A STUDY OF THE RIBONUCLEIC ACID-POLYPHOSPHATE COMPLEXES ISOLATED FROM ANABAENA VARIABILIS AND SYNCHRONIZED CHLORELLA PYRENOIDOSA

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

David L. Correll

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This is to certify that the

thesis entitled

A STUDY OF THE RIBONUCLEIC ACID-POLYPHOSPHATE COMPLEXES ISOLATED FROM ANABAENA VARIABILIS AND SYNCHRONIZED CHLORELLA PYRENOIDOSA

presented by

David L. Correll

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Fisheries and Wildlife and Agricultural Chemistry

Robert C. Bell
Major professor
M. E. Tolbert

Date March 20, 1961

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ABSTRACT

A STUDY OF THE RIBONUCLEIC ACID-POLYPHOSPHATE COMPLEXES ISOLATED FROM ANABAENA VARIABILIS AND SYNCHRONIZED CHLORELLA PYRENOIDOSA

by David L. Correll

Although considerable literature concerning polyphosphate has appeared in recent years, no acceptable metabolic function of these compounds has been demonstrated. This study was undertaken to better characterize the structure and physiology of the RNA-polyphosphate complexes. These complexes seemed to be a potential mechanism for the metabolic utilization of the polyphosphates.

RNA-polyphosphates were isolated from Anabaena and Chlorella which had been raised in chemically defined media under carefully controlled conditions. The isolated complexes were fractionated on DEAE-cellulose columns. The complexes were shown to be free from serious contamination by other phosphate compounds, amino acids, protein, other carbohydrates, or DNA. Changes in the complexes during the growth curve in Anabaena and the life cycle in synchronized Chlorella were studied.

An effect of light on the ratio of total RNA to total polyphosphate was found. High light levels depressed the RNA level and increased the polyphosphate level. Low light brought about unusually high amounts of RNA. Under normal light conditions both organisms were found to have about 15

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micromoles of polyphosphate-phosphorus per micromole of RNA-ribose. This relationship was surprizingly consistent.

Changes in six fractionated RNA-polyphosphates were studied in synchronized cultures of Chlorella. The six complexes underwent independent changes in amount, dialysis properties, and metachromatic character.

The complexes isolated from Chlorella showed the same characteristics as those isolated from Anabaena. Chemical and physical evidence was obtained that both a hydrogen-bond system and covalent bonds are involved in the linkage of the RNA to the polyphosphate. The hydrogen-bond system was found to be most stable at pH 7.4 to 7.6 and at salt concentrations above 0.2 M. Exhaustive dialysis against distilled water resulted in the cleavage of this system. Before dialysis the metachromatic reaction of the complex with toluidine blue was very small. After dialysis a pronounced metachromatic reaction was produced. This metachromatic reaction was not released by boiling, freezing, mild acid or basic pH, EDTA, or RNase.

Yeast polyphosphatase was not very active on undialyzed complex, but was very active on the dialyzed complex. RNase and snake venom phosphodiesterase both reduce the metachromasy of dialyzed complex. After short-time treatment with polyphosphatase, RNase, or snake venom phosphodiesterase; the polyphosphate involved in the complex only dialyzed to a small degree. This, coupled with the low specific metachromasy, was interpretted as evidence that only short polyphosphate

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chains were left, but that these chains were covalently bonded to the RNA. This type of material also resembled the low molecular weight complexes, as isolated from Chlorella.

The data obtained were consistent with a structure involving a polyphosphate of variable length, which had both of its terminal secondary hydroxyls esterified to RNA-ribose. In the "native" complex the polyphosphate chain might be involved in a hydrogen-bond system with the nitrogen bases of the RNA.

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by

David L. Correll

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The author wishes to acknowledge the technical help of Dr. N. E. Tolbert and, perhaps more important, his guidance into the climate of modern plant biochemistry. I wish to thank Dr. R. C. Ball for the original stimulation to do research in this area and for his continuing interest in my intellectual development.

Without the use of Dr. Harold Sell's laboratory equipment, much of the work would have been more difficult. Dr. R. R. Schmidt of Virginia Polytechnic Institute deserves a great deal of credit for his unselfish and scientific spirit in sharing research ideas.

I also wish to acknowledge the support of the National Science Foundation and Michigan State University during the course of this research.

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LITERATURE REVIEW

Polyphosphates in Microorganisms with Special Reference to the Algae

Importance of Polyphosphates in Algae

In the cell of a microorganism the phosphorus contents can be divided into six main pools; namely nucleic acids, phosphoproteins, phospholipids, small molecular weight esters which are mainly sugar phosphates, orthophosphate, and polyphosphates. The amount of phosphorus in the cell can undergo many-fold alterations. In the process of these alterations it has been established that the ortho- and polyphosphate pools play the principle role.

It is possible to separate these groos pools from each other. The bulk of the phospholipids can be extracted with a solvent such as ethanol-ether (3:1). It is also possible to extract, quantitatively, the ortho- and polyphosphates along with some organic phosphates by using such solvents as hot five percent trichloroacetic acid or 0.1 N NaOH. Heating polyphosphates at 100° in 1 N HCl for seven minutes will completely hydrolyze these compounds to orthophosphate, which then can be determined accurately and selectively. This hydrolysis is mild enough that only minor amounts of organic phosphates are hydrolyzed.

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An application of these techniques coupled with qualitative tests has been made in a study of Chlorella ellipsoidea (105). These algae were raised in a synchronous, mass culture and the phosphorus in various pools was determined at different stages of the life cycle. The total cellular phosphorus ranged from about 1.5 to 2.2 percent of the dry organic weight. The amount of phosphorus in the organic phosphorus pools remained fairly constant at about 0.4 to 0.5 percent, and the ortho- and polyphosphates accounted for the rest. By a division of the inorganic phosphates into low and high polymers it was shown that most of the polyphosphate was in the high polymer fraction. In a study of the phosphate compounds of Euglena gracilis, Albaum (4) found at least 18 percent of the total phosphorus to be acid-soluble polyphosphate. In the case of Acetabularia, Schweiger (122) found over half of the total phosphorus in the acid-soluble polyphosphate. These studies and many others of a less comprehensive nature have established that polyphosphates are a normal and important part of the healthy algal cell.

Definition

In order to clarify the term polyphosphate, we shall consider a polyphosphate as a linear polymer of inorganic orthophosphate. There is some confusion in the literature, especially the older literature, due to the former use of the term metaphosphate. The latter term is now reserved for cyclic polymers of orthophosphate. A thorough up-to-date classification of poly- and metaphosphates can be found in

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Mattenheimer (99). Both poly- and metaphosphates are known to occur in microorganisms (73). However, with the possible exception of yeasts, the polyphosphates appear to be the important form.

Occurrence

At the present time it would seem that very few generalizations about the occurrence of polyphosphates are possible. One which seems valid is that most nonvascular plants synthesize polyphosphates. No exceptions to this rule have been found at present.

Among the algae polyphosphates have been reported in Chlorella (125); Euglena (4); Phormidium (34); Chara, Cosmarium, Spirogyra, Mougeotia, Zygnema, Navicula, Fragillaria, Vaucheria, Ulothrix, Oedogonium, Oscillatoria (66); Acetabularia (129); and Pseudoanabaena (54). Polyphosphates have been reported in the following fungi: brewer's yeast and baker's yeast (143), Aspergillus (97), Neurospora (51), Penicillium (81), Merulius (44), and Agaricus (82). Among the bacteria polyphosphates have been reported in Corynebacterium (14), Azotobacter (37), Mycobacteria (148), Clostridium (131), and Aerobacter (144).

In the animal kingdom polyphosphates have been reported in the fat bodies of the butterfly Deilephila (53), and the excreta of the wax moth Galleria (103).

Polyphosphatase enzymes are known to occur in such vascular plants as rice (153), barley (113), and peas (106).

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However, Keck (66) failed to demonstrate polyphosphates in such vascular plants as Pinus, Polypodium, Osmunda, and Psilotium. Ebel (34) also failed to demonstrate polyphosphates in Escherichia coli, Bacillus anthracis, sheep liver, beef muscle, and calf thymus. This failure to demonstrate polyphosphates must be qualified and reconsidered as a result of other investigations. An enzyme has been isolated and characterized from Escherischia coli (71) which carries out the following reaction in vitro:

$$X\ ATP + (PO_3^-)_n \rightleftharpoons X\ ADP + (PO_3^-)_{n+x}$$

Also, another enzyme from E. coli (76) catalyzes the reaction:

$$dGTP \rightleftharpoons P\text{-}P\text{-}P + dGR$$

Furthermore, cell-free extracts of beef skeletal muscle (85) have been shown to carry out the reaction:

$$ADP + P\text{-}P \rightleftharpoons AMP + P\text{-}P\text{-}P$$

Thus, it seems reasonable to doubt the complete absence of these compounds in E. coli and beef muscle, even though they may be found only in small amounts.

Location in Cell

Polyphosphates have been detected in the cell by the metachromatic reaction, which is reviewed in a subsequent section. This procedure doesn't necessarily detect the RNA-polyphosphates which will be discussed in this thesis.

In the marine alga Acetabularia the polyphosphate was found in spheres in the cytoplasm (129). These spheres varied from 0.1 u to 400 u in diameter depending upon the physiological condition of the algae. They were concentrated

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in the tip of the filament and the tips of the rhizoids and were even found in the gametes and zygotes. In a more comprehensive study, Keck (66) found that in members of the family of green algae, Zygnemataceae, the polyphosphate was found in the chloroplasts along with a high concentration of RNA. The pyrenoids were also surrounded by a sphere of polyphosphate granules. He also found that Vaucheria, a coenocytic form, accumulated polyphosphate in small bodies in the cytoplasm of unfertilized oogonia. Some diatoms were found to have polyphosphate granules in the cytoplasm and Oedogonium had polyphosphate dissolved in the cytoplasmic vacuole. Oscillatoria had polyphosphate dissolved in the pseudovacuole. Herbst (54) reports cytochemical evidence of nuclear equivalents in Oscillatoria and Pseudoanabaena which contain both polyphosphates, RNA, and DNA. In contrast are the findings of Krieg (79), who illustrated nuclear equivalents in Nostoc and Oscillatoria and showed metachromatic granula to be present in the periplasm. Krieg could show no cytological relationship between these metachromatic granula and the nuclear equivalents.

Several studies of Corynebacterium diphtheriae (101, 116) have shown the presence of polyphosphate-containing granules which varied in size with physiological conditions and seemed to be localized around a bacterial equivalent of mitochondria.

In a study of living and fixed Mycobacterium thamnopheos (70) it has been shown that the polyphosphate was localized

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in granules which divide in the living cell, were feulgen positive, and gave a Millon test. These granules were observed to line up at the plane of division in dividing cells and undergo division. They were also shown to contain RNA.

Using Aerobacter aerogenes, Widra (144) obtained cytochemical evidence that the volutin granules contained polyphosphate, RNA, and lipids. These granules were also masked with basic protein. He concluded that the polyphosphate was linked to RNA and the RNA was linked to protein.

Isolation

Mann (97) in 1944, working with homogenates of the mold Aspergillus, extracted polyphosphates with ice-cold 10 percent trichloroacetic acid (TCA) for 15 minutes followed by 5 percent TCA for 12 hours at 0°. In 1949 Wiame (143) distinguished yeast polyphosphates of two types. One, made up of orthophosphate and polymers of low molecular weight, was soluble in cold 10 percent TCA; and the other, made up of higher polymers, was soluble only in hot acid or dilute bases at room temperature. Lohmann (91) found that heating a solution of polyphosphates and nucleic acids in 0.1 N NaOH for five minutes at 100° would hydrolyze the glycosidic bond of the nucleic acid, but would not hydrolyze the polyphosphate. Lohmann also found that he could extract all of the polyphosphates of brewer's yeast with boiling water, but it was necessary to use 2.5 percent TCA in the case of baker's yeast. Another technique developed by Lohmann was the extraction of all polyphosphates with 0.1 M salt solution

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and subsequent extraction of the orthophosphate as ammonium phosphomolybdate with isobutyl alcohol. $NaClO_4$, $NaNO_3$, NaNCS, or NaI may be used.

Liss and Langen (88) have developed the most refined isolation for yeast polyphosphates. They obtained an acid-soluble fraction with a chain-length of three to eight, a concentrated salt and acid pH soluble fraction with an average chain-length of 18-22, a fraction soluble at 0° and pH 10 with an average chain-length of 50-60, and an organically bound fraction soluble at room temperature on lengthy exposure to 0.05 N NaOH.

Once the polyphosphates are extracted they may be precipitated with any of a number of reagents. (A) Mann (97) precipitated polyphosphates with $Pb(NO_3)_2$ at pH 1.8, and with $Ba(NO_3)_2$ at pH 2, but this procedure lost most of the polyphosphate due to hydrolysis. (B) He also treated an extract with an equal volume of ethanol, discarded the precipitate and adjusted the supernatant to pH 5.5 with either NaOH or NH_4OH. The resultant precipitate contained about 13-16 percent phosphorus. By repeated reprecipitations he obtained a material with a phosphorus content of 23 percent. (C) Wiame (143) adjusted a TCA extract to pH 4.5 with NaOH and then added sodium acetate until the solution was 1 M with respect to acetate. This was followed by the addition of $Ba(NO_3)_2$ which precipitated a good yield of polyphosphate. (D) Inorganic phosphates including polyphosphates have been precipitated by various monoamines, diamines, hydrazines, dibasic

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amino acids, dipeptides, and guanidine compounds (35). This precipitation by amines was selective for highly polymerized polyphosphate at pH's of 2.0 to 4.0. Completeness of precipitation varied from 50 to 95 percent. If the extract were first mixed with an equal volume of ethanol and the resulting precipitate removed, the polyphosphate could be precipitated from the supernatant by adding $MgCl_2$ and alcohol (91).

Application of various combinations of these methods has resulted in phosphorus fractionation data for Acetabularia (136) of a fairly comprehensive nature. These data indicate that in the case of this alga there are no detectable levels of polyphosphates between orthophosphate and polymers of a length of ten phosphates. They also indicate that polyphosphate accounts for the bulk of the total phosphorus of the cell.

Physical Properties

At present no one has isolated polyphosphate from an algal source and determined such properties as molecular weight. A water soluble, non-dialyzable polyphosphate has been isolated from Aspergillus niger and an average molecular weight of 6000-7000 obtained (59). Similarly two types of polyphosphate were characterized from yeast. One had an average chain length of about ten phosphates and the other had an average length of about 50 phosphates (155). The larger polymer was found in greater amounts. Liss and Langen

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(88), as discussed earlier, characterized four types of polyphosphate in brewer's yeast. In a further study of the organically bound fraction (89) they showed that 75 percent of this fraction had an average chain length of 165 phosphates. Electrophoretic behavior of this polyphosphate indicated that it was composed of a variety of chain lengths.

One study has been done on the heat of hydrolysis of the polyphosphate from yeast (154). The results were:

Yeast polyphosphate	$\Delta H = 10$ Kcal./anhydride bond
Graham's salt	$\Delta H = 11$ Kcal./anhydride bond
Pyrophosphate	$\Delta H = 9$ Kcal./anhydride bond

From these data we classify the polyphosphate anhydride linkage as equivalent to the "high energy" anhydride linkage in ATP.

It has been shown that low-frequency ultrasonic waves irreversibly break the phosphate anhydride bonds of polyphosphates in aqueous solutions (23). In the same study the dialyzability of various polyphosphates and metaphosphates was tested. It was found that the metaphosphates dialyzed slightly faster than the corresponding polyphosphates. Graham's salt (wt. aver. mol. wt. = 1545) was found to dialyze fairly rapidly and a series of other polymers with weight average molecular weights of up to 17,580 showed gradually decreasing rates of dialysis.

In a study of long-chain polyphosphates Malmgren (96), made use of the electron microscope and streaming birefringence in addition to ultracentrifugation and diffusion rates.

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He concluded that the shape of the molecule changes with the medium, that the molecules are largely solvated, and that the shape of the molecule is not a perfect sphere, but rather a wrinkled chain. Thilo (137) demonstrated by X-ray diffraction that the phosphate tetrahedra of various salts of long-chain polyphosphates are wrinkled and spiraled in various ways such that the sodium salt has three phosphates in a distance of 7.3 A and the silver salt has four phosphates in a distance of 6.1 A.

In a study of diffusion rates of synthetic and yeast polyphosphates, Katchman (65) found a marked effect of electrolytes. Diffusion was more rapid in water than in salt solutions and the rate was also more rapid in dilute than in concentrated solutions of polyphosphate.

Methods of Analysis

The analysis of polyphosphates is a difficult problem which has not been completely solved to date. The qualitative analysis may be divided into chromatographic, electrophoretic, colorimetric, and spectroscopic methods.

It has been known for some time that very dilute solutions of polyphosphates give a metachromatic reaction with the basophilic dye, toluidine blue (142). With a given polymer a maximum color production takes place at a certain ratio of phosphate to dye and color production declines on either side of this ratio. This color development is inhibited by salts. The metachromatic reaction is also given by

sulfuric esters of polysaccharids, by nucleic acids, and by polyanions in general (66). A weak reaction is given by ATP (13). Bradley (12) has made a study of the chromatic reactions of a variety of dyes with several polyanions.

If cells are dyed with toluidine blue and fixed on microscope slides, the nucleic acid color can be extracted with 0.1 N HCl and the polyphosphates can be extracted with cold 10 percent TCA. Any remaining colored cell material can be assumed to be sulfuric esters of polysaccharides. These reactions can be used to qualitatively identify polyphosphates in cells or extracts. It has been shown that methyl green and pyronine give almost identical cytological reactions (36). One attempt has been made to utilize the metachromatic reaction as a quantitative method by controlling temperature, pH, salt concentration, and time of reaction (28). This method was still only semi-quantitative due to interference from other biologically occurring materials and the variation in color development with the length of the phosphate polymer. This variation was thought to be due to the interference of the doubly-charged end phosphates (135).

One-dimensional paper chromatographic procedures for polyphosphates have been developed (9, 33, 49, 137), and two-dimensional paper chromatograms have been used to separate metaphosphates from polyphosphates (36). Attempts to elute spots and analyze them by micromethods have only succeeded in giving semiquantitative results, and there is some doubt

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if they are any improvement over the measurement of the density of spots after spraying with a color-developing reagent. Continuous paper electrophoresis has also been developed and under ideal conditions it can yield a quantitative separation of various phosphate polymers (117). The use of Dowex-1 ion-exchange resin to adsorb the polyphosphates and dilute HCl-KCl mixtures to elute the components has limited usefulness for the separation of the oligopolyphosphates (72).

The infrared spectra of several polyphosphates have been determined (26). Orthophosphate had a broad absorption between 8.7 and 10.0 u, and a linear, long-chain polyphosphate was found to have a strong band at 7.7 to 7.9 u and a weaker band at 11.5 to 11.8 u.

Upon exposure to ultraviolet light in the region of 260-280 m u or 296-313 m u, polyphosphates fluoresced at about 350-360 mu rather markedly, and a tendency for the intensity of the fluorescence to be dependent upon the degree of polymerization has been noted (2).

The state of quantitative analysis of polyphosphates is not very well advanced. Of the many methods of analysis for total phosphorus, that of King (68) applies well to this type of material. Orthophosphate may be determined quite accurately (92). Furthermore if the only inorganic phosphates present were orthophosphate and pyrophosphate, these compounds could be accurately determined (39). Beyond this simple beginning a certain amount of guess work enters the

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picture. It is known that all inorganic polyphosphates are quantitatively hydrolyzed to orthophosphate by boiling in 1 N HCl for seven minutes. Most organic phosphates are stabile to these conditions, but this is not universally true. Lohmann (91) determined orthophosphate (Δ_0^0), orthophosphate after seven minutes hydrolysis at 100° in 1 N HCl (Δ_0^7), and orthophosphate after 30 minutes hydrolysis at 100° in 1 N HCl (Δ_7^{30}). He then postulated on the basis of some experimental work that (Δ_0^7) - (Δ_7^{30}) = polyphosphate.

Ecological Considerations

The existence of polyphosphates in natural populations of algae and the quantitative importance of this type of phosphate has not been directly demonstrated. However, in view of the following literature reports, such an occurrence of polyphosphate should not be in serious doubt. Lund (94) in his studies on the annual spring blooms of Asterionella formosa in England found the blooms to be almost entirely composed of this algae. In this case available orthophosphate was usually in the range of one to four parts per billion (p.p.b.) and silicon was shown to be the limiting factor in growth. It was found that the total phosphorus per cell was much higher at or near the maximum period of the bloom. Ketchum (67) in studies of a pure culture of Chlorella has shown that under ordinary culture conditions the phosphorus to nitrogen ratio was about one to three, but under conditions of deficient phosphate the ratio became about one to 17.

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Algae

Such variations in total phosphorus are probably due to changes in the polyphosphate pool. It has also been found that Chlorella accumulates polyphosphate in the absence of CO_2 (150).

