, DNA and lipid.

Therefore, it is considered that acid-insoluble polyphosphate is principal acceptor of phosphorus from exogenous phosphate through photochemical reactions of the cell, and the RNA-polyphosphate complex is a final reservoir of both phosphate and energy.

Ionizing radiation inhibited the incorporation of phosphorus from acid-insoluble polyhosphate into DNA, and the biosynthes of phosphoprotein and RNA decreased owing to either this indirect effect on DNA synthesis or the direct effect of gamma-ray itself, and the growth of irradiated cells was consequently retarded. In general, the incorporation of phosphorus from the external medium into RNA in the algal cells which were growing in each micronutritional element-deficient medium, was inhibited more or less, and biosynthesis of protein and algal growth were consequently retarded.

## 摘 要

蛋白質, DNA, RNA 및 燐脂質 等의 生合成 過程에 있어서의 inorganic polyphosphate 와 RNA-polyphosphate complex 의 役割을 $^{32}P$ 로 미리 均等히 label 한 *Chlorella* 細胞를 使用하여 追跡하고 電離放射線의 影響과 微養元素 缺乏効果等을 觀察하였다.

$^{32}P$-labeled *Chlorella* 를 正常的인 "cold" medium 에서 培養하였을 때 蛋白質, DNA 및 RNA-polyphosphate complex 의 $^{32}P$ 및 total P 는 增加하였으나 酸不溶性 포리燐酸의 $^{32}P$ 는 顯著히 減少하였다. $^{32}P$로 label 한 細胞를 P-free medium 에서 培養하였을 때는 酸不溶性 포리燐酸 및 RNA-polyphosphate complex 의 $^{32}P$의 量은 顯著히 減少하였으나 DNA 및 蛋白質 fraction 의 $^{32}P$의 量은 相對的으로 增加하였다. 따라서 酸不容性 포리燐酸은 細胞의 光化學的反應을 通한 外部 燐酸의 受容體로서의 作用을 하고 RNA-polyphosphate complex 는 燐 및 energy 의 貯藏源이 되는 것으로 生覺된다.

約 70,000 r의 감마線에 照射된 細胞는 DNA, RNA. nucleotide 및 蛋白質의 形成能은 減少하였으나 酸不溶性 포리燐酸의 含量은 對照區에 비해서도 增加하였다.

모리브덴-缺乏培地에서의 alkali-stable protein 및 RNA의 生合成은 減少하였으나 細胞의 酸可溶性 全燐酸 및 nucleotides의 含量은 對照區에 비해 增加하였다. 보론-缺乏培地에서 자란 細胞의 燐脂質 및 alkali-labile protein 의 形成能은 減少하였으나 酸可溶性燐酸의 量은 對照區보다도 增加하였다. 그러나 鐵 및 망강 缺乏細胞에서는 酸可溶性 全燐酸量이 顯著히 減少하는 것으로 보아 鐵 및 망강은 각각 光合成過程에서 보다 重要한 作用을 하는 것으로 生覺된다.


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