It is interesting to contrast the type of environment favorable to the growth of blue-green algae with that favorable for such algae as the Asterionella discussed above. Guseva (58) found that under natural conditions Anabaena lemmermanii required 870 p.p.b. of phosphate-phosphorus for optimal development and Aphanizomenon flos-aquae required 260 p.p.b. These values may be affected drastically by such factors as the nitrogen source. There is evidence that lake water contains a factor which aids in the uptake of phosphate which is not present in synthetic media. This factor may be a peptide (58).

The absorption of orthophosphate and formation of polyphosphate in yeast can be carried out anaerobically but requires potassium ions (119, 143). This potassium requirement is due to its effect on the yeast cell wall permeability (153). In the case of diatoms, $Fe(OH)_3$ is believed to play a role in the uptake of orthophosphate. $Fe(OH)_3$ forms a colloid which adsorbs phosphate and is, in turn, attracted to the cell membrane of the diatom (52). It has been suggested that algae precipitated to the bottom of the sea were the source of the present-day sedimentary phosphate rocks (22).

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pools by inhibiting their growth with the heavy-metal salts of polyphosphate (112).

Gross Physiological Effects on Higher Animals

Labeled polyphosphates, when injected intravenously into dogs, were stored in the liver and spleen. They were then degraded to orthophosphate (47). When trimetaphosphate and tetrametaphosphate were injected intravenously into rabbits, they were excreted intact in the urine (45). The linear polymers were hydrolyzed to orthophosphate. All phosphate polymers were hydrolyzed when taken orally. When high concentrations were injected intravenously into rats, polyphosphates caused severe acidosis, the formation of a calcium complex, and cardiac arrest (46).

It has also been shown that both pyro- and tripolyphosphate significantly inhibited oxidative phosphorylation in isolated mitochondria (138). Phosphate glass failed to have an effect.

Biosynthesis

In order for an organism to carry out the biosynthesis of polyphosphates it must generate "high-energy" phosphate carriers such as ATP. The formation of this carrier may be accomplished by photosynthetic phosphorylation or respiratory oxidative phosphorylation.

In the photosynthetic bacteria which do not have their grana enclosed in a chloroplast, CO_2 fixation takes place in the cytoplasm (42). This may also be the case in blue-green

algae. The ATP which is produced by photosynthetic phosphorylation is very rapidly utilized by two competitive mechanisms: CO_2 fixation and polyphosphate formation. Wassink (140) found evidence for the accumulation of a phosphate compound in Chlorella other than ATP after two minutes exposure to light. Wintermans (151) found the uptake of orthophosphate by Chlorella to be rapid in the light and more rapid in the absence of CO_2. He concluded that there was a conversion of photosynthetic energy to stored polyphosphate. He supported this conclusion by extracting polyphosphate from the cultures.

Early workers assumed that the mode of synthesis of polyphosphate chains was the addition of phosphate units one at a time to a short starter or to orthophosphate itself. In 1949, Wiame (143) postulated that two systems might exist, one for the biosynthesis of the longer polymers and one for the formation of the shorter ones. This came as a result of his studies of the acid-soluble and acid-insoluble polyphosphate pools in yeast which had been exposed to various changes in their environment.

Unfortunately no enzymatic studies have been carried out on polyphosphate formation in photosynthetic organisms. However, some studies have been done on yeast and bacteria. In 1954 Hoffman-Ostenoff (55) reported the finding of an enzyme in yeast which carried out the following reaction with P^{32}-labeled polyphosphate:

$$ADP + (NaP^{32}O_3)_n \rightleftharpoons ATP^{32} + (NaP^{32}O_3)_{n-1}$$

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The optimum pH was found to be 6.7, but the reversal of the reaction was not demonstrated due to a lack of P^{32}-labeled ATP. In 1955 Yoshida (155) mentioned "metaphosphate synthesized from orthophosphate or ATP by the enzyme juice extracted from yeast" and referred to a previous obscure paper (152). Finally in 1956 Kornberg (71) reported the isolation of a relatively stabile enzyme from Escherischia coli for the synthesis of polyphosphate. This enzyme required magnesium ion (4×10^{-3}); was unaffected by potassium, arsenate, or dinitrophenol; had an optimum pH of 7.2; was completely inhibited by fluoride; and carried out the reaction:

$$X\ ATP + \underset{\text{primer}}{(PO_3^-)_n} \rightleftharpoons X\ ADP + (PO_3^-)_{n+x}$$

In a later paper (75) Kornberg also illustrated the reversal of this reaction and showed that CDP, UDP, GDP, and AMP had little or no reactivity in this system. A very interesting finding was that low concentrations of ADP inhibited the formation of polyphosphate in this system. This fact places considerable doubt on the role of such an anzyme system in the biosynthesis of polyphosphate. Kornberg accepted the possibility that two systems are in operation for the synthesis of low and high polymers as discussed above. Kornberg's final product was quite large, since it was quantitatively precipitated by albumin at pH 4.0.

Kornberg (72) suggested a mechanism for the formation of tripolyphosphate:

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$$P\text{-}P + ADP \xrightarrow[\text{kinase}]{\text{adenylate}} P\text{-}P\text{-}P + AMP$$

He also suggested that the large amounts of pyrophosphate formed by pyrophosphorylase reactions might undergo reactions such as:

$$P\text{-}P + (PO_3^-)_n \rightleftharpoons P_i + (PO_3^-)_{n+1}$$

An enzyme system similar to that of Kornberg's has now been isolated from Azotobacter by Zaetseva (157). It can not utilize UTP or ITP in the forward direction or IDP, GDP, or UDP in the reverse direction. Since total polyphosphate was measured, we can be sure that net synthesis was demonstrated. This enzyme was tested for activity on polyphosphates of various sizes and only showed activity in both reaction directions with those of high molecular weight. ADP was found to inhibit the forward direction of the reaction.

It has been thoroughly demonstrated that the metabolic turnover of all types of polyphosphate is very rapid (7, 80, 84, 130). By adding P^{32}-labeled orthophosphate to the media for various lengths of time and at various stages of the life cycle, and by intricate polyphosphate fractionation procedures; two groups of investigators working with different organisms have arrived at the same conclusions (80, 84, 156). One conclusion was that the largest polymers of polyphosphate were bound rather tightly to an organic compound. Another conclusion was that, on the basis of both specific activity and total activity, this type of poly-

phosphate was the first to become P^{32}-labeled and thereafter the other lower-weight polymers became labeled in descending order until the classical acid-soluble fraction was finally labeled.

As a result of studies of RNA-polyphosphate complexes, several workers have postulated that polyphosphates might be synthesized on the surface of RNA or a nucleoprotein (6, 20, 31). These long polymers could then separate and become degraded either by means of transphosphorylation to ADP or AMP or by means of polyphosphatases.

Two reports in the literature tend to support a conflicting view that very low molecular weight polymers of phosphate might be made directly from phosphate in some cases. Szulmajster and Gardiner (131) using a homogenate of a mutant of Clostridium found that 37.5 percent of the P^{32}-labeled orthophosphate was incorporated as pyrophosphate, 23 percent as tripolyphosphate, and 22 percent as unidentified polyphosphate. This mutant uses creatinine as its major energy and nitrogen source and CO_2 as a major carbon source. A possible reason for these results could be a very active polyphosphatase system and a rather severe extraction procedures which might have broken down the higher molecular weight polyphosphates to pyro- and tripolyphosphate before they were identified. More work is needed to elucidate this point.

In the second case Goksoyr (44) found that the basidiomycete fungus Merulius lacrymans, upon exposure to P^{32}-labeled

phosphate for various time intervals, labeled pyrophosphate first and the pyrophosphate had a higher specific activity than ATP at 30 minutes. It was also found that high molecular weight polyphosphate was not labeled rapidly while both GDP and UDP were labeled rapidly.

Several conditions other than CO_2 deficiency have been shown to promote unusually high polyphosphate levels. Winder (146) found that Mycobacteria tripled their inorganic phosphate content in five hours and underwent a tremendous enlargement of their metachromatic granules when 2.5 percent tetrahydrofurfuryl alcohol was added to their medium. In a later study Winder (149) found lesser but similar effects upon the addition of ethanol, tetrahydrofuran, or butanol to their medium. Propylene glycol, butyrate, and chloromycetin had no effect.

Wiame (143) studied the effects on the polyphosphate in yeast of alternately placing the yeast in phosphate-free and phosphate-containing media. Upon exposure to phosphate, phosphorus-deficient yeast, which had very little polyphosphate, formed large quantities of both low and high polymers of phosphate. Next the orthophosphate and high polymers were reduced. Then the low polymers were rapidly utilized and the yeast returned to their normal condition with regard to phosphorus pools. Thus, exposure of phosphorus deficient organisms to high phosphate concentrations could be a method of improving isolation yields.

It has been shown in cultures of Corynebacterium

diphtheriae (101, 115), that glucose in the medium depressed polyphosphate and increased RNA, but dinitrophenol or malate had the opposite effect.

There is some evidence that calcium might be involved in the biosynthesis of polyphosphate. It has been shown (37), that calcium was required for both nitrogen fixation and polyphosphate synthesis in Azotobacter vinelandi.

The phosphorus metabolism of Acetabularia mediterranea has been studied under normal and enucleated conditions (122). It was found that growth, polyphosphate synthesis, protein synthesis, and phosphorus metabolism in general proceeded without the nucleus, but at a decreased rate.

Catabolism

The catabolism of polyphosphates in microorganisms has been shown to proceed by both energy conserving and phosphatase mechanisms. The energy conserving mechanisms will be discussed first. It was found by Winder (147) that in cell-free extracts of Mycobacterium under anaerobic conditions the reaction:

$$(NaPO_3)_n + \text{Glycerol} \xrightarrow[ADP]{ATP\ or} \text{glycerol phosphate} + (NaPO_3)_{n-1}$$

took place if polyphosphate, glycerol, and ATP or ADP were added. A further purification of this enzyme system was carried out (149) and resulted in the characterization of the enzyme polyphosphate-AMP-phosphotransferase, which catalyzed the reaction:

$$AMP + (NaPO_3)_n \rightleftharpoons ADP + (NaPO_3)_{n-1}$$

The enzyme was located in the 25,000 g supernatant fraction, was stimulated by 3×10^{-4} M Mg^{++}, would not utilize pyrophosphate, and had a pH optimum of 6.3.

If we consider the previously discussed enzymes which carry out the transphosphorylation to AMP and ADP and which seem to have equilibria in the direction of ATP formation (55, 71, 75, 152, 155, 157), they constitute a system to explain the breakdown of polyphosphates with conservation of their "high-energy" bonds as either ADP or ATP.

The only report of an energy conserving enzyme for the metabolism of metaphosphates is that of Rafter (107). This enzyme was found in yeast and carried out the phosphorolysis of trimetaphosphate to tetrapolyphosphate. Since yeast is the only organism in which cyclic metaphosphates have been demonstrated as a major constituent, this was the most likely source for such an enzyme. This is the only case reported at this time of an enzyme which carries out the phosphorolysis of a cyclic compound.

Another interesting enzymatic system, which has been reported by Rafter (108), utilized pyrophosphate and glucose to form glucose-6-phosphate and orthophosphate. Such a system, if it is found to occur widely, could explain another method of utilizing the energy of the oligopolyphosphates as they are broken down. Thus far it has only been demonstrated in mouse mitochondria.

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Poly- and metaphosphatases have been found widely occurring. Mattenheimer (98) found frog muscle extracts and dried yeast to be capable of splitting all phosphate polymers, but human and rat liver could not split polyphosphates above tripolyphosphate and human kidney doesn't act upon Graham's salt. Poly- and metaphosphatases were found in rice (153) and barley (113). No work has been done with algae, but Mann (97) found a polyphosphatase in both the cells and the media of Aspergillus niger. He found the optimum pH to be 3.7 to 4.2 Lindeberg (86) found that ferrous and cupric ions stimulated this enzyme's production, while high Zn^{++} stopped its production in A. niger. Malmgren (96) studied the polyphosphatase complex of A. niger after a 20-fold purification. He applied this complex to a polyphosphate with a molecular weight of one million and the action seemed to stop at about the level of tetra- or pentapolyphosphate if the pH was controled and manganous ion was added. Manganous ion was an activator for the high molecular weight polyphosphatases but not the lower molecular weight ones. It increased activity 30-fold. Mg^{++}, Ca^{++}, and Zn^{++} may act as activators also. Fluoride, phloretin phosphate, and citric acid inhibited the low molecular weight polyphosphatases. The high molecular weight polyphosphatases were more heat sensitive. Phosphatase activity was found to have different pH optima for different polyphosphate substrates.

Kunitz (83) has crystallized the pyrophosphatase from baker's yeast. It is a protein of the albumin type with a

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molecular weight of about 100,000, and an isoelectric point of about pH 4.8. It requires Mg^{++}, Mn^{++}, or Co^{++} for an activator, and is inhibited by Ca^{++}. Optimum pH is 7.0, temperature 40°, substrate concentration 3-4 X 10^{-3} M $Na_4P_2O_7$, and activator concentration 3 X 10^{-3} M Mg^{++}. Maximum rate is 1000 moles of pyrophosphate per mole of enzyme per second.

In 1956 Kornberg (74) illustrated the following system in yeast extracts:

trimetaphosphate $\xrightarrow{\text{trimeta-phosphatase}}$ tripolyphosphate

tripolyphosphate $\xrightarrow{\text{tripoly-phosphatase}}$ pyro-P + ortho-P

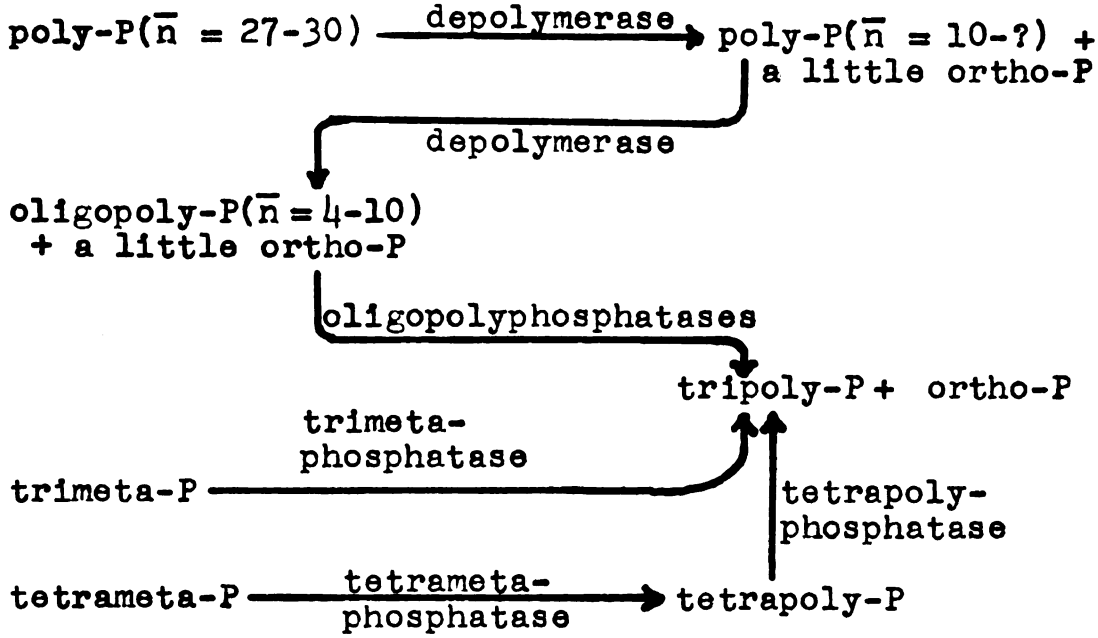
pyrophosphate $\xrightarrow{\text{pyro-phosphatase}}$ 2 orthophosphate

In the same year Mattenheimer (99) published the results of a more extensive study. Working with brewer's yeast, primarily, he found the optimum pH to be close to 7 for metaphosphatases and close to 8 for polyphosphatases. In the case of pyrophosphatase he postulates the following mechanism of action:

$$\begin{array}{ccccc} E + MgPP & \rightleftharpoons & EMgPP & \rightleftharpoons & E + Mg + 2P \\ \pm PP \Big\downarrow\Big\uparrow & & \pm \Big\downarrow\Big\uparrow MgPP & & \\ EPP & & E(MgPP)_2 & & \end{array}$$

By means of ammonium sulfate fractionations Mattenheimer obtained two fractions with specifically higher activity on pyrophosphate and tripolyphosphate respectively. Similar separations were made on alumina, calcium bentonite, and by means of electrophoresis. By studying the products of polyphosphatase activity on high molecular weight substrates,

Mattenheimer was able to show the following scheme of breakdown:



From tripolyphosphate Mattenheimer's scheme proceeds in the same manner as that of Kornberg's. In this manner yeast are able to hydrolyze high molecular weight polyphosphates to orthophosphate.

An enzyme from Corynebacterium xerosis (102) has been purified 100-fold which is unusual in that it causes no formation of short polyphosphate polymers, but rather only orthophosphate. This suggests that it operates specifically on the terminal phosphates of the chain. It is also unusual in that all metal ions tested, including Mg^{++}, inhibited activity and EDTA stimulated activity.

Another unusual enzyme or enzyme complex has been studied in peas (106). In this case no separation could be achieved between phosphomonoesterase, and polyphosphatase activity. The enzyme attacks trimetaphosphate, Graham's

salt, tripolyphosphate, nucleoside triphosphates, and β-glycerol phosphate but not tetrametaphosphate. It is inhibited by fluoride and molybdate, and is unaffected by Mg^{++}.

One possible physiological role of polyphosphatases would be as a mechanism for the breakdown of external polyphosphates in order to make the phosphate available to the cell.

In studying either biosynthesis or catabolism of polyphosphates several facts should be kept in mind. Ca^{++} and other alkaline earth ions are known to catalyze the non-enzymatic breakdown of high, but not of low molecular weight polyphosphates in alkaline solutions (89). It has also been shown (136) that there is a chemical equilibrium in neutral aqueous solutions at 60° as follows:

trimeta-P + ortho-P $\rightleftharpoons$ tetrapoly-P

Similar interconversions between other polyphosphates and metaphosphates at elevated temperatures should be expected.

Organic Complexes

Under this general heading will be discussed a number of compounds and complexes which have been shown to contain polyphosphates or which may contain polyphosphates.

In the presence of magnesium ion; pyrophosphate, tripolyphosphate, ATP, and Graham's salt all form dissociable complexes with actomyosin and the actomyosin is split into myosin and actin (1).

The longer polyphosphates are coprecipitated with the polycations of proteins when TCA is added, but the short polymers are not (64). This accounts for the "insolubility" of the higher polyphosphates in cold TCA.

From a physiological point of view, the most interesting of the polyphosphate complexes studied to date are the RNA-polyphosphate complexes. For many years a close cytological connection has been noted in a wide variety of organisms between RNA and polyphosphate. One of the earliest direct approaches to the relationship was in 1953 (32). At that time it was reported that isolated RNA contained excess phosphorus and that this phosphorus was acid labile. If this RNA was incubated with myokinase and ATP, the excess phosphorus could be increased. In 1956 Winder (148) reported the isolation from Mycobacteria of a fraction containing both RNA and polyphosphate. In an attempt to separate these two components neither of the following was effective:

1. Extraction for 24 hours at 19° with 0.01 M bicarbonate buffer, pH 9.0.

2. Extraction for 24 hours at 37° with saturated aqueous urea, followed by 1 M NaCl at 19°.

About the same time Belozersky (6) reported finding that the acid-soluble polyphosphate of yeast combined with pentose polymers and RNA. In 1957 the same group (7) showed that the phosphorus entering into the cellular composition of Aspergillus niger was first found in the acid-insoluble polyphosphates and that this fraction contained a large

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amount of RNA. After many reprecipitations the RNA still contained three times as much phosphorus as to be expected and the excess phosphorus was shown to be in the form of polyphosphate. This group also showed that mixtures of yeast-RNA and polyphosphate could be separated by treatment with magnesia mixture, but that the natural material was not affected.

In 1958 Langen (84) reported the same general type of data for yeast, but he did not investigate the nature of the organic material combined with the polyphosphate. In 1959 Liss and Langen (88) reported that the release of this organically bound high molecular weight polyphosphate into solution with 0.05 N NaOH followed first order kinetics and was quite temperature dependent. He suggested that a chemical reaction was involved in the release. In 1960 the same group (89) further characterized this fraction of organically bound polyphosphate. If the yeast were pretreated to remove RNA either by the action of RNase or dilute alkali and salt extraction, the kinetics of the polyphosphate extraction were unaffected. When 75 percent of this polyphosphate fraction was carefully purified, its average chain length was 165 phosphate units, corresponding to a molecular weight of 19,500. The authors expressed the belief that there was no connection between RNA and polyphosphate on the basis that:

1. Most of the RNA can be removed by salt extraction without simultaneously extracting polyphosphate.

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2. The 75 percent of the organically bound fraction of polyphosphate which can be extracted by dodecylsulfate can then be treated with charcoal, which removes the RNA and not the polyphosphate.

These facts did not prove that an RNA-polyphosphate complex did not exist. They did indicate that the yeast which Liss and Langen studied probably contained a considerable amount of RNA and polyphosphate which was not complexed.

In another case Schweiger (122) applied charcoal adsorption in a fractionation of the phosphorus compounds of Acetabularia. He found that varying percentages of polyphosphates, both acid-soluble and acid-insoluble, were adsorbed by the charcoal. In these cases there may have existed a complex between the RNA, which was adsorbed, and the fraction of polyphosphate which was adsorbed.

In 1958 Griunberg-Manago (48) reported the isolation of an enzyme from yeast which synthesized RNA polymers. It was not typical of this type of enzyme since exchange was inhibited by Mg^{++} and the enzyme was able to utilize GDP. Polymer formation required Mg^{++} and was specific for nucleoside diphosphates. Another unusual property was the fact that orthophosphate was taken up rather than released in the reaction and an enzyme-RNA-phosphate was precipitated. The product had a different ultraviolet spectrum than yeast RNA, would not form a triple helix, would not react with formaldehyde, and the products of hydrolysis did not correspond to 3' AMP, 5' AMP or adenine. Whether or not the excess phosphorus in the complex was polyphosphate was not tested.

Intercellular Physiological Functions

When the preceding discussions are considered it becomes obvious that one gross physiological function of polyphosphates in the algal cell might be the storage of phosphate or energy. A possible use of the large amount of energy of the polyphosphates would be for the synthesis of proteins or nucleic acids, especially during cell division. Polyphosphates would provide a pool of energy which could be stored over a period of time and kept available for limited and specific metabolic uses.

Keck (66) found that members of the family Zygnemataceae in the green algae characteristically have polyphosphate and RNA in the chloroplasts. Both were found in high concentrations and he suggests a possible protein synthesis in the chloroplasts with the polyphosphate providing the necessary ATP. In a study on the yeast Torulopsis utilis Chayen (24) found the cellular polyphosphates reached a minimum at exactly the same time that protein synthesis reached a maximum. At this same time nucleic acid content reached a maximum. It was found that nucleic acid extracted from actively growing yeast was capable of supporting luminescence in firefly extracts. This phenomenon may have been due to utilization of the polyphosphates which were associated with the nucleic acid.

Bringman (14) in a study of Corynebacterium found them to have "nucleoids" containing DNA in young cells and both

DNA and RNA in older cells. In the young cells the cytoplasm contained RNA but the older cells had no free RNA. Polyphosphate was demonstrated to be present in these nucleoids. This was also shown to be the case in Actinomycetes, and blue-green algae. In Mycobacterium these particles were found to lie at the plane of cell division and were also observed to undergo division at this time (70).

In a cytological study of cell division in yeast Lindegren (87) showed by cytological dye techniques that budding in yeast was invariably preceded by polyphosphate deposition on the chromosomes even when grown in low phosphate media. During rapid division the polyphosphate may temporarily disappear, but this division can not be maintained without polyphosphate on the chromosomes.

In 1947 Albaum (3) reported the isolation of a compound from oat seedlings which seemed to be made up of two units of adenine, two units of arabinose, and four units of phosphate. Stich (129) in his work with Acetabularia, using dye techniques, believed that the polyphosphates, which are found in spheres within the cell, were combined with an organic component since the metachromatic reaction did not take place in vivo.

It would seem pertinent to give careful consideration to the possible role of polyphosphates in the synthesis of protein, RNA, and DNA as a result of these rather scattered and fragmentary findings.

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studied Chlorella at the stage of nuclear division. He found the uptake of CO_2 was greatly reduced at this stage, the uptake of orthophosphate was accelerated, and the oxygen evolution was relatively constant. Further analysis showed that the bulk of the phosphate taken up was converted to a high molecular weight polyphosphate.

More recently it has been reported that Chlorella pyrenoidosa, when synchronized 90 percent, take up 80-90 percent of their total phosphorus immediately prior to nuclear division and that this is found as polyphosphate at that time (121). If these cells were synchronized and then put into phosphate deficient medium, they would not divide although they had some polyphosphate. Apparently the polyphosphate must reach a certain level before cell division is triggered. The same report confirmed the observation of Stich (129), that the metachromasy of living cells is very low compared with fixed cells.

Sall (116) has reported that 80 percent synchronized Corynebacterium diphtheriae showed a marked increase in polyphosphates immediately prior to cell division.

Several workers (51, 120) have reported that conditions which cause a decrease in nucleic acid synthesis result in increased levels of polyphosphate.

No definitive evidence exists for the function of the RNA-polyphosphate complexes, but it has been shown that they are the first compounds other than ATP to be labeled with radioactive phosphate (80), that the polyphosphate-phosphorus

has a higher specific activity in this complex than the RNA-phosphorus, and that this complex probably accumulates just before cell division.

In the field of speculation Belozersky (6) proposed that high molecular weight polyphosphate is synthesized by phosphorylation of RNA. Gruinberg-Manago (48) suggested that the excess phosphate serves as a phosphorylating agent in the course of protein biosynthesis and perhaps has an influence in the determination of amino acid sequence along the polynucleotide chain.

In 1952 Dounce (31) proposed a hypothetical mechanism which could be adapted to either protein, nucleic acid, or polyphosphate biosynthesis:

1. Formation of diphosphonucleic acids (by splitting out water between orthophosphate and the free hydroxyl of the phosphates in the nucleic acid)
2. Reaction with either amino acids or nucleotides to release orthophosphate and form a complex
3. Cleavage of protein by transamidations or nucleic acids by transphosphorylations or (by elimination of step 2) polyphosphate by transphosphorylations

In 1959 Dounce reported the use of artificially phosphorylated RNA and mixtures of RNA and polyphosphates for the synthesis, in vitro, of copolymers of histidine and cysteine which were nondialyzable and gave positive biuret reactions (118). Orthophosphate was liberated in the reaction and the product was attacked by carboxypeptidase.

Separation of RNA into Chemically or Physiologically Distinct Fractions

A large amount of experimental effort has been directed toward the goal of isolating relatively undenatured RNA which is chemically and physiologically homogeneous. This work is based upon the hypothesis that within one organism there are a number of RNA molecules which differ in such parameters as nucleotide sequence and molecular weight. Some limited progress has been made in attaining this goal by the use of differential centrifugation of broken cells, solvent extraction procedures, countercurrent distribution, and ion-exchange chromatography. At present there are not many criteria by which the separated fractions can be distinguished. Base ratios, molecular weight, the infectivity of virus RNA, and the amino-acid-incorporating activity of "soluble" RNA are currently used to distinguish various types of RNA.

An example of the application of differential centrifugation is seen in the work of Iwamura (62). In this study he showed that the various fractions obtained from Chlorella ellipsoidea had different base ratios. Although this was the first time this had been done on algal material, the same type of results were obtained earlier on mammalian tissues. One example is De Lamirande's study of rat liver (29).

In a study of rabbit thymus, and calf thymus, spleen,

and liver, Logan (90) combined a centrifugal separation of nuclei and cytoplasm with solvent extraction. He found that one fraction of the nuclear RNA was citrate soluble and that the base ratios and metabolic turnover of the two nuclear RNAs and the cytoplasmic RNA were different. Calf thymus nuclei were also shown by Frick (41) to contain two RNA fractions with different base ratios, one of which was soluble in $MgCl_2$.

Recently Kirby (69) and Holley (56) have reported the separation of amino acid acceptor activity in "soluble" RNA by counter current distribution.

A number of attempts have been made to utilize ion-exchange resins to separate nucleic acids. RNA has been fractionated on calcium phosphate (139) and Ecteola-cellulose (12, 134). DNA has also been fractionated on histones coupled to cellulose (19) and DEAE-cellulose (18). In all of these studies no clear-cut separations of nucleic acids, other than artifacts produced by discontinuous gradients, have been reported. In some chromatographic procedures two somewhat separated areas have resulted, but the results are usually a wide band of continuously eluting nucleic acid. When the band is analyzed for base ratios and sedimentation coefficients at various points, differences can be shown. Brown found a decreasing percentage of guanine as ionic strength increased (19) in the case of his histone-cellulose columns. Both Bradley (12) and Taussig (134) found that higher sedimentation coefficients corresponded to higher ionic strengths

in the case of Ecteola cellulose columns.

Some Properties of Isolated RNA

The Shape of the Isolated RNA Molecule

Although the double-helical structure of DNA has found wide acceptance, the structure of RNA is not very well established. Rich (100, 111) found that synthetically prepared poly-AGUC had about the same sedimentation constant as natural RNA and that a copolymer of poly-A and poly-U showed an X-ray diffraction pattern indicating a double helix with 10 nucleotides per turn. Natural RNA shows a different X-ray diffraction pattern. Recently Doty (30) stated that RNA doesn't have a regular helical structure as in DNA but has ". . . irregularly coiled, relatively compact, single polymeric chains." He also stated that at alkaline pH all RNA hydrogen bonding systems are denatured. Similarly Cox (27) calculated that bacterial RNA that had been isolated at a neutral pH had about 30 percent hydrogen-bonded nature. These calculations were based on the amount of discrepancy between potentiometric titrations and back titrations. He also decided that the molecule was not branched on the basis of a lack of inflection at the pK for the ionization of the phosphate secondary hydroxyl.

Hyperchromicity of RNA

RNA, which has an optical density maximum near 260 mu, is known to undergo various changes in its extinction coefficient. A general working theory is that these fluctuations are due to an "uncovering or covering" of the resonating double bond system in the nitrogen bases. A model system in which anthracene is coupled to bovine plasma albumin by means of a mercury-sulfhydryl linkage has been studied by Williams (145). He found that by changing the pH, and thus the shape of the albumin molecules, the extinction coefficient of the anthracene moiety could be varied. He then hypothesized that this change had been caused by a nonspecific interaction of the anthracene with its protein environment.

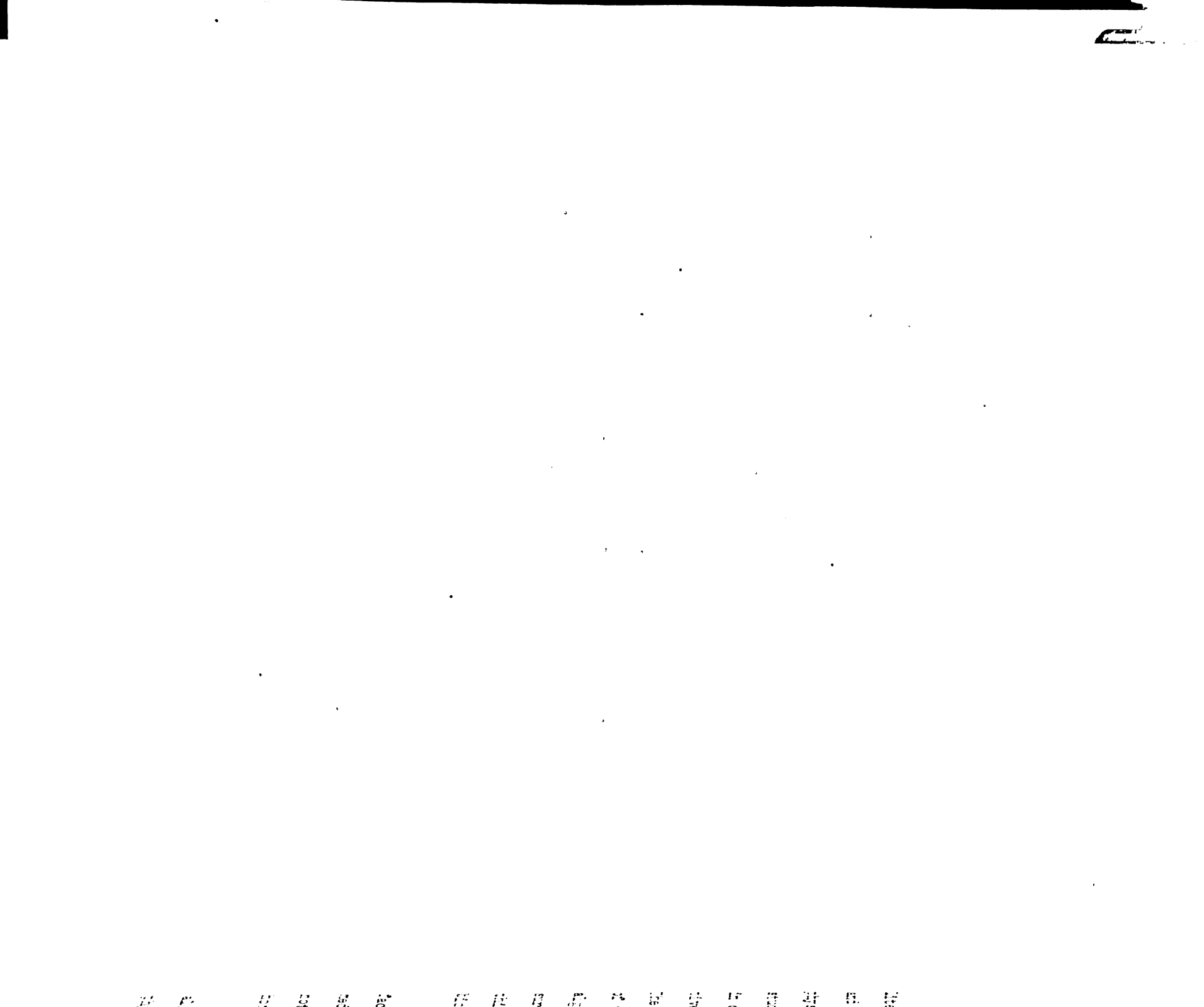
The above idea agrees well with the experimental observations of extinction coefficients at 260 mu of ribonucleotides, oligoribonucleic acid and high molecular weight "native" and synthetic RNA. Doty (30) reported increases in the extinction coefficients of 32 percent for tobacco mosaic virus RNA, 22 percent for calf liver microsomes, and 50 percent for synthetic poly (A+ U) or poly (I+ C) when heated or adjusted to an alkaline pH. When denatured yeast RNA was subjected to the action of ribonuclease, a further increase of 20 percent was reported by Mallet (95).

Various Nonnucleotide Moieties Which Have Been Shown to be Attached to RNA

Several studies have found evidence that amino acids and peptides are a normal component of RNA. Haberman (50) found a peptide component in the RNA isolated from baker's yeast, tumour cells, mouse liver, and mouse brain. This component contained many amino acids and made up 0.2 to 1.5 percent of the RNA by weight. Ishihara (60) found numerous amino acids and some peptides in yeast RNA which could be released by vigorous acid hydrolysis.

Shuzo (123) isolated a material from the bacteria Alcaligenes faecalis which had a high molecular weight. Its phosphorus was located 26.7 percent in DNA, 50.7 percent in RNA, and 15.1 percent in Δ_7-phosphate. The nucleic acids had a high guanine content and a very low amount of thymine. By mild acid hydrolysis a glycopeptide could be released and the isolated glycopeptide was shown to contain glucosamine and ten amino acids.

With these findings in mind the previously discussed polyphosphate containing RNA materials should seem less unique (7, 148). In the course of their metabolic activities RNA molecules probably become complexed and bonded to many different moieties.



INTRODUCTION

In view of the somewhat confusing data reported in the literature concerning a possible polyphosphate-RNA complex and the wide-spread speculation and theorizing as to the physiological role of such a complex, it seemed that a study of the structure and properties of this material was in order. Two biological systems were chosen as representatives of the two phyla of algae, Cyanophyta and Chlorophyta. The reasons for working with algae were two-fold. The first of these was a special interest in algal physiology. The algae are the dominant plants over most of the earth's surface and occur in a great variety of forms. Despite this fact very little is known about their physiology and biochemistry.

The second reason for choosing these organisms was the fact that both can be mass-cultured in chemically defined media under uniform conditions. Essentially the cells do not differentiate when cultured in this way and a relatively homogeneous biological material is produced.

A review of the physiology and biochemistry of blue-green algae is given by Fogg (40) and a more recent review of the physiology of fresh water algae is given by Krauss (78).

Some preliminary experiments were carried out to find

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whether polyphosphates accounted for a significant amount of the total phosphorus in Anabaena and whether the turnover of the polyphosphates was very rapid. The location of the polyphosphate in the cell was studied and a method of isolating reasonably pure RNA-polyphosphate was arrived at. In an effort to obtain complexes with reproducible characteristics, cultures were isolated at various points on the growth curve. This work indicated that the type of complex and the degree of complexing were functions of cell division.

To test this Chlorella pyrenoidosa was raised in mass, synchronized cultures and isolations of the total RNA and polyphosphate were made. These isolates were then chromatographed on DEAE-cellulose columns in a standard way and compared.

Concurrent with this work an effort was made to learn as much as possible about the properties of the isolated complexes.

Other authors have reported changes in the metabolism of various species of synchronized Chlorella. Among these authors Stange (127) found that the $C^{14}O_2$-incorporation pattern changed, Sorokin (126) studied changes in respiration rate, and Iwamura (61) reported changes in the base ratio of total RNA.

Before discussing the experimental methods and results I shall present a reference framework which was derived from the experimental results. The results of the study indicate

that a large but variable portion of the cellular phosphorus in these algae is in the form of polyphosphates. Some of this polyphosphate is complexed with RNA. During certain periods in the life cycle and under the proper conditions, all of the polyphosphate and much, if not all, of the RNA is involved in these complexes. There seems to be a close relationship between the synthesis and utilization of polyphosphate and RNA in synchronized Chlorella. The properties of the larger complexes are consistent with the theory that the RNA and polyphosphate are linked by both a hydrogen-bond or salt-bonding system and covalent bonds. The smaller complexes seem to involve only covalent bonds.

METHODS

Culture

The Semi-mass Culture of Anabaena

Agar slant cultures of Anabaena variabilis Kütz were obtained from the Algal Culture Laboratory, Botany Department, University of Indiana (128). For liquid culture, medium C as described by Kratz (77) was used. This medium was made up in 12 l. batches and autoclaved for one hour in Pyrex carboys as illustrated in Fig. 1a.

125 ml. Erlenmeyer flasks containing 50 ml. of liquid medium were stoppered with cotton and sterilized. They were then inoculated with algae from an agar slant using a flamed wire. These sub-cultures were kept at 30-35° C. and illuminated with a tungsten lamp (200 watts) with occasional swirling. In several weeks a considerable growth usually would have occurred.

The entire semi-mass culture apparatus was suspended in a large aquarium to act as a warm water bath (Fig. 2). Aquarium heaters and a thermostat were used to maintain a 30° C. temperature in the bath and an excess of $CuSO_4$ was added to the bath to inhibit the growth of microorganisms. Above the apparatus two 300 watt tungsten lamps in reflectors were fastened to a bar suspended from the ceiling by cords.

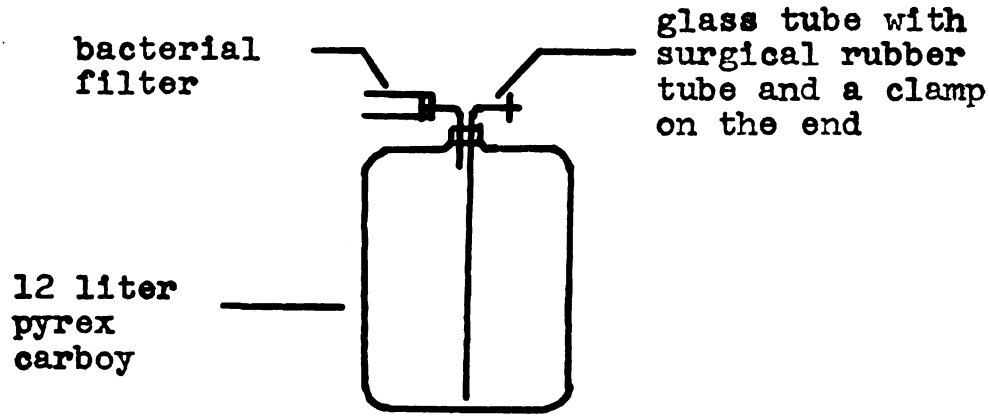

Figure 1a, medium carboy

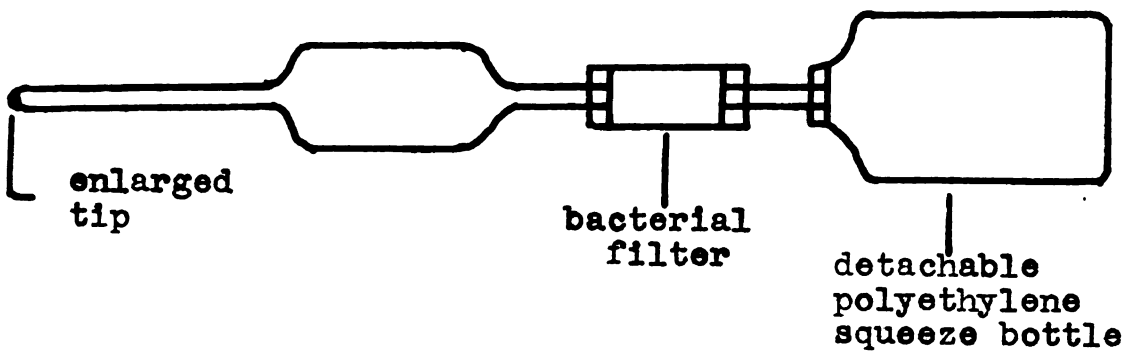

Figure 1b, Anabaena inoculator
(a modified pipet)

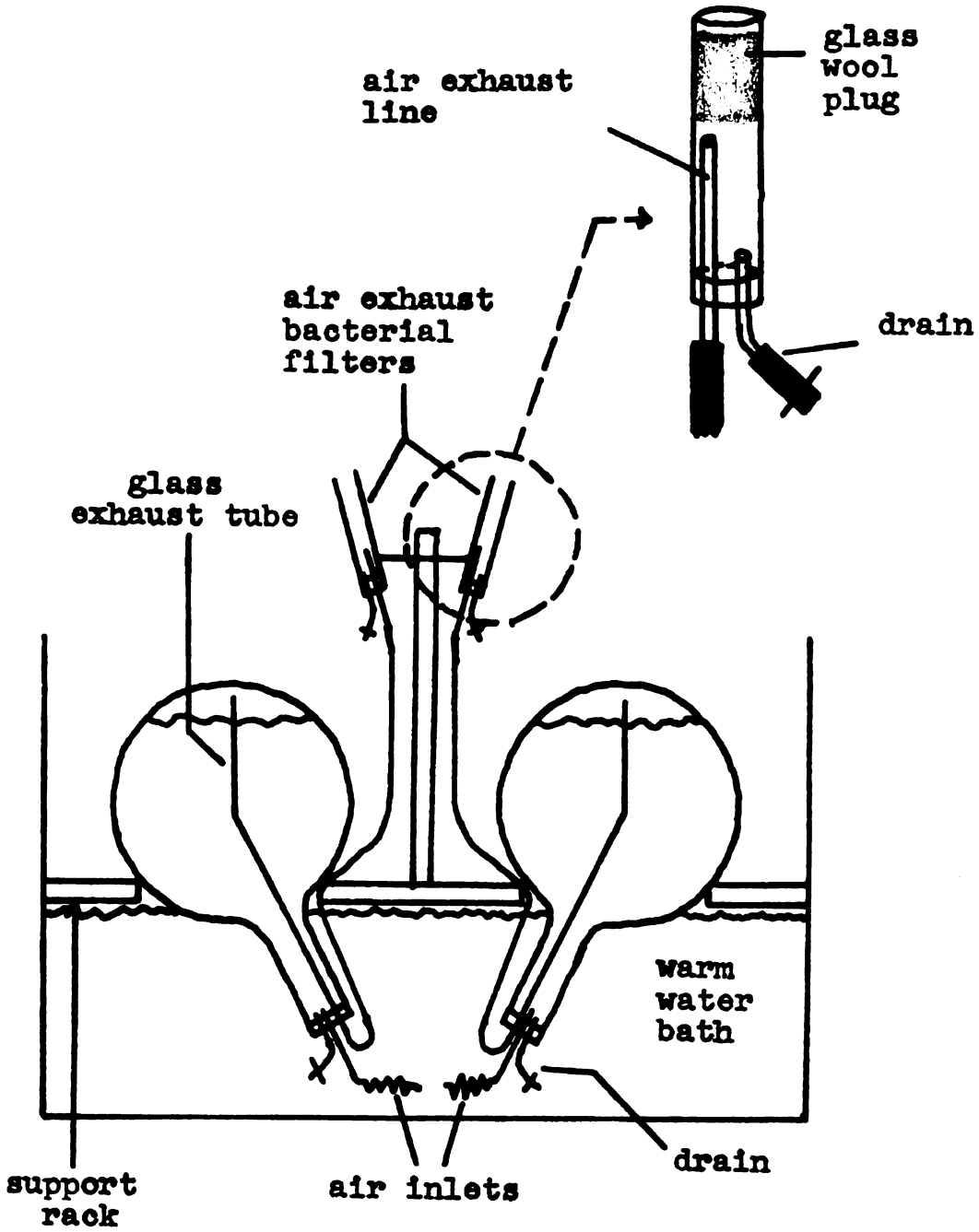

Figure 2, partial diagram of the Anabaena culture apparatus

This allowed for adjustment in height until an intensity of about 500 footcandles was obtained. A compressed air line and a carbon dioxide cylinder with reduction valves were used for aeration of the algae cultures and circulation of the water bath (as explained later).

For the culture flasks themselves two six-liter Florence flasks were used in an inverted position. This was the largest practical size since light rapidly became limiting in a dense culture. This type of flask was necessary because of the ecology of the organism which, without rapid turbulence, formed plate-like masses on any surface to which it was exposed.

The air line bubbler was a one-liter Erlenmeyer flask with a four-hole stopper, Figure 3. This flask was maintained about one-half full of distilled water and it both moistened the air stream and gave a method of adjusting the relative volumes of carbon dioxide and air. Carbon dioxide should never exceed one percent by volume. The water bath bubbler valve could be used as a fine adjustment of the amount of carbon dioxide flow.

The portion of the apparatus enclosed within the block labeled "sterile zone" was sterilized as a unit. This was done as follows:

1. The two Florence flasks were put in an upright position and filled with medium.
2. The stoppers and everything else involved in the "sterile zone" were assembled and all clamps were closed.

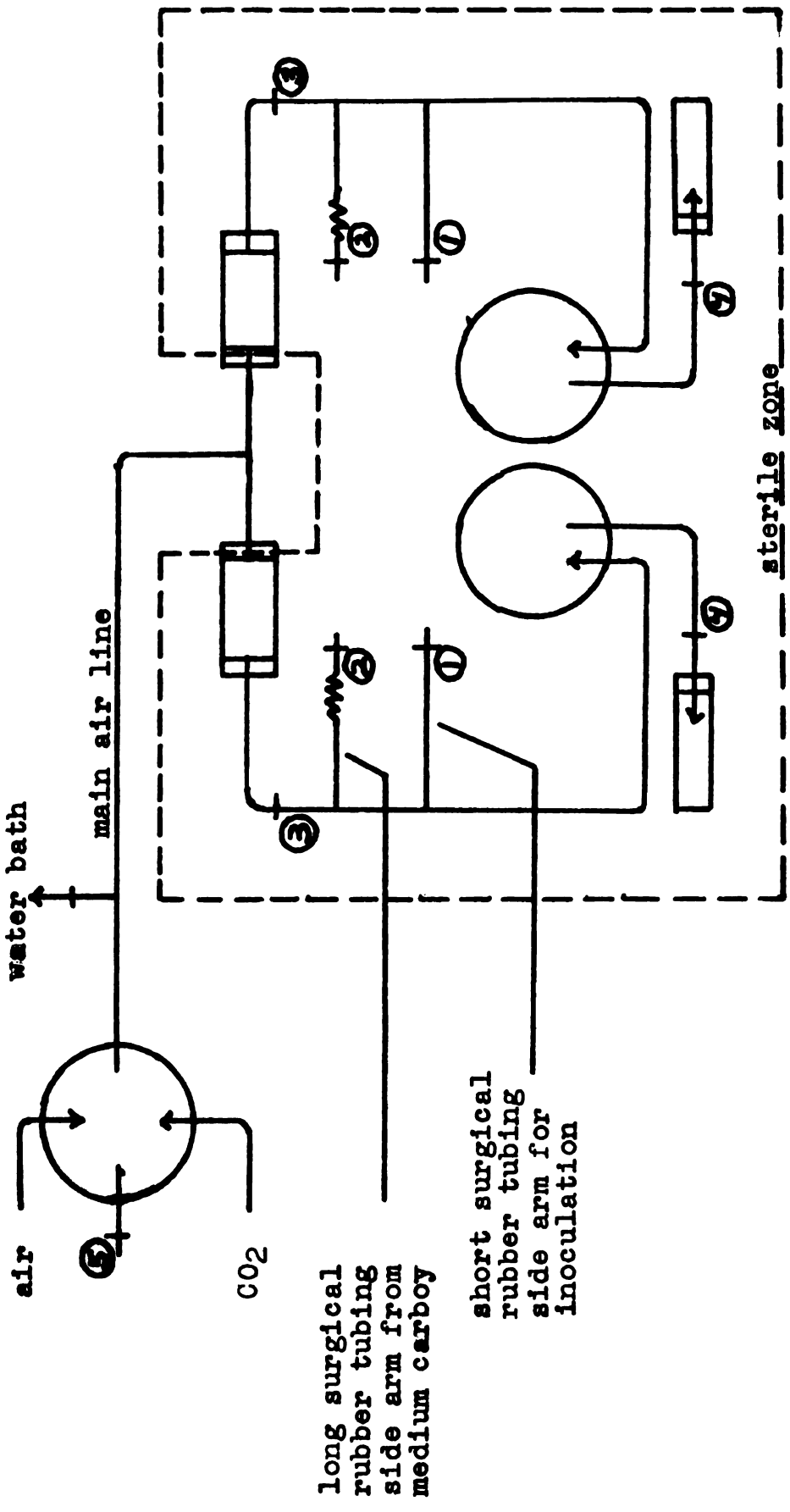

Figure 3, schematic diagram of the *Anabaena* culture system

3. Clamp 3 was opened.

4. The whole "sterile zone" was autoclaved one hour and cooled to room temperature.

5. Clamp 3 was closed and the Florence flasks were inverted and placed in operating position. The bacterial filters were connected to the line from the air-line bubbler and clamp 3 was opened. After several hours of circulation the medium should be completely in solution.

The Florence flasks were inoculated as follows:

1. Clamp 3 was closed and the clamp 1 on the inoculating side arm was opened.

2. The inoculator (Fig. 1b) was used to take up some of the suspension of algae from one of the subcultures. The tip of the inoculator was pushed into the surgical rubber tubing of the inoculation side arm, and the squeeze bulb was used to force the solution well into the main air line. Clamp 1 was then replaced in a position immediately adjacent to the main air line and the inoculator was removed.

3. Clamp 3 was opened and the inoculation was swept into the Florence flask by the gas stream.

Vigorous aeration was maintained until a dense culture was achieved. During this period the following points should be checked routinely:

1. level of distilled water in the air line bubbler

2. level of water in the constant temperature bath

3. temperature of constant temperature bath

4. drainage of exhaust line filters, which accumulate condensation rapidly

5. proper speed of aeration

When a culture was to be harvested, the following procedure was followed:

1. Clamp 5 was opened partially, clamp 3 was closed, and the connection at the nonsterile end of the bacterial filter for the air inlet was broken.

2. The Florence flask, in the inverted position, was then raised out of the water bath with a simple, prearranged hoist and the drain clamp was opened. Vigorous swirling at this time will clean off any clinging algae.

3. About five and a half l. of culture was drained out and the drain clamp was closed. The Florence flask was then replaced in operating position and the air line filter reconnected.

4. The refilling side arm was connected to a 12 l. carboy of sterile media as follows:

    a. The surgical rubber tube and clamp (Fig. 1a) were slipped off.

    b. The refilling side arm was cut off just on the inside of clamp 2 and the end was immediately slipped over the glass tube on the media carboy.

    c. Medium was siphoned into the Florence flask until it was filled to within about one inch of the top, then clamp 2 was replaced on the refilling side arm and closed.

    d. Clamp 3 was opened.

The "starter" of culture left in the Florence flask from the previous culture rapidly multiplied and the whole process could be repeated many times without reinoculating the culture.

By following the procedures above it has been possible to obtain 50-100 ml. of packed cells per week. The apparatus has been maintained in continuous operation for as long as six months at a time without loss of culture.

When a culture was harvested, the volume of medium removed was recorded and the algae were centrifuged in an

International refrigerated centrifuge, model PR-2, using 250 ml. buckets in the number 259 head. The cells were then resuspended in distilled water to wash off the residual medium, and centrifuged in graduated glass tubes for six minutes at 3,000 r.p.m. in the number 824 head (1,770 g). The packed cell volume (p.c.v.) was then recorded and the cells were either utilized immediately or frozen until needed.

The Mass, Synchronized Culture of Chlorella

An agar slant culture of Chlorella pyrenoidosa Van Niel 211 was obtained from Dr. R. R. Schmidt, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute. The liquid medium formula, given below, was also obtained in a personal communication from Dr. Schmidt. The medium was made up and sterilized in 12 l. pyrex carboys (Figure 1a).

The medium was made up by dissolving 30 gm. KNO_3, 30 gm. KH_2PO_4, and three gm. $MgSO_4 \cdot 7\ H_2O$ in eight l. of distilled water. Then 120 ml. of each of the four stock solutions listed below was added, the solution was diluted to 12 l. with distilled water and the pH was adjusted to 6.75 with two normal KOH solution.

1. micronutrient stock solution

   11.4 gm. H_3BO_3

   0.89 gm. $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$

   1.58 gm. $CuSO_4 \cdot 5\ H_2O$

   8.82 gm. $ZnSO_4 \cdot 7\ H_2O$

0.48 gm. $CoSO_4 \cdot 7\ H_2O$

1.44 gm. $MnCl_2 \cdot 4\ H_2O$

dissolve in one 1. distilled water

2. acidified ferrous sulfate stock solution

Dissolve 5.0 gm. $FeSO_4 \cdot 7\ H_2O$ in one 1. distilled water and add 20 drops of concentrated sulfuric acid.

3. calcium chloride stock solution

Dissolve 11.1 gm. $CaCl_2 \cdot 2\ H_2O$ in one 1. of distilled water.

4. chelate stock solution

50 gm. EDTA (free acid) and 31 gm. KOH are brought to one 1. with distilled water.

During subculturing it was found to be necessary for rapid growth to add one percent glucose to the usual liquid medium. This addition was suggested by the studies of Reisner (110). 50 ml. of medium was added to each 125 ml. Erlenmeyer flask and an aerator containing a bacterial filter was inserted in a cotton plug. Then the subculture flasks were sterilized and inoculated with algae using a flamed wire. The subcultures were immersed in a water bath at 20-25° C. and aerated with compressed air. A 30 watt fluorescent light was used for illumination.

Subcultures were used to inoculate a 650 ml. semi-mass culture apparatus which was illuminated by an immersed neon tube. This second-stage apparatus was aerated with one to five percent carbon dioxide in compressed air. It was maintained at 20-25° C. and was connected to a carboy of

sterilized medium. Before inoculation the apparatus was flushed with 70 percent alcohol and then several times with sterile medium.

The apparatus used for mass synchronization was specially designed and constructed from three-eighths inch plexiglass in such a way that saturation light intensities could be maintained with dense cultures. The apparatus is diagrammed in Figure 4. It consisted of three parallel compartments four cm. thick by 76 cm. square. The outer two contained baffles and were used as constant temperature baths. An American Instrument Company constant temperature circulator, number 701-481, was used to circulate distilled water at 25° C. through these two compartments. The middle compartment was the actual algae chamber. It had an operating capacity of 20 l.

Circulation was maintained by a flow of compressed air and five percent carbon dioxide from a series of small holes in a polyethylene tube, which was held across the bottom of the algae chamber.

Two banks of 48 inch, 40 watt, daylight-type fluorescent lights provided the lighting. On one side a bank of seven tubes was adjusted so that the average light intensity on the algae chamber surface was about 800 foot-candles. On the other side a bank of six tubes was adjusted so that the average light intensity on the algae chamber surface was about 700 foot-candles. Thus by turning both banks on at the same time, an intensity of 1500 foot-candles could be

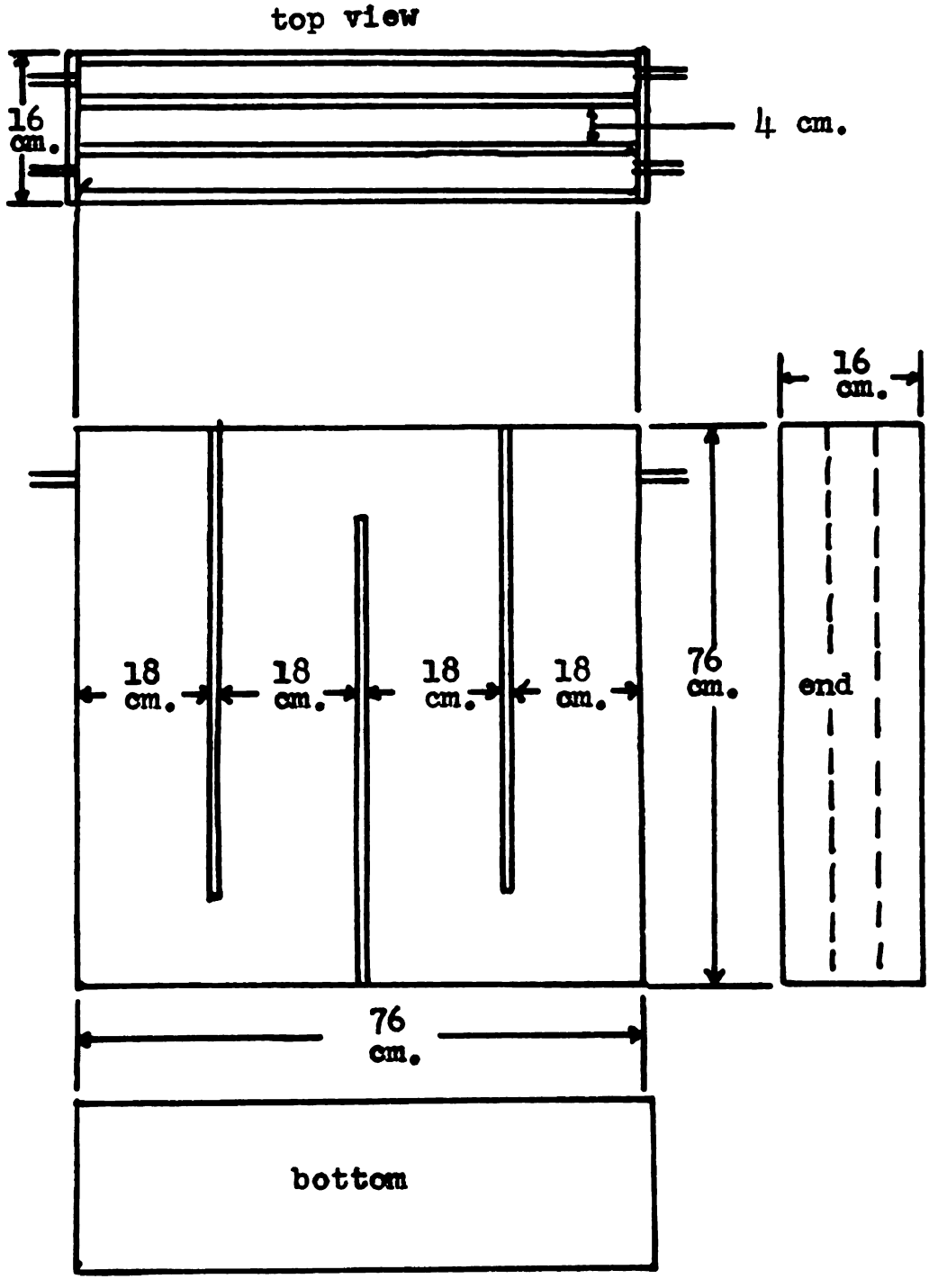

Figure 4, diagram of the plexiglass chamber of the mass, synchronized culture apparatus

obtained. Light intensity was measured with a Weston illuminator meter, model 756.

A siphon was used to remove the algae and, simultaneously, while this was going on, the algae were run through a Sharples continuous centrifuge, model M-41-24-8CY-34, using a large jet. Only twenty minutes were required to process 18 l. of algae suspension. The algae were then transferred to 100 ml. tubes and centrifuged in an International refrigerated centrifuge, model PR-2, washed once with distilled water, and finally centrifuged again in the high-speed head, number 824, at 3,600 r.p.m. for 10 minutes (2,120 g). The algae were then frozen until needed. Volume of suspension harvested and packed cell volume (p.c.v.) of Chlorella were recorded for each harvest.

The basic method in initiating synchrony in Chlorella has been reported by Schmidt (121) and further details have been received in a personal communication. In his work Schmidt reports a degree of synchrony of approximately 90-95 percent. No attempt was made in the present study to determine percent synchrony, but visual microscopic checks were made at key times to determine whether the cultures were growing normally and whether daughter cell release occurred at the right time.

In order to initiate synchrony, a culture would be grown in the second-stage apparatus described above and, after a rapidly dividing culture was achieved, it was allowed to grow until a cell density of about one ml. p.c.v. per

100 ml. of medium was reached. By frequent microscopic checks a period could be found, during which about 80 percent of the culture was centrifuged at 10°C. and resuspended in fresh media. This cell suspension was then used to inoculate the algae chamber of the synchronization apparatus. This chamber had been previously flushed out with 70 percent alcohol followed by sterile medium.

The dilute cell suspension in the synchronization chamber was then subjected to 800 foot-candles of light for 18 hours. The Chlorella were then darkened for 12 hours by turning the light banks off and covering the apparatus with several layers of black cloth. During this dark period the algae tend to accumulate at the young, daughter cell stage. Then the Chlorella were subjected to periods of 1500 foot-candles of light for 18 hours and 12 hours of darkness alternately as long as necessary to complete the desired experiment. After four light cycles, the algae were considered adequately synchronized and harvests were begun. Usually only 15 or 16 l. were harvested so that only one cycle would be required before another harvest could be made.

In his studies Schmidt (121) found most of the phosphate uptake from the medium to occur between six and nine hours of light, and nuclear division to begin at about 14 hours. The synchronized Chlorella life cycle is shown diagrammatically in Figure 5. Under the conditions described most mother cells were observed to release four daughter cells.

Inhibition of Metabolism with Chloramphenicol

In one case 40 parts per million (p.p.m.) of chloramphenicol was added to the synchronization chamber after nine hours of light and the Chlorella were harvested after three additional hours of continued light period. The chloramphenicol for this experiment was obtained through the courtesy of Parke, Davis and Company, Ann Arbor, Michigan. It was added as a solution of 100 mgm. of chloramphenicol in one l. of fresh nutrient over a period of 15 minutes. This experiment and the concentrations used were suggested by the report of Takeda (132) that 40 p.p.m. of chloramphenicol completely stopped net protein synthesis in Pseudomonas fluorescens, and the report of Brock (15) that 50 p.p.m. caused an immediate halt in protein synthesis and growth in Escherischia coli.

Isolation and Characterization of a Bacterium From The Anabaena Culture

Dense liquid cultures of the bacteria were obtained from liquid cultures of Anabaena by inoculating into flasks of the regular Anabaena medium which had been enriched with four gm. glucose, one gm. peptone, and one gm. of yeast extract per liter. These flasks were then incubated at 30° C. in the dark with occasional swirling for 12 hours.

Some of the liquid from these flasks was used to streak the surface of agar plates made by adding 15 gm. agar, eight gm. glucose, four gm. peptone, and four gm. yeast extract per

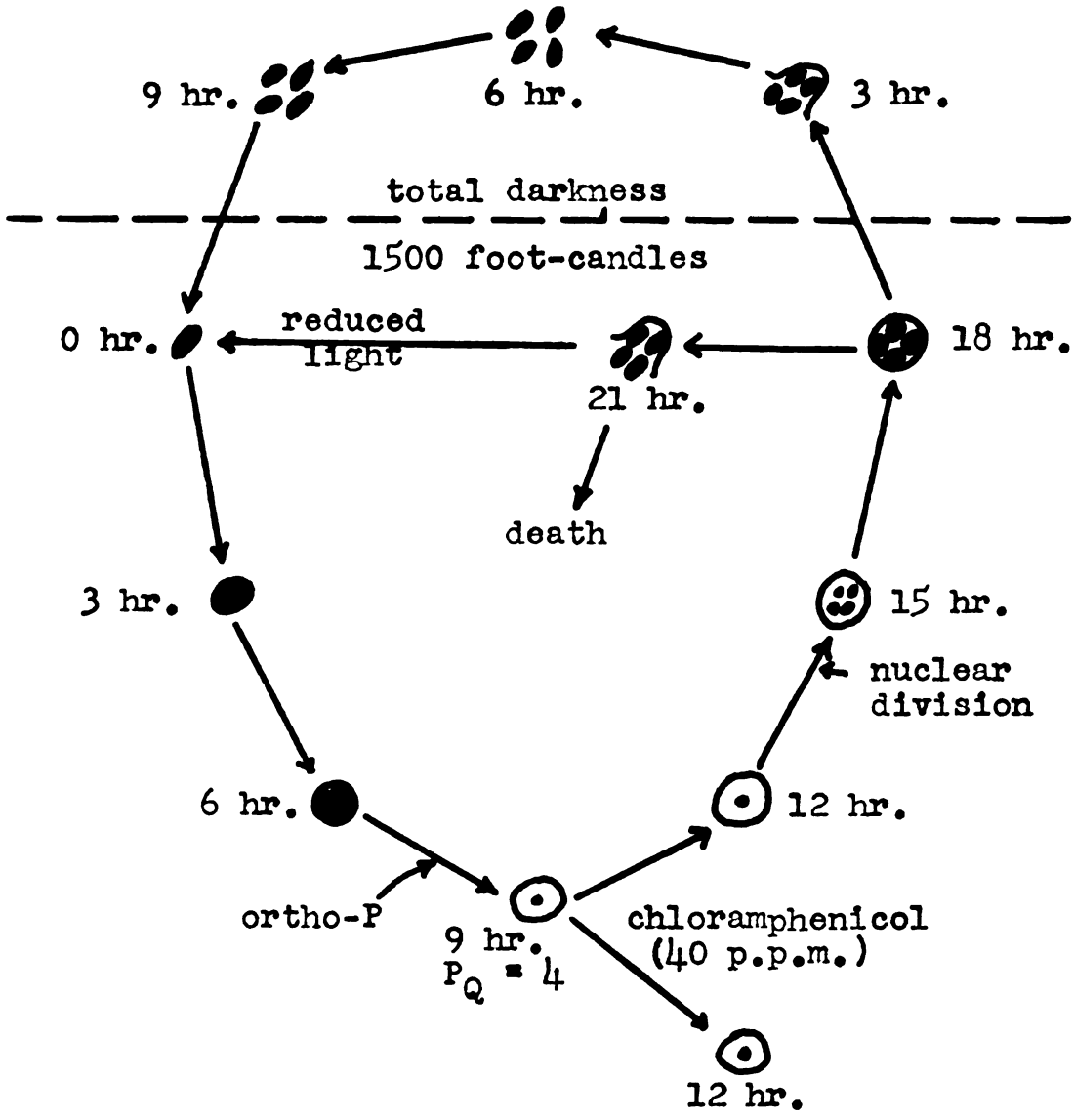

Figure 5, synchronization cycle of Chlorella pyrenoidosa (Van Niel 211) at 25° C.

liter to regular Anabaena medium. One type of colony developed. A Bacto Unidisk for antibiotics, high concentration, was then placed on the plate to test for sensitivity to various antibiotics.

Isolations

Fractionation of the Phosphorus Components of Anabaena

Figure 6 outlines the fractionation procedure. The procedure, up to the crude preparation, was a slight modification of the procedures reported by Kulaev (80) and Juni (63). The phenol extraction has been described by Gierer (43).

Standard Isolation of the RNA-Polyphosphate of Chlorella

A frozen sample was thawed in 300 ml. of distilled water which was adjusted at intervals to pH 11.5 with KOH. After one hour at room temperature the pH was adjusted to 7.4 with HCl and 300 ml. of cold, water-saturated phenol was added. The mixture was shaken for five minutes and then centrifuged for eight minutes at five degrees.

The upper (aqueous) phase was saved, 300 ml. of water saturated phenol was added and the process repeated. The upper phase was then extracted four times with ether and evaporated under reduced pressure for a short time to remove the residual ether.

One percent sodium acetate was dissolved in the solution, and one drop of 2 N HCl and four volumes of alcohol were

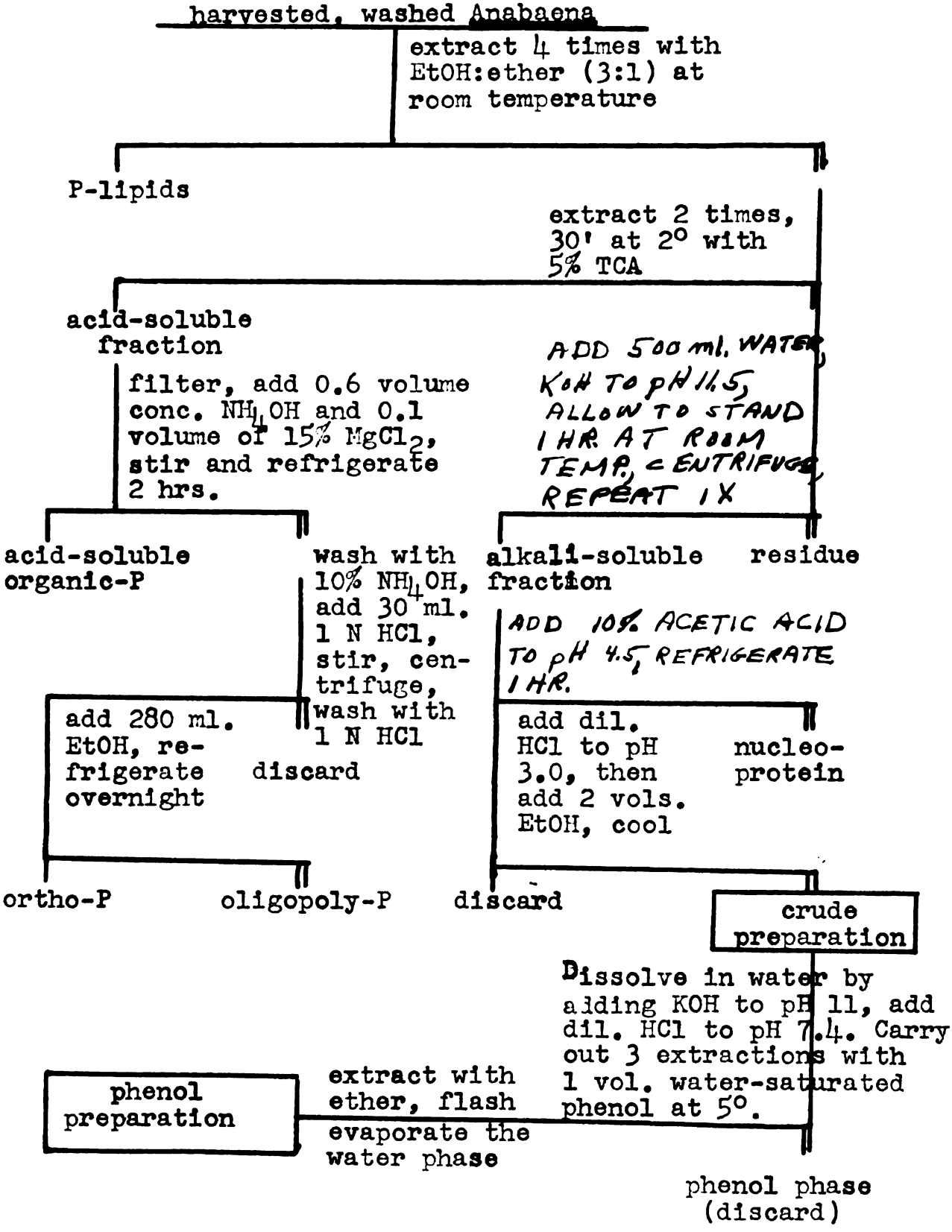

Figure 6, fractionation of phosphorus components of Anabaena

added. The solution was stirred and more sodium acetate was added slowly until a flocculent precipitate formed. The solution was then put in a freezer for one hour with occasional stirring. The precipitate was centrifuged at 0°, washed with alcohol, and then dried at reduced pressure. The material thus obtained was dissolved in 30 ml. of 0.01 M tris buffer, pH 7.6 and was ready to be applied to a column of DEAE-cellulose.

Preparation and Elution of Diethylaminoethyl Cellulose Columns

The resin was placed in a large column and was washed with saturated NaCl solution, then with 0.1 N NaOH, and finally with a large volume of 0.01 M tris buffer, pH 7.6 until the pH of the eluate is 7.6. The columns used for the fractionation of RNA-polyphosphate were packed with only very gentle pressure. After the resin was packed it was washed with a small volume of buffer and the sample was applied. The column was then washed again with 20 ml. of buffer. In the case of the material from Anabaena, a column with a resin bed about 20 cm. tall and two cm. in diameter was used. In the case of the material from Chlorella, a resin bed about 20 cm. tall and one cm. in diameter was used.

The columns were connected to a 500 ml. fixing flask and this was filled with 0.01 M tris, pH 7.6. The mixing flask was then connected to a reservoir to which a salt

solution was added. In the case of the work with Anabaena, 2 M NaCl was added to the reservoir, and 10 ml. fractions were collected from the columns with a Rinco fraction collector.

In the case of the work with Chlorella, a series of NaCl solutions were added to the reservoir: first 200 ml. of 1 M NaCl, then 200 ml. of 2 M NaCl, 200 ml. of 3 M NaCl, and finally 200 ml. of M NaCl. Five ml. fractions were collected from the columns with a Rinco fraction collector.

Miscellaneous Chemical Determinations

Cytochemical Location of Polyphosphate

The location of polyphosphate in the Anabaena cells was carried out with toluidine blue by the technique reported by Keck (66).

P^{32}-Phosphate Incorporation

In order to determine if the polyphosphate pool had a rapid turnover, 0.9 ml. of packed Anabaena cells were suspended in the usual liquid medium which had been diluted ten fold. The suspension was equilibrated at 30-35° C. with a 1000 watt flood-lamp. Occasional aeration was accomplished by bubbling a stream of air through the suspension. About 25 microcuries of P^{32}-phosphate were added and after three hours the suspension was centrifuged. The supernatant and precipitate were boiled separately for 10 minutes. An aliquot of each was then chromatographed in both the two-

dimensional system of water-saturated phenol and butanol-propionic acid- water described by Benson (8) and the one-dimensional system for inorganic phosphates, described later. In both cases radioactive spots were located by authoradiography using no-screen X-ray film.

Phosphorus

Total phosphorus was determined by the technique of King (68). Acid-labile phosphate-phosphorus was determined by hydrolyzing samples for seven minutes with one normal HCl in a boiling water bath and then determining orthophosphate by the method of Fiske and Subbarow (38). In all cases results are reported in terms of weights of phosphorus.

Ribose and Deoxyribose

Ribose was determined by the orcinol reaction (25) using a 40 minute hydrolysis with 6 N HCl in a boiling water bath. A solution of adenylic acid was used as a standard. Deoxyribose was tested for by the diphenylamine reaction (21).

Total Nucleic Acids

Total nucleic acid was determined by the method of Webb (141).

Protein and Amino Acids

Protein was determined by both the Lowry method (93), using bovine serum albumin as a standard, and by the ninhydrin reaction. The ninhydrin reaction was carried out by heating one ml. of sample with 0.2 ml. of a 0.1 percent solution of triketohydrindene hydrate in a boiling water bath.

Total Carbohydrates

Total carbohydrates were determined by the indole reaction for total carbohydrates (25) using adenylic acid as a standard.

Metachromasy

Metachromasy was determined by a modification of the method of Damle (28). Two ml. of a solution of 30 mgm. toluidine blue in one l. of distilled water was added to the sample and the volume was diluted to 10 ml. with distilled water. The maximum decrease in optical density at 630 mu was measured with a Beckman, model B, spectrophotometer and compared with a standard curve. This curve was constructed by carrying out the reaction with a series of concentrations of a synthetic polyphosphate obtained from Monsanto Chemical Company, St. Louis, Missouri (marketed as sodium hexametaphosphate). This polymer has been determined to have an average chain length of 16 phosphates.

Care was always taken to make sure that the sample concentration was low enough to be on the ascending side of the standard curve.

Ultraviolet Spectra

Complete ultraviolet spectra between 220 mu and 340 mu were recorded using a Beckman, model DK 2, recording spectrophotometer and one cm. quartz cells. In cases where optical densities at individual wave lengths were determined, a

Beckman, model DU, spectrophotometer and one cm. quartz cells were used.

Infrared Spectra

Samples of RNA-polyphosphate complex were prepared in two ways. In the first an effort was made to avoid denaturation of the complex. Ten ml. of complex was dialyzed against 0.5 M KBr and the KBr solution was changed several times. In the second, a ten ml. sample was dialyzed exhaustively against distilled water to denature it and then it was dialyzed against 0.5 M KBr. Both samples were then lyophilyzed and stored in a desiccator. Aliquots of 400 mgm. of the powder were weighed out and used for pressing pellets in a hydrolic press. Infrared spectra of the pellets were obtained with a Beckman, model IR 5, recording spectrophotometer.

Qualitative and Quantitative Nitrogen Base Determinations

The first method involved the alkaline hydrolysis of RNA by incubation for 18 hours with 0.5 N KOH at 40° C. This resulted in the free nucleotides. Subsequent neutralization with cold 36 percent $HClO_4$ resulted in the precipitation of $KClO_4$ which was filtered off. The filtrate was subjected to column chromatography by the method of Hurlbert (57). The separated nucleotide solutions were then lyophilized to remove the formic acid. Ultraviolet spectra were determined at both acid and basic pH to verify their identities.

In a second method samples were hydrolyzed in sealed tubes with 1 N HCl for one hour in a boiling water bath as described by Smith (124). This results in the free purine bases and the pyrimidine nucleotides. The hydrolyzed material was then chromatographed on Whatman number one paper by the descending technique using the isobutyric acid-NH_4OH-water (66/1/33) solvent described in circular OR-10, Pabst Laboratories, Milwaukee, Wisconsin. Known standards were also chromatographed to determine Rf values.

In the column chromatography when base ratios were calculated, the areas under the 260 mu optical density (O. D.) elution curves and the extinction coefficients published by Pabst Laboratories in circular OR-10 were used.

In the case of paper chromatography, the spots were eluted by immersing the cut out spots in five ml. of 0.1 N HCl for one hour and measuring the O. D. at 257 mu for adenine, 256 mu for guanine, 280 mu for cytidylic acid, and 262 mu for uridylic acid. Base ratios were calculated using the extinction coefficients in Pabst Laboratories circular OR-10.

Paper Chromatography of Polyphosphates

A modification of the system reported by Thilo (137) was used. The solvent was prepared by adding 20 ml. of 20 percent trichloroacetic acid and 10 ml. of water to 70 ml. of isopropyl alcohol, and then titrating to pH 4.0 with concentrated NH_4OH. Whatman No. 1 paper was used and the

chromatograms were developed by the descending technique for 18 hours at room temperature.

Spots were located by spraying the paper with Hanes-Isherwood reagent which was composed of five ml. of 60 percent $HClO_4$, 10 ml. 1 N HCl, 25 ml. four percent ammonium molybdate, and 60 ml. of water. The sprayed paper was placed in an air oven at about 70° C. for several minutes and was then exposed to ultraviolet light for several minutes to develop the spots.

Preparation and Use of Charcoal

Norite was treated by exhaustive washing with 0.1 N HCl and then, as suggested by Liss (89), with a large volume of four percent potassium-EDTA at pH 7.0. Then the charcoal was washed with 0.01 M tris, pH 7.6 to which one percent NaCl had been added.

In each adsorption study about three gm. of charcoal was added to between 5 and 10 ml. of sample and the mixture was stirred occasionally for 20 minutes. Then the mixture was filtered with a Büchner funnel and a vacuum flask, recycling the filtrate several times.

Enzymes

Crystalline ribonuclease was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Snake venom phosphodiesterase was obtained through the

courtesy of Dr. Charles Mead, who isolated the enzyme free of 5'-nucleotidase activity from Crotalus terrificus venom. Lyophilyzed Crotalus terrificus venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

A polyphosphatase complex was isolated from dried brewer's yeast, obtained from Budweiser Breweries, Inc., St. Louis, Missouri. The isolation used was reported by Mattenheimer (99) and the 20-70 percent ammonium sulfate fraction was used for the studies to be reported.

Specific reaction conditions for each enzyme are reported with the individual sets of experimental results.

RESULTS AND DISCUSSION

Anabaena

Distribution of Phosphorus

When cultures of various ages were extracted first with alcohol:ether (3:1) and then with KOH at a pH of 10, the data in Table I was obtained. It can be seen that although the total phosphorus remained about the same, the percent which was in the form of orthophosphate declined sharply with older cultures.

The results of two fractionations of the phosphorus compounds of Anabaena are shown in Table II. In both cases polyphosphates constitute about 40-50 percent of the total phosphorus. The bulk of the remaining phosphorus was orthophosphate.

Incorporation of P^{32}-Phosphate

P^{32}-labeled phosphate was utilized to determine whether the polyphosphate had a rapid turnover. After three hours incubation no labeled phosphorus compounds other than orthophosphate could be found in the medium. Upon paper chromatoraphy of the hot-water extract of the cells, a small amount of radioactivity was found in sugar-phosphates, AMP, ADP, and orthophosphate. The origin, which is occupied by polyphosphate in both systems, was very radioactive.

Table I. Changes in the orthophosphate content of the alkali-soluble fraction of Anabaena

culture density (ml. p.c.v*/l.)	total-P (ug.P/ml. p.c.v.)	ortho-P (ug.P/ml. p.c.v.)	% ortho-P
0.5	598	598	100
1.8	565	485	86
7.8	540	240	44

* p.c.v. = packed cell volume

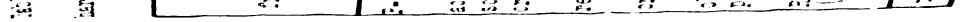

Table II. Distribution of phosphorus in Anabaena harvested at two culture densities

fraction	culture # 1 (density = 5.6 ml. p.c.v./l.)		culture # 2 (density = 12 ml. p.c.v./l.)	
	ug.P/ml. p.c.v.	%	ug.P/ml. p.c.v.	%
P-lipids	2	0.3	8	1.4
acid-soluble organic-P	68	10.2	5	0.9
residue	29	4.3	77	13.1
ortho-P	224	33.5	175	29.9
oligo-poly-P	130	19.5	153	26.1
RNA-poly-P (crude prep)	215 (134)*	32.5 (20.2)*	168	28.7
total	668		586	

* figures in parenthesis are Δ_7-P values

These data, although not conclusive, indicated that Anabaena exhibits the same type phosphate metabolism as Aspergillus (80) and brewer's yeast (84). Since no radioactive ATP was detected it seems plausible to assume that newly synthesized ATP was being utilized rapidly in polyphosphate synthesis.

Location of Polyphosphate in the Cell

The metachromasy of Anabaena cells was found to be located completely within the pseudovacuoles, whether the cells were fixed before staining or not. This ability to give the metachromatic reaction could be destroyed by exhaustive extraction with ice-cold 10 percent trichloroacetic acid. The reactivity was not removed by short treatment in 0.1 N HCl, indicating that the reaction was not due to nucleic acids or to sulfonated polysaccharides. However, as a result of further studies reported below it is likely that this technique locates only free polyphosphates or denatured RNA-polyphosphate since native complexes give no metachromatic reaction. Thus their location in the cell is still unknown.

Characteristics of the Bacterium Isolated from the Culture

This bacterium was found to be nonmotile, rod-shaped, and about one micron long. It was gram-negative and was strongly inhibited by such tetracycline antibiotics as aureomycin, terramycin, and tetramycin. It was not inhibited

noticeably by penicillin, chloromycetin, polymyxin, erythromycin, or distreptomycin.

Characteristics of RNA-Polyphosphate Phenol Preparations

Data obtained from a series of phenol preparations is recorded in Table III. In addition, protein was determined on some phenol preparations. Unless great care was taken to remove every trace of phenol the protein values were erratic, since phenol itself gives a positive reaction with the Lowry reagent. In a few cases protein was determined to be either absent or only present in such small amounts as to be insignificant. A rough correspondence can be seen within experiments between difference phosphorus and nucleic acid phosphorus, as determined by multiplying total nucleic acid values by a gravimetric factor. A similar correspondence occurs between ribose as determined with the orcinol reaction and ribose as calculated by multiplying difference phosphorus by the appropriate gravimetric factor. However, a considerable variability between experiments was obtained in all of the parameters. This fact indicated that a variation in the composition of the harvested Anabaena existed.

In two cases crude preparations of RNA-polyphosphate were tested for deoxyribose and none was found. One residue fraction was tested and found to contain 17 ug. deoxyribose per ml. p.c.v. It should be remembered that this test is only given by the purine deoxynucleotides.

When acid hydrolysates of crude preparations of RNA-

Table III. Parameters of typical phenol preparations of RNA-polyphosphate from Anabaena

parameter	experiment							
	1	2	3	4	5	6	7	8
total-P (ug.P/ml. p.c.v.)	95	64	83	41	216	116	451	77
Δ_7-P (ug.P/ml. p.c.v.)	81	40	71	33	134	61	366	52
difference-P (ug.P/ml. p.c.v.)	14	24	12	8	82	55	85	25
(Δ_7-P)/(diff.-P)	5.8	1.6	6.0	3.9	1.7	1.1	4.3	2.1
(diff.-P)(150/31)	67	-	60	40	-	-	-	-
ribose (ug./ml. p.c.v.)	105	-	69	42	-	-	-	-
total nuc. acid (ug./ml. p.c.v.)	-	-	210	56	1060	272	1,380	228
(nuc. acid)($\frac{1}{10.3}$)	-	-	20	8	103	26	134	22
$\frac{\text{O.D. @ 260 mu}}{\text{O.D. @ 280 mu}}$	1.43	1.24	1.92	1.37	1.51	1.68	1.41	1.46
specific metachromasy	1.8	2.7	1.5	1.1	-	-	-	-

polyphosphate were separated by paper chromatography, adenine, guanine, cytidylic acid, and uridylic acid were identified. No thymidylic acid was detected.

When an aliquot of the phenol preparation used in experiment 3, Table III, was subjected to alkaline hydrolysis and the nucleotides were resolved by ion-exchange chromatography, the data in Tables IV and V were obtained. When considering these results it is important to remember that under the hydrolysis conditions used, RNA yields 2' and 3' nucleotides and very little hydrolysis of polyphosphate occurs. The latter is illustrated by the synthetic polyphosphate control, which still had a high specific metachromasy after hydrolysis. No nucleoside di- or triphosphates were detected but any nucleoside phosphates with higher degrees of phosphorylation would probably not have been eluted from the resin. Reference to the significance of these results will be made in later sections.

Changes Occurring as Cultures Reach Higher Densities

The variation in the properties and amounts of RNA-polyphosphate in phenol preparations could be caused by a number of factors. Of these the one that seemed the most likely was the change in light intensity due to shading in older cultures. This effect could have been caused either by influencing the types and speed of metabolism in the cells, or indirectly by causing an accumulation of cells at some critical energy-requiring stage of the life cycle.

The first of these possibilities is suggested by

Table IV. Nucleotide composition of the alkaline hydrolysate of an Anabaena RNA-polyphosphate phenol preparation

nucleotide	mole percent
CMP	22.8
AMP	20.9
GMP	31.6
UMP	24.7

Table V. Effects of mild alkaline hydrolysis on an RNA-polyphosphate phenol preparation and on synthetic polyphosphate

	specific metachromasy	% (Δ_7-P) - (ortho-P)
RNA-polyphosphate before hydrolysis	1.5	85.5
RNA-polyphosphate after hydrolysis	0.1	84.5
synthetic poly-P before hydrolysis	1.2	-
synthetic poly-P after hydrolysis	0.9	-

changes in the respiration rate. Brown (16) found the respiration rate to change abruptly in Anabaena upon exposure to light after a dark period and Sorokin (126) showed a light effect on the rate of respiration in Chlorella, independent of the changes induced by the life cycle.

The second possibility is the basis of present methods for obtaining synchronized algae cultures. One of the early reports was that of Tamiya (133). He induced synchrony in Chlorella by allowing a culture to become very dense. About 80 percent of these cells accumulated at the "nascent" young daughter-cell stage.

Figure 7 illustrates the increase in packed cell volume and percent of cells in a visible state of cell division with time in a typical culture. The increase in packed cell volume after nine or ten days is largely a reflection of the production of an excessive amount of sheath material encasing the filaments. The accumulation of up to 80 percent of the cells in a state of cell division indicates that this could be a factor in the observed variation of isolated RNA-polyphosphate. It could also happen that such a dense culture, when diluted after a harvest, might retain some degree of synchrony for several days and thus give erratic results in the case of algae harvested within that period.

Changes in RNA-Polyphosphate with Increasing Culture Density

Phenol preparations were isolated from a series of cultures of different densities. These phenol preparations

Figure 7. Changes occurring in a typical *Anabaena* population during growth

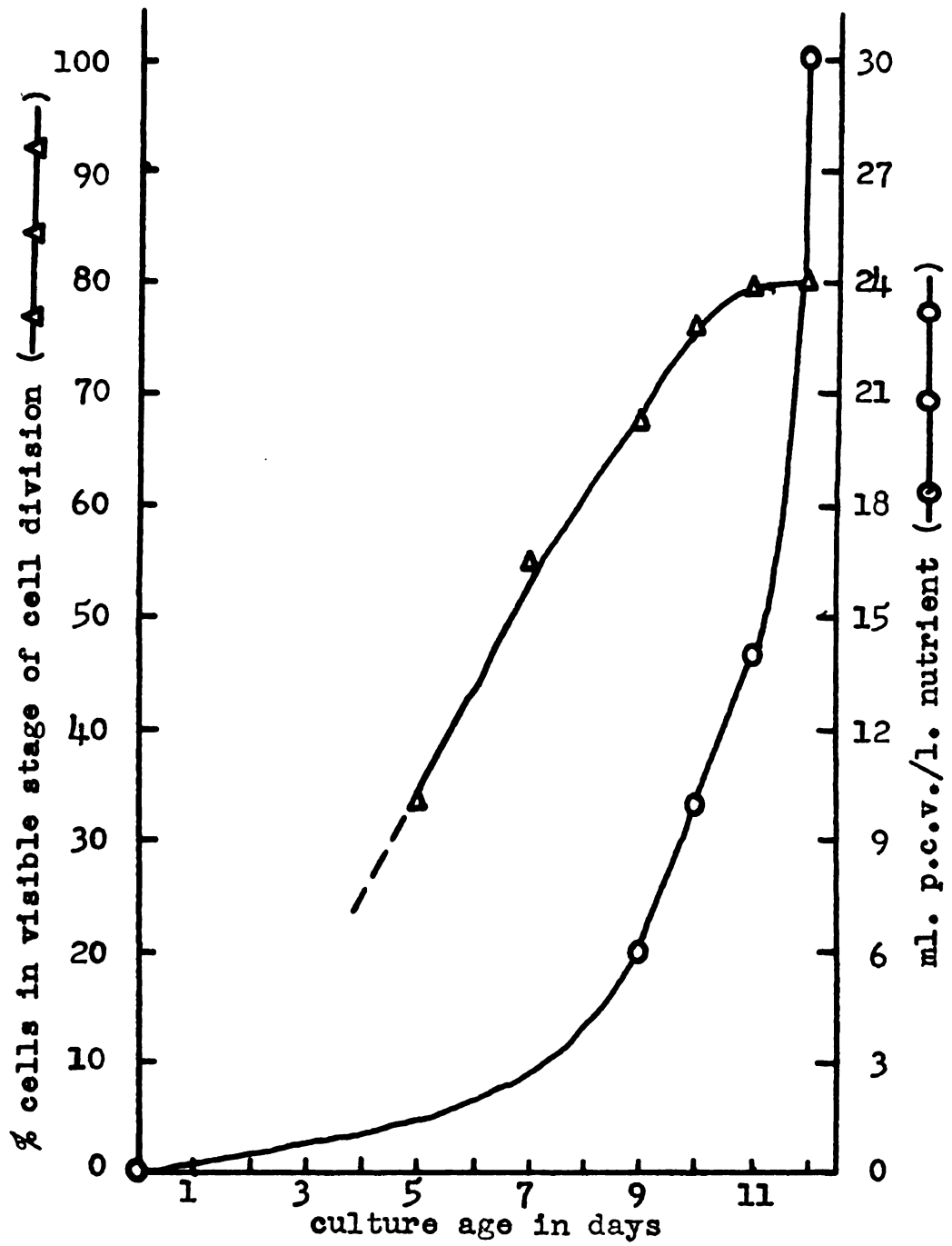
% cells in visible stage of cell division (—△—△—△—)
ml. p.c.v./l. nutrient (—○—○—○—)
culture age in days
100
90
80
70
60
50
40
30
20
10
0
30
27
24
21
18
15
12
9
6
3
0
1
3
5
7
9
11

Figure 7

were then fractionated on DEAE-cellulose columns. A number of eluate areas containing RNA and polyphosphate were separated by this technique. The elution pattern as measured by the optical densities at 260 mu. and by total phosphorus are shown in Figures 8-14. The amount of polyphosphate in Figure 8 is much greater than in Figure 14. In Figure 14 there is also a large RNA peak, which apparently contains very little polyphosphate.

One very interesting result of calculations made from these curves is illustrated in Figure 15. The culture density series could be broken up into three parts. In the first part there was an excessive amount of polyphosphate, and even though there was also a relatively large amount of RNA, the ratio of O. D. units of RNA to mgm. of total phosphate was very small. This low ratio was characteristic of young cultures.

In the second part this ratio was fairly stabile between 16 and 21. Finally in the third part, composed only of the oldest of cultures, the ratio increases rapidly due to an increase in the amount of RNA. An interpretation of these results will be discussed in a later section with similar data from Chlorella.

The specific metachromasies and ultraviolet spectra of a series of tubes from each elution were determined before and after exhaustive dialysis (24 hours) against distilled water. In this case specific metachromasy was determined by dividing the metachromasy by total phosphorus. The

Figures 8 - 14. Elution patterns of acid-insoluble RNA and phosphorus from Anabaena cultures of various densities (solid line= ug. total-P/ml./10 ml. p.c.v.; dotted line= optical density at 260 mu/10 ml. p.c.v.)

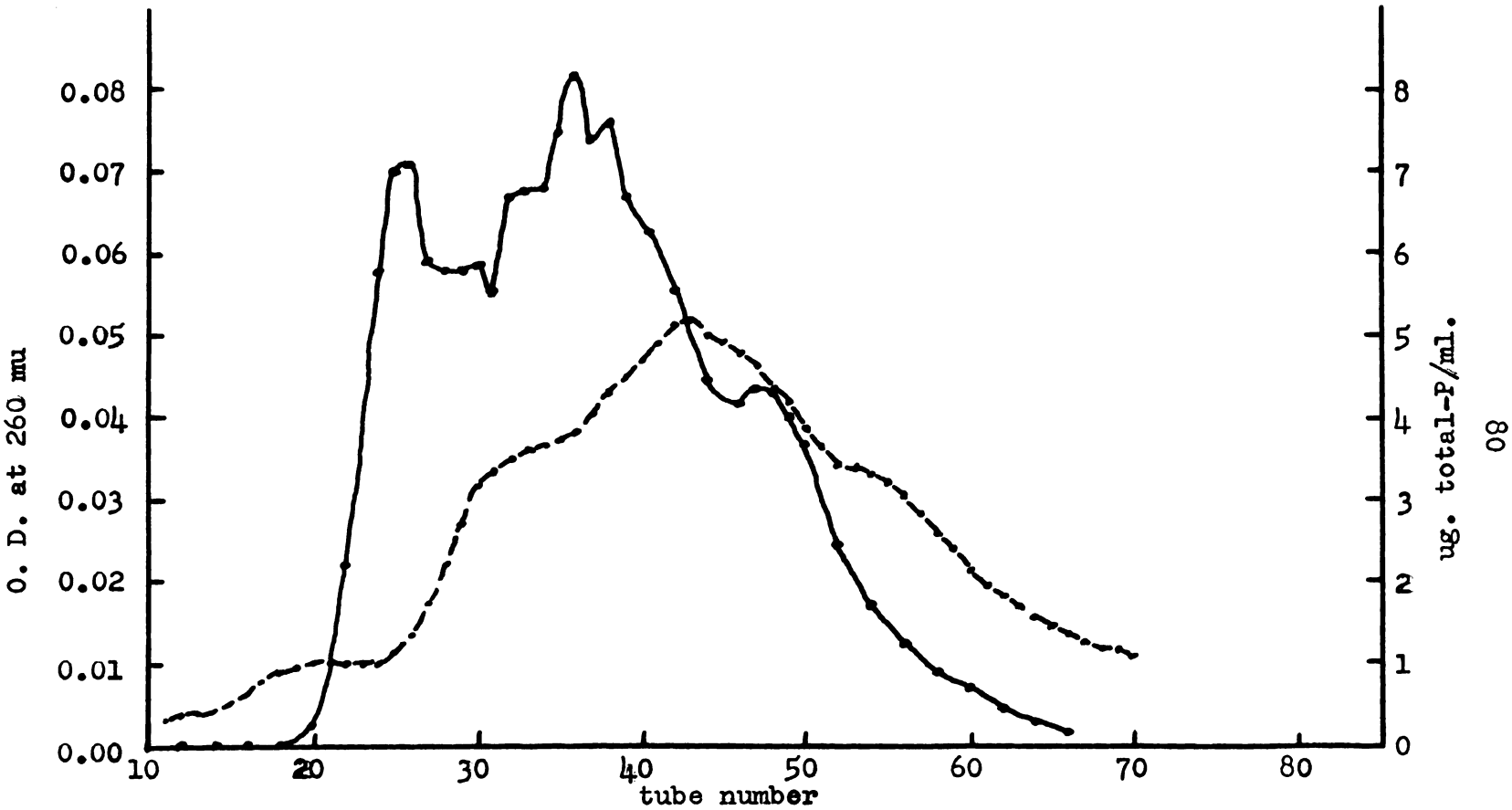

Figure 8, culture density = 3.6 ml. packed cells per liter of medium

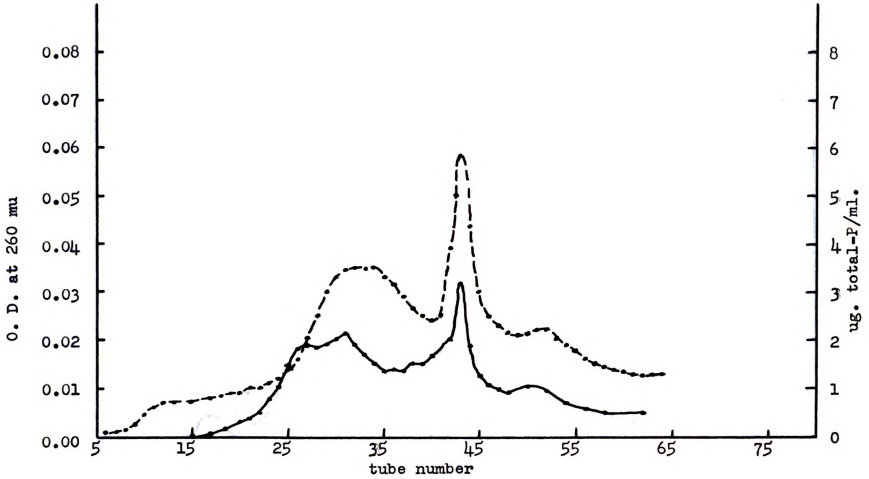

Figure 9, culture density = 7.2 ml. packed cells per liter of medium

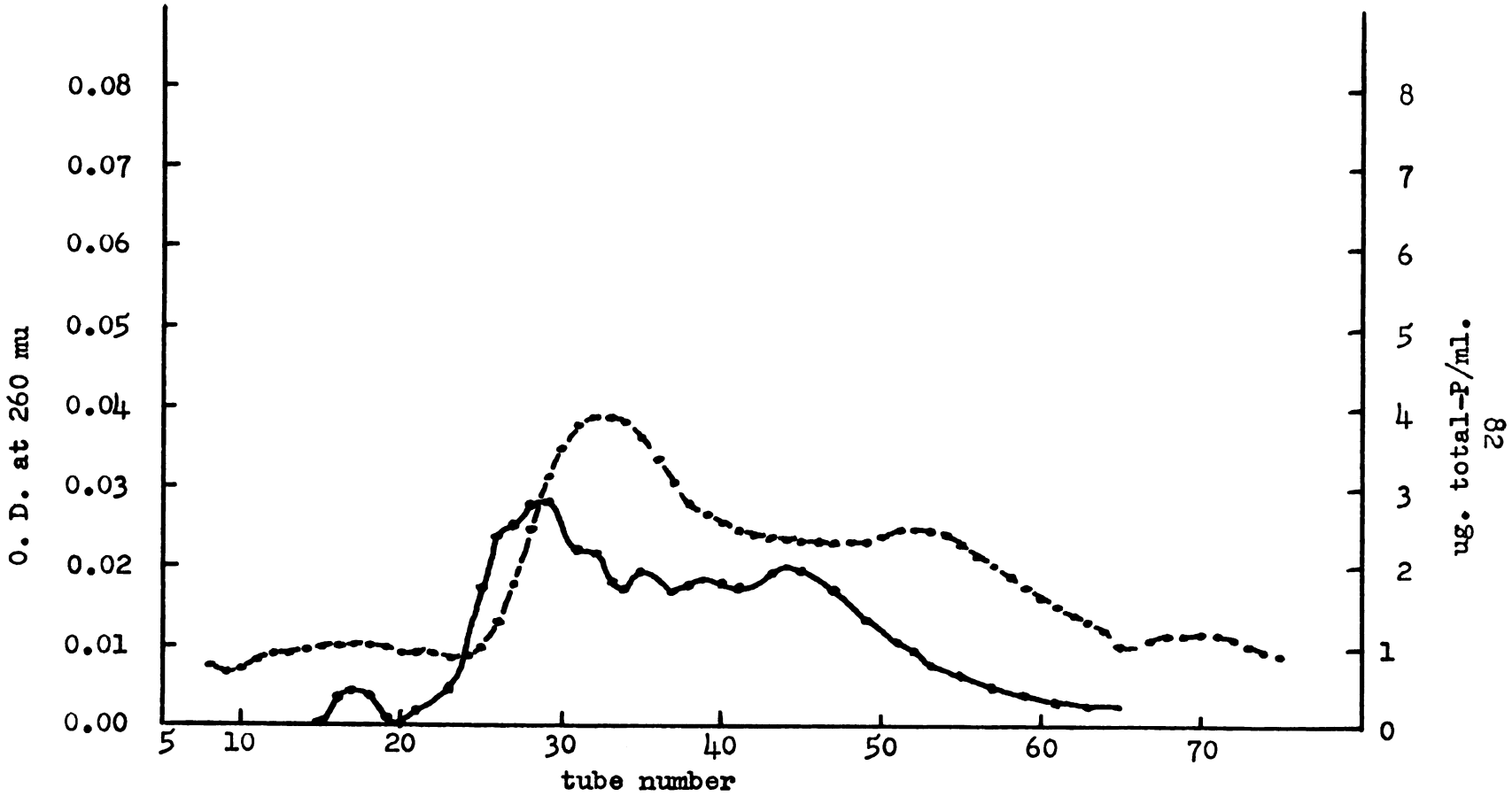

Figure 10, culture density = 8.0 ml. packed cells per liter of medium

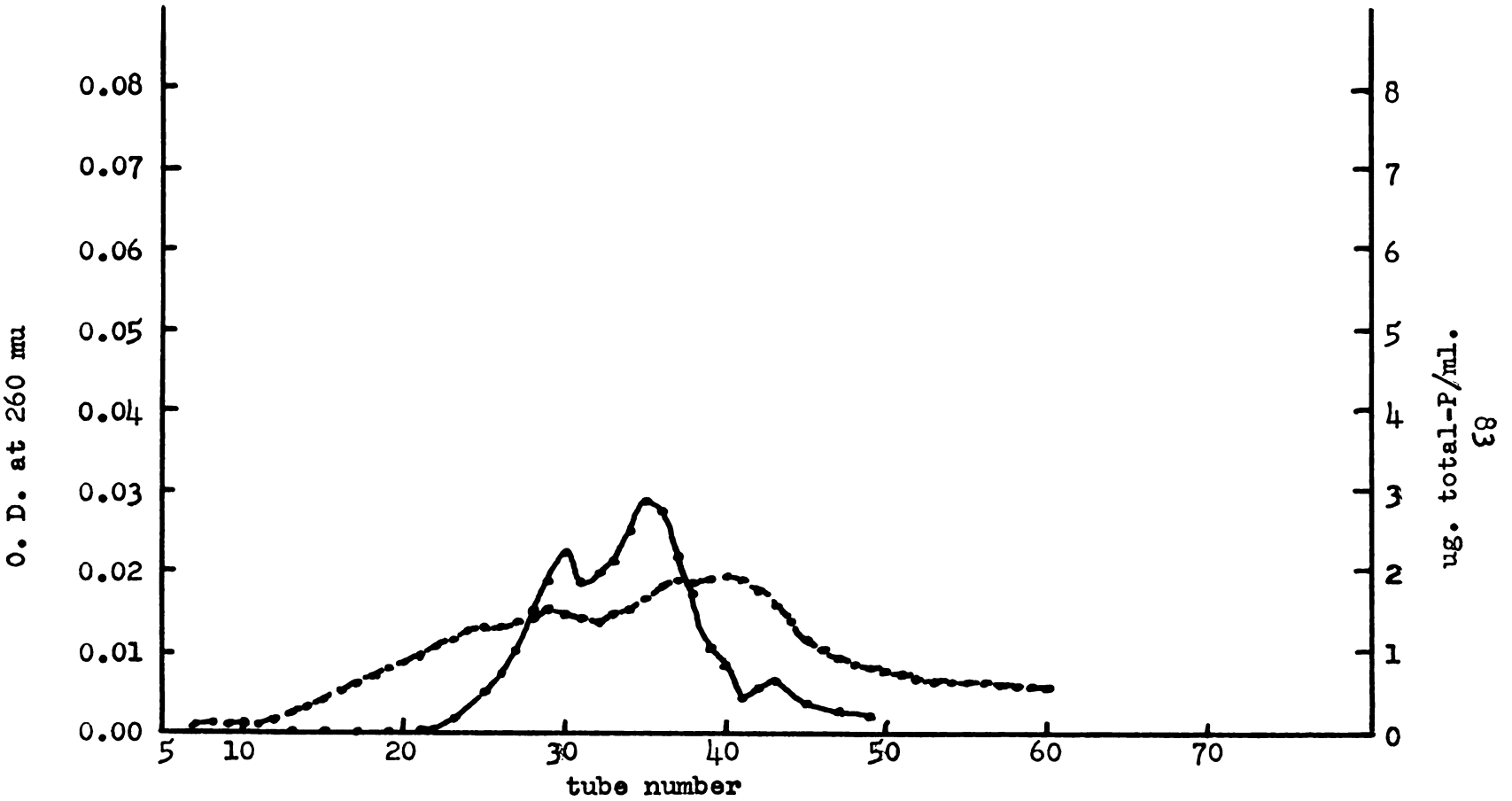

Figure 11, culture density = 11.2 ml. packed cells per liter of medium

Figure 12, culture density = 13 ml. packed cells per liter of medium

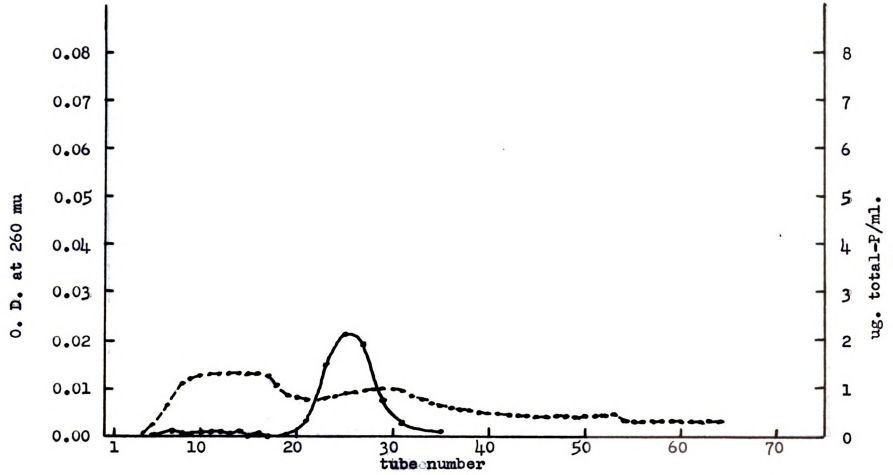

Figure 13, culture density = 25 ml. packed cells per liter of medium

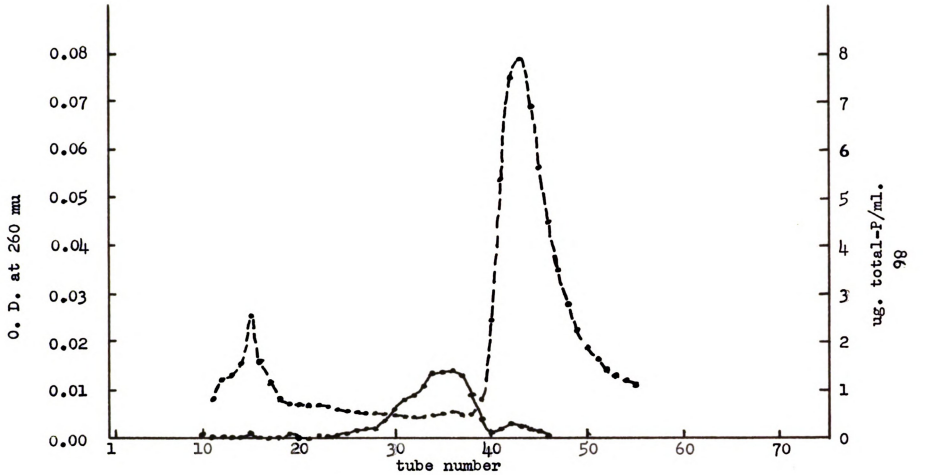

Figure 14, culture density = 29.6 ml. packed cells per liter of medium

Figure 15. Total acid-insoluble RNA and phosphorus in Anabaena cultures of different densities (one O. D. unit of RNA is equivalent to one ml. of solution with an absorbance of 1.0 at 260 mu in a one cm. cell)

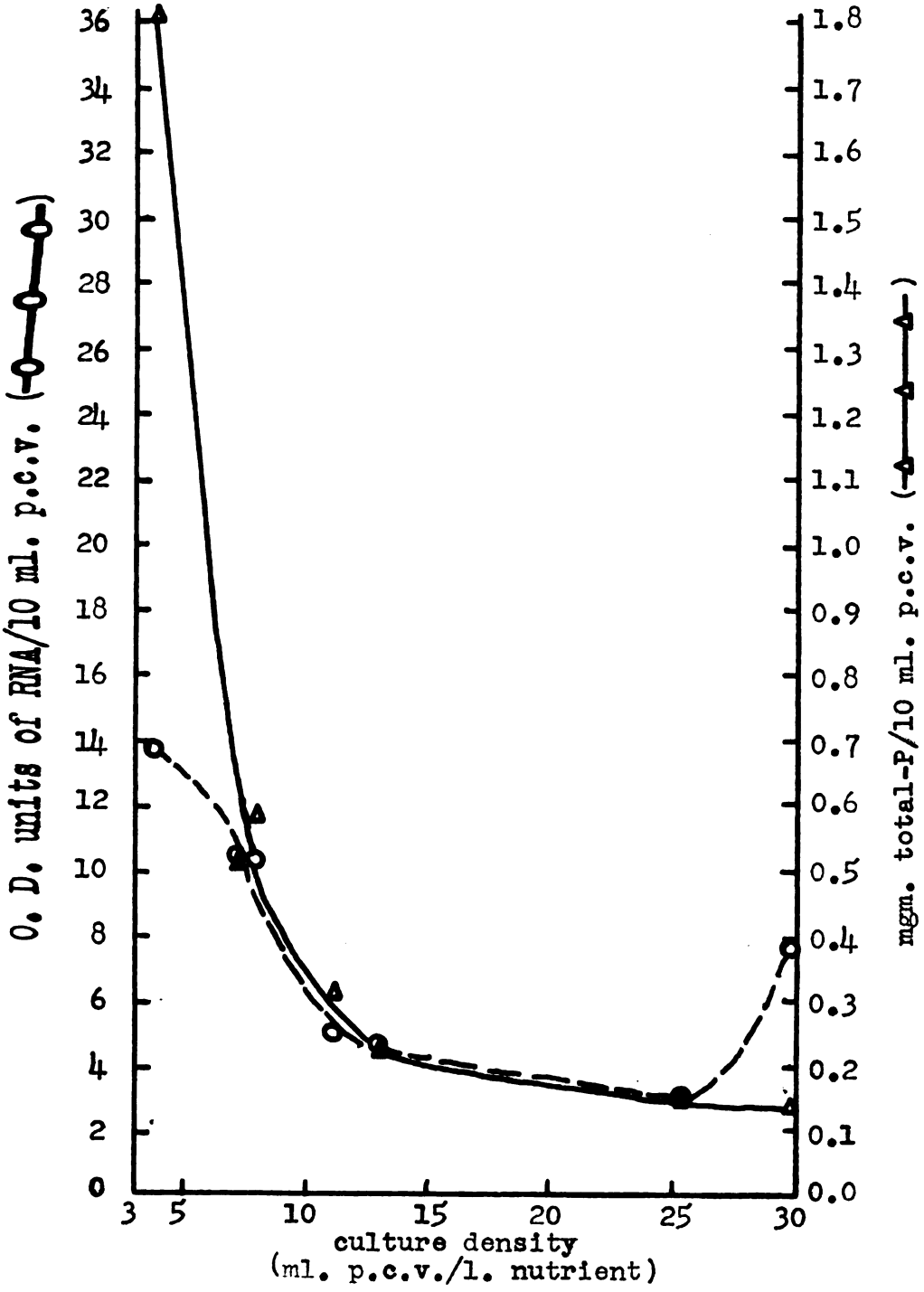
O. D. units of RNA/10 ml. p.c.v. (—o—)
mgm. total-P/10 ml. p.c.v. (—Δ—)
culture density
(ml. p.c.v./l. nutrient)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
3
5
10
15
20
25
30

Figure 15

Table VI. Changes induced by exhaustive dialysis against distilled water in the metachromasy and ultraviolet absorption of separated areas from the elution patterns in Figures 8-14

Fig.	tube #	specific metachromasy		O.D.@ 260 mu		O.D.@ 260 mu / O.D.@ 280 mu	
		before dial.	after dial.	before dial.	after dial.	before dial.	after dial.
8	25	0.65	1.2	0.032	0.119	1.8	1.1
"	32	0.95	1.5	0.225	0.310	2.0	1.3
"	36	0.84	1.7	0.201	0.220	2.1	1.6
"	42	0.66	1.5	0.274	0.307	2.1	1.6
"	48	0.45	1.3	0.504	0.576	2.2	1.6
"	56	0.13	1.4	0.160	0.211	2.1	1.5
9	26	0.38	1.3	0.026	0.068	1.2	1.1
"	32	0.33	1.4	0.294	0.400	1.8	1.4
"	40	0.29	1.4	0.133	0.226	1.9	1.4
"	43	0.17	1.3	0.499	0.621	2.0	1.6
"	52	0.15	1.1	0.124	0.192	1.6	1.4
10	17	0.44	0.1	0.080	0.104	0.9	1.2
"	25	0.34	1.1	0.079	0.095	1.2	1.3
"	28	0.50	1.1	0.278	0.296	1.6	1.5
"	32	0.11	1.2	0.314	0.304	1.9	1.8
"	35	0.22	1.1	0.311	0.307	1.8	1.7
"	39	0.02	1.2	0.219	0.264	2.0	1.6
"	44	0.00	1.1	0.412	0.476	1.9	1.7
"	53	0.00	1.2	0.198	0.209	2.0	1.7
"	71	0.83	1.3	0.083	0.096	2.0	1.7
11	29	0.41	1.0	0.122	0.217	1.5	1.1
"	36	1.00	1.0	0.090	0.193	1.4	1.3
"	41	1.20	1.8	0.096	0.183	1.7	1.3
"	44	0.40	1.4	0.067	0.166	1.7	1.3
12	15	0.05	0.5	0.193	0.148	2.1	1.2
"	20	0.18	1.1	0.227	0.324	1.8	1.3
13	14	-	-	0.095	0.216	1.3	1.1
"	27	-	-	0.090	0.224	1.4	1.2
14	36	1.90	-	0.078	-	-	-
"	42	1.80	-	1.090	-	-	-

results are given in Table VI. No consistent correlation could be seen between the increases in specific metachromasy, which were observed, and the increases in 260 mu optical densities.

A few generalizations can be made about the ultraviolet spectra. The spectra varied all the way from those in which the maximum was at about 258 mu and a deep valley was present in the region of 230 mu to those in which no valley occurred at 230 mu and only a plateau could be observed in the region from 250 to 270 mu. In general dialysis brought about a decrease in the 230/260 and 260/280 ratios. The increase in O. D. at 260 mu upon dialysis was usually much greater in those samples whose spectra lack a 230 mu valley. In some cases this increase was at least four-fold. Another characteristic of these spectra was a very rapid increase in optical density in the low wave length end of the spectrum.

Such ultraviolet spectra and changes in the extinction coefficents at 260 mu could be explained on the basis of the assumption that a complex between RNA and some other component was being broken by dialysis. This breakage would have to be irreversible since addition of NaCl to the dialyzed samples only caused minor changes. Blout (10) has reported this type and magnitude of spectral changes in calf thymus DNA when it is mixed with small amounts of plasma albumin. The changes observed could not be explained on the basis of the hyperchromic effects resulting from the denaturating of the hydrogen-bond system of the RNA, since such effects are

not of this magnitude.

Further Characterization of the Fractionated RNA-Polyphosphate

In one case a series of seven tubes at intervals in the elution from a DEAE-cellulose column were tested for ninhydrin reactivity before and after hydrolysis for one hour in 2 N HCl. No ninhydrin reaction could be detected in any of the tubes, although the same amount of 0.001 M alanine was easily detected. This indicated that the phenol extraction had completely removed all peptides and proteins.

A series of ribose determinations on elution tubes from one column are shown in Table VII. It can be seen that the amount of orcinol-positive material was about the same before and after dialysis.

In Table VIII a series of total carbohydrate and ribose determinations are recorded from the elution tubes of another column. These carbohydrate analyses may be too high, because small particles of the DEAE-cellulose sometimes elute from the column and cause an increase in the total carbohydrate values. Therefore it is not known whether polysaccharides in small amounts are involved in the RNA-polyphosphate, as it is isolated.

In the case of one column, five groups of tubes were pooled and used to determine base ratios. The values in Table IX are the averages of paired determinations. No consistent changes could be seen.

Table VII. Effect of dialysis upon the ribose content of the eluates from a DEAE-cellulose column

tube #	ug. ribose/ml.	
	before dial.	after dial.
12	1.3	1.3
18	6.0	4.8
25	4.8	5.0
31	8.7	10.6
36	6.6	3.2

Table VIII. Concentration of ribose and total carbohydrates in a series of elution tubes from a DEAE-cellulose column

tube #	ribose (ug./ml.)	total carbohydrates (ug./ml. as ribose)
43	0.6	12.5
50	3.0	4.7
53	2.3	12.7
57	2.5	2.2
62	3.5	5.5
70	5.0	12.0

Table IX. Approximate base composition of a series of areas of RNA from a DEAE-cellulose column

	mole percent			
tube numbers	adenine	guanine	CMP	UMP
10-12	35.8	22.5	12.7	28.9
18-20	29.5	17.9	21.2	31.4
24-26	27.6	25.5	18.4	28.6
29-31	31.7	31.0	16.0	21.2
35-37	28.8	29.8	13.6	27.8

Stability of RNA-Polyphosphates

It has already been noted that most samples show greatly increased specific metachromasy after dialysis against distilled water. The release of this metachromasy was utilized in a series of studies on the stability of the RNA-polyphosphate complexes, after purification on a DEAE-cellulose column. The results are reported in Table X. Mild acid or mild alkaline hydrolysis resulted in decreased metachromasy. Incubation with ethylene-diamine-tetraacetic acid (EDTA) did not significantly increase the metachromasy. A most interesting fact was the failure of boiling temperatures to release the metachromasy, since such treatment will cause the complete separation of the hydrogen-bond systems of native DNA and synthetic RNA double helices. Alkaline pH's such as used in the extraction procedure would also have completely denatured ordinary RNA (30).

The action of crystalline RNase on synthetic polyphosphate, yeast RNA, and both "native" and dialyzed RNA-polyphosphate was measured by the effects on metachromasy as presented in Table XI. Aliquots of experiments 2, 3, 7, and 9 were chromatographed, but no release of any free orthophosphate or oligopolyphosphates ($n < 7$) was detectable.

Aliquots of experiments 4, 5, 7, and 9 were chromatographed to see if any free nucleotides or oligonucleotides were released. In experiment 4 none were detected. In experiments 5, 7, and 9 ultraviolet-absorbing material was

released, which had a small Rf and was probably a di- or trinucleotide. In experiment 5 a faint spot was detected with an Rf of about 0.7, which would be characteristic of a nucleotide.

In summary, RNase seemed to attack both yeast-RNA and RNA-polyphosphate complexes whether dialyzed or not, but the enzyme did not affect synthetic polyphosphate. In the case of both RNA-polyphosphate and dialyzed RNA-polyphosphate, free low-molecular-weight polyphosphates were not released but some low-molecular-weight oligonucleotides were released and the specific metachromasy was changed significantly. More will be said later about the interpretation of these data.

Attempts to Produce Synthetic or Artificial RNA-Polyphosphate Complexes

An effort was made to determine whether RNA-polyphosphate complexes were artifacts. This possibility was not likely, since the changes produced by dialysis against distilled water were not reversible.

No effects could be observed on the optical density at 260 mu of solutions of yeast-RNA or salmon-sperm DNA, when synthetic polyphosphate was added. These experiments were done in distilled water and in 2 M NaCl. In another experiment RNA was isolated from tobacco leaves by the same method as used in isolating RNA-polyphosphate from Anabaena, but synthetic polyphosphate was added at the beginning of the

Table X. Effects of various treatments on the specific metachromasy of Anabaena RNA-polyphosphate

treatment	specific metachromasy
none	0.20
dialysis against distilled water	1.13
10 min. boiling	0.18
30 min. incubation with Na.EDTA	0.23
12 hrs. in 0.5 N NaOH, room temp.	0.09
10 min. boiling in 0.5 N NaOH	0.03
2 min. boiling in 0.1 N HCl	0.05
30 min. boiling in 0.1 N HCl	0.15
5 min. in 1 N HCl, room temp.	0.09

Table XI. Effects of RNase on free polyphosphate, yeast-RNA, and RNA-polyphosphate

assay: 5 ml. substrate and 1 ml. of enzyme or buffer; incubate 30 min. at 25°; boil 5 min.

substrates: Enough yeast-RNA was dissolved in 0.5 M tris, pH 7.6, to have an O. D. @ 260 mu of 1.0.

For synthetic polyphosphate 33 mgm. sodium hexametaphosphate per 100 ml. 0.5 M tris at pH 7.6 was used.

enzyme solution: 1.3 mgm. crystalline RNase in 10 ml. 0.5 M tris, pH 7.6

experiment	substrate	enzyme	specific metachromasy
1	buffer	+	0.0
2	synthetic polyphosphate	-	1.0
3	synthetic polyphosphate	+	1.0
4	yeast RNA	-	0.0
5	yeast RNA	+	0.0
6	RNA-polyphosphate	-	0.1
7	RNA-polyphosphate	+	0.2
8	dialyzed RNA-poly-P	-	1.15
9	dialyzed RNA-poly-P	+	0.6

alkaline extraction step (Figure 6). The resulting phenol preparation was then fractionated on a DEAE-cellulose column and no material could be found which exhibited the properties of the RNA-polyphosphate complexes isolated from Anabaena.

Chlorella

Changes in RNA-Polyphosphate During the Life Cycle

Samples of synchronized Chlorella, taken at three-hour intervals in their life cycle, (Figure 5), were used to isolate and fractionate RNA-polyphosphate, (Figures 16-25). In addition the effects produced by 21 hours of light are given in Figure 26. Figure 27 illustrates the result obtained when chloramphenicol was added after nine hours of light and the Chlorella were harvested three hours later.

Six elution areas of RNA could be distinguished, since they eluted at about the same places from column to column and their magnitudes showed systematic rather than erratic changes. Boundaries for these six areas were arbitrarily established and the areas were labeled A through F as can be seen in Figures 16-27. Six areas of polyphosphate, which roughly correspond to the six RNA areas, were also delineated and labeled I through VI. Studies and calculations were then carried out of the properties, amounts, and changes in these areas during the course of the life cycle.

Figure 28 shows the changes in amounts of total phosphorus and total RNA. A striking correspondence between the changes in these two components can be seen. This could be interpretted as evidence that the synthesis and utilization of polyphosphate and RNA are somehow interrelated. Those points at which this relationship was not very close

Figures 16-27. Elution patterns of total RNA and phosphorus from synchronized Chlorella cultures at various times in the life cycle (solid line = ug. total-P/ml./10 ml. p.c.v.; dotted line = optical density at 260 mu/10 ml. p.c.v.; A-F = RNA areas, I-VI = polyphosphate areas)

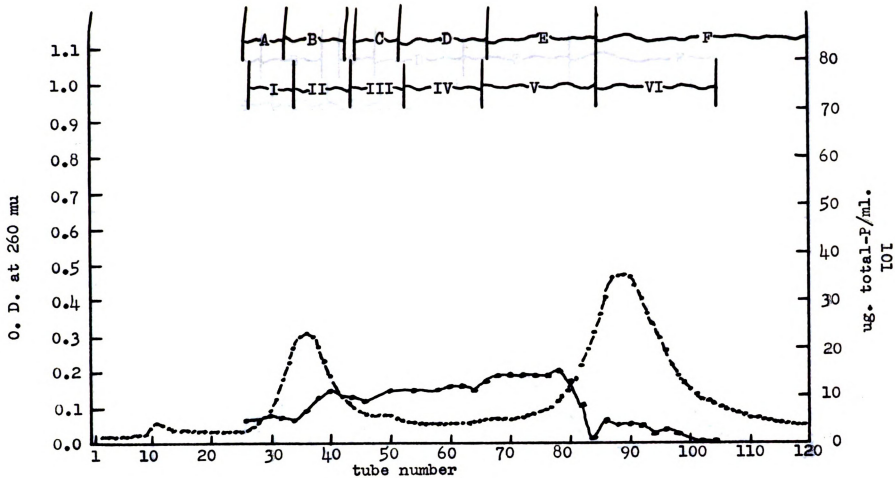

Figure 16, 0 hours in light

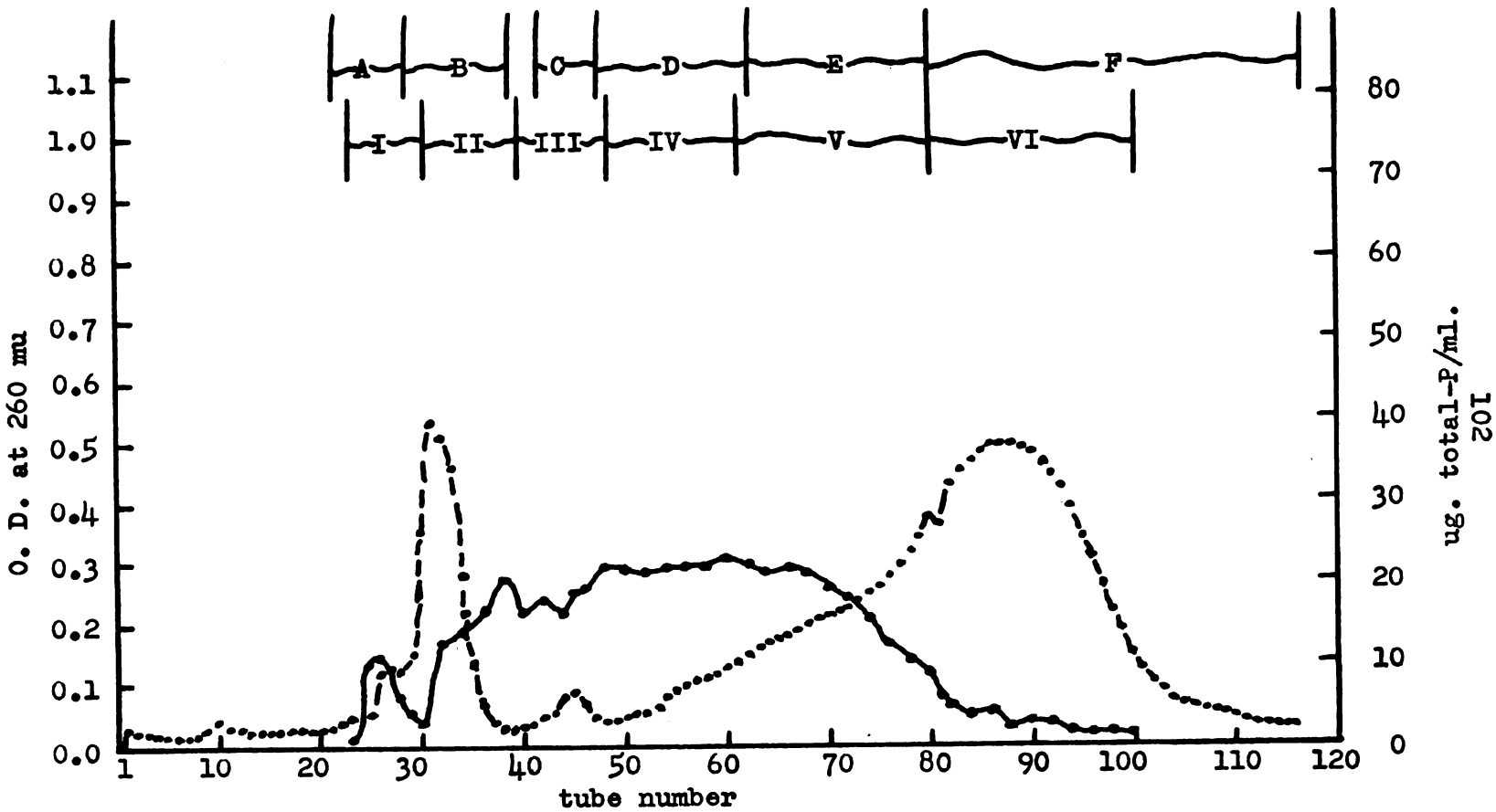

Figure 17, 3 hours in light

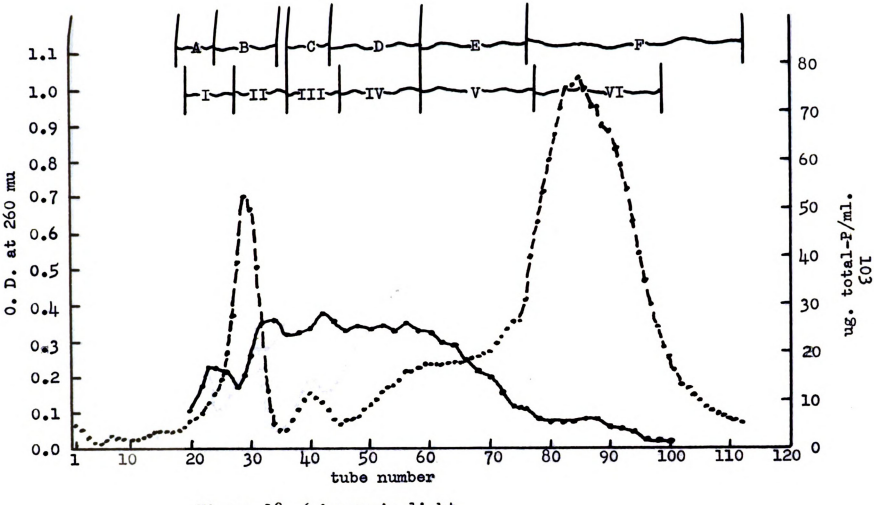

Figure 18, 6 hours in light

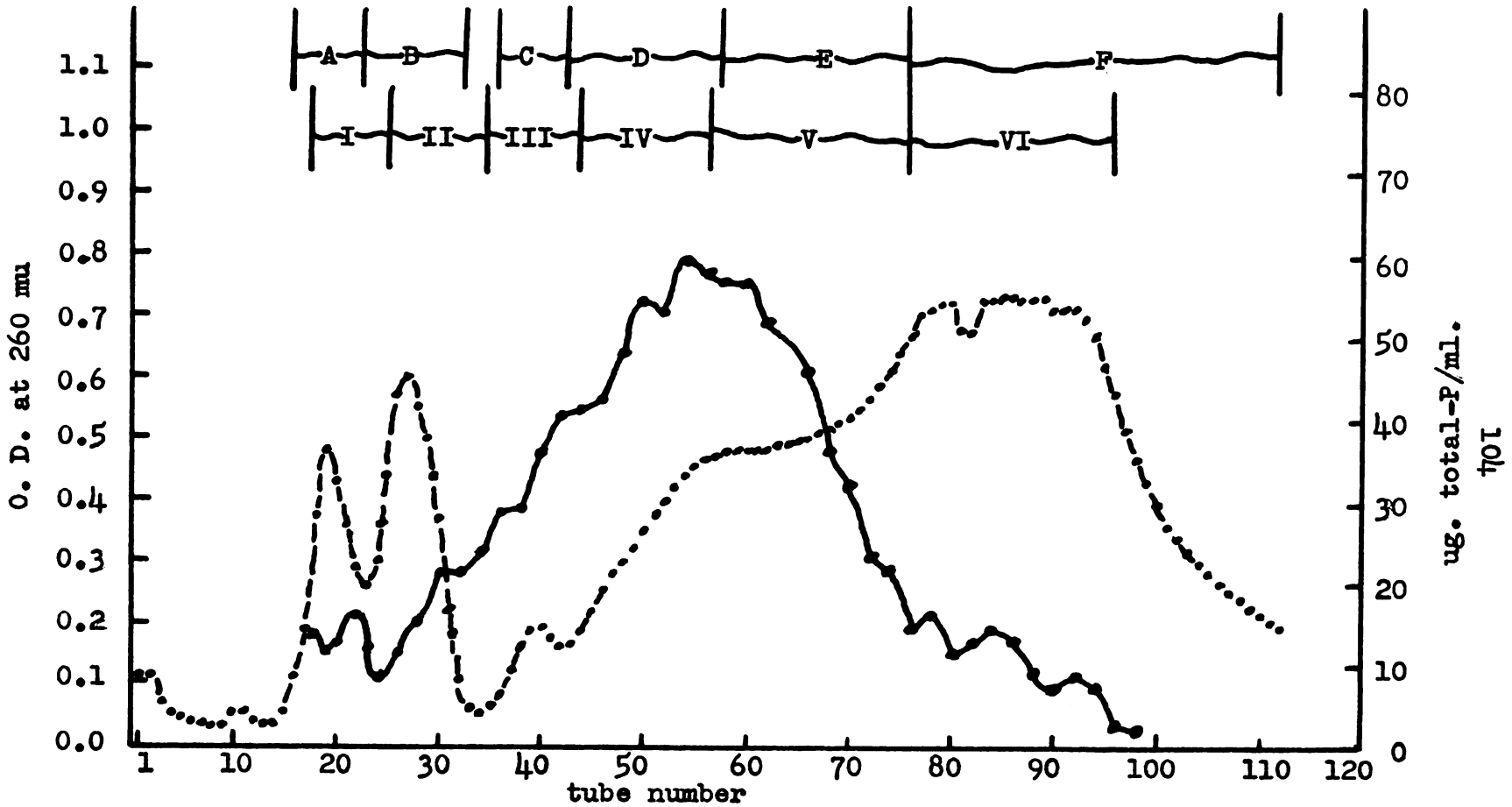

Figure 19, 9 hours in light

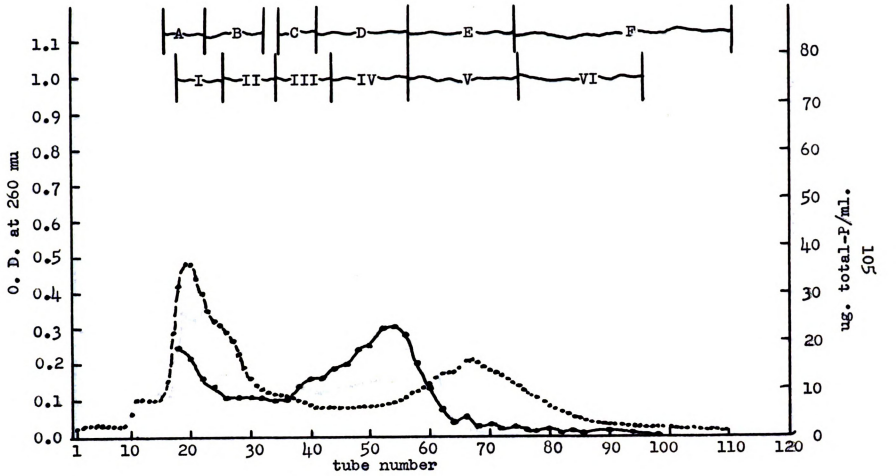

Figure 20, 12 hours in light

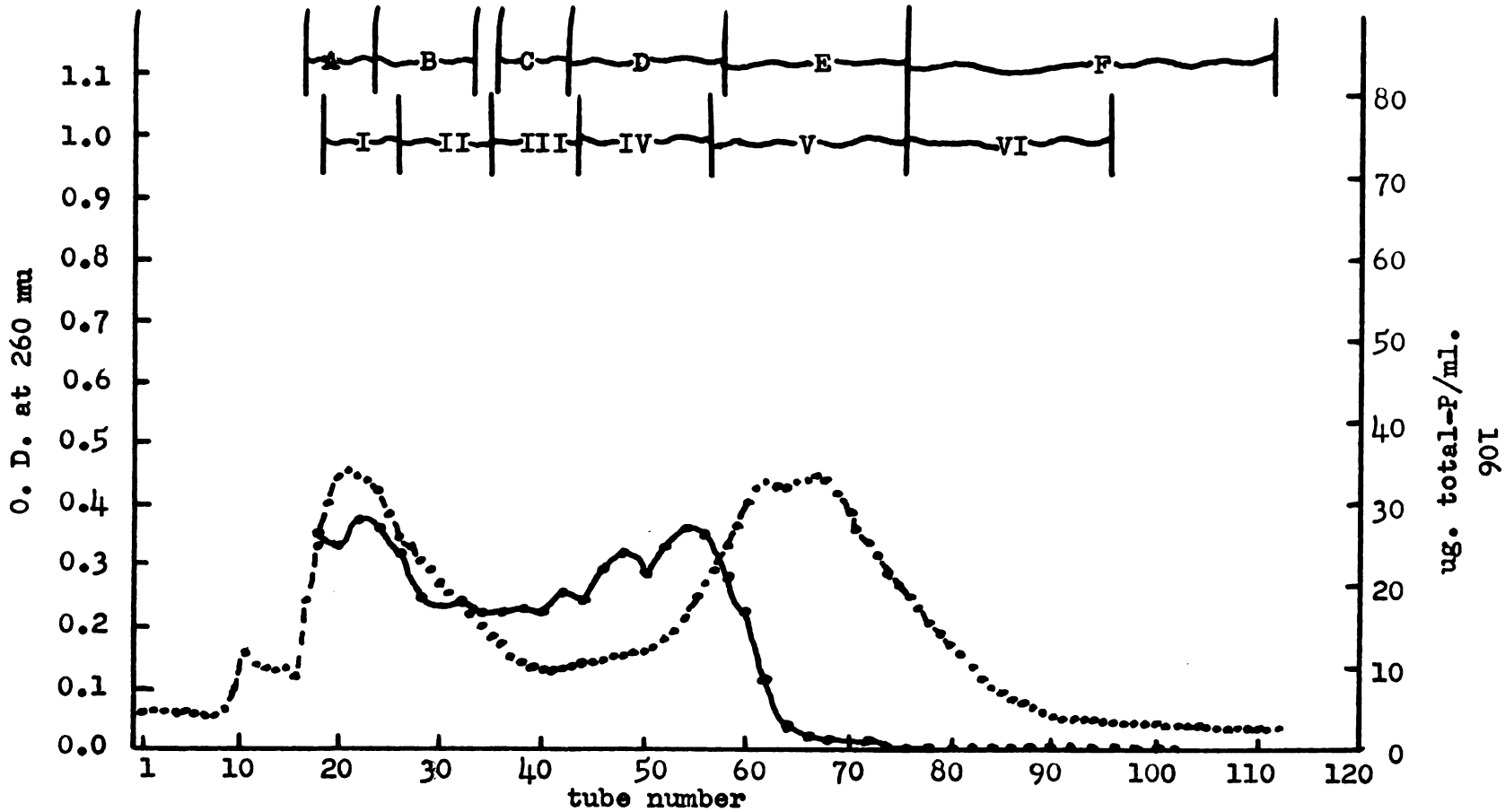

Figure 21, 15 hours in light

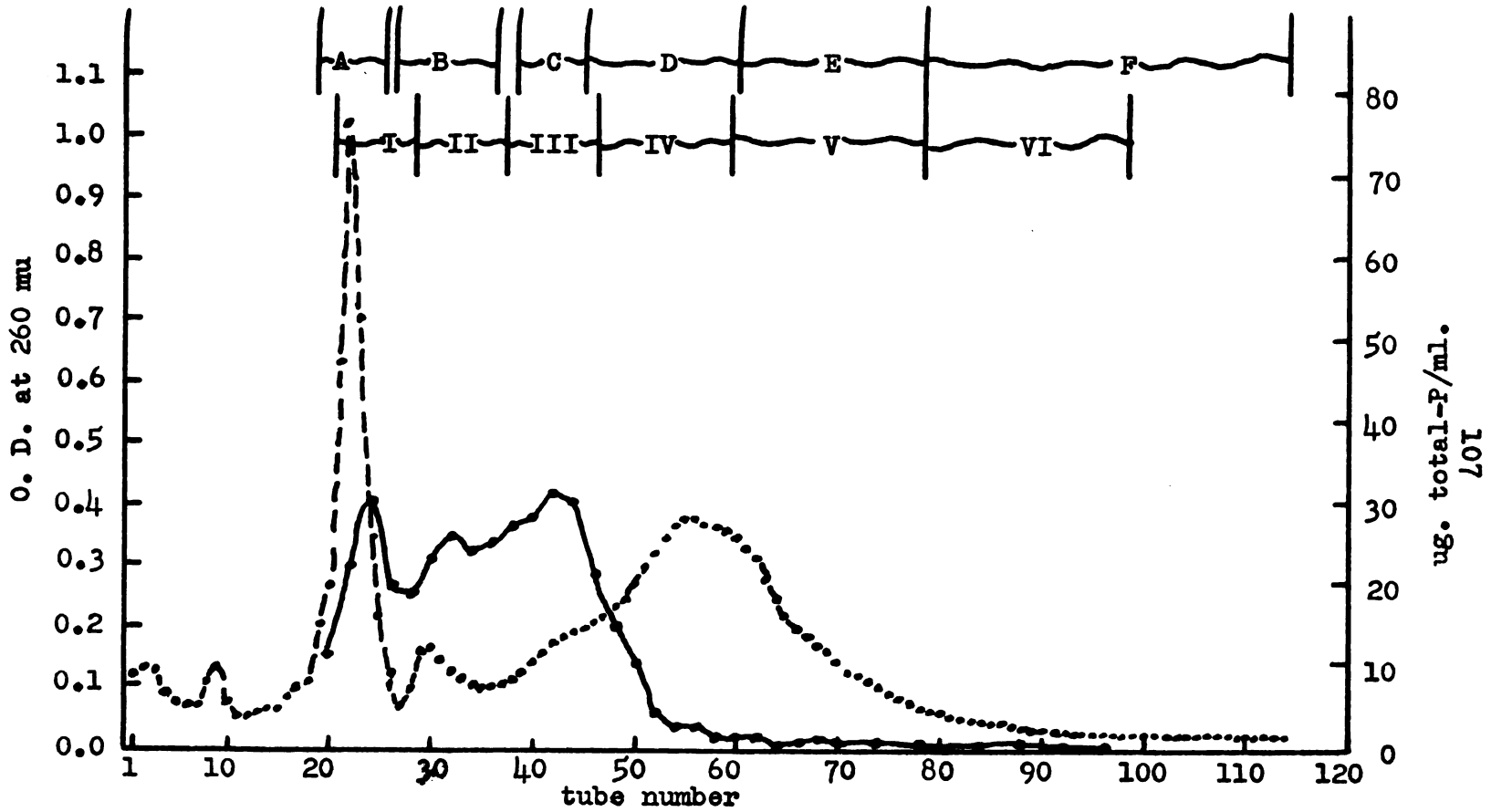

Figure 22, 18 hours in light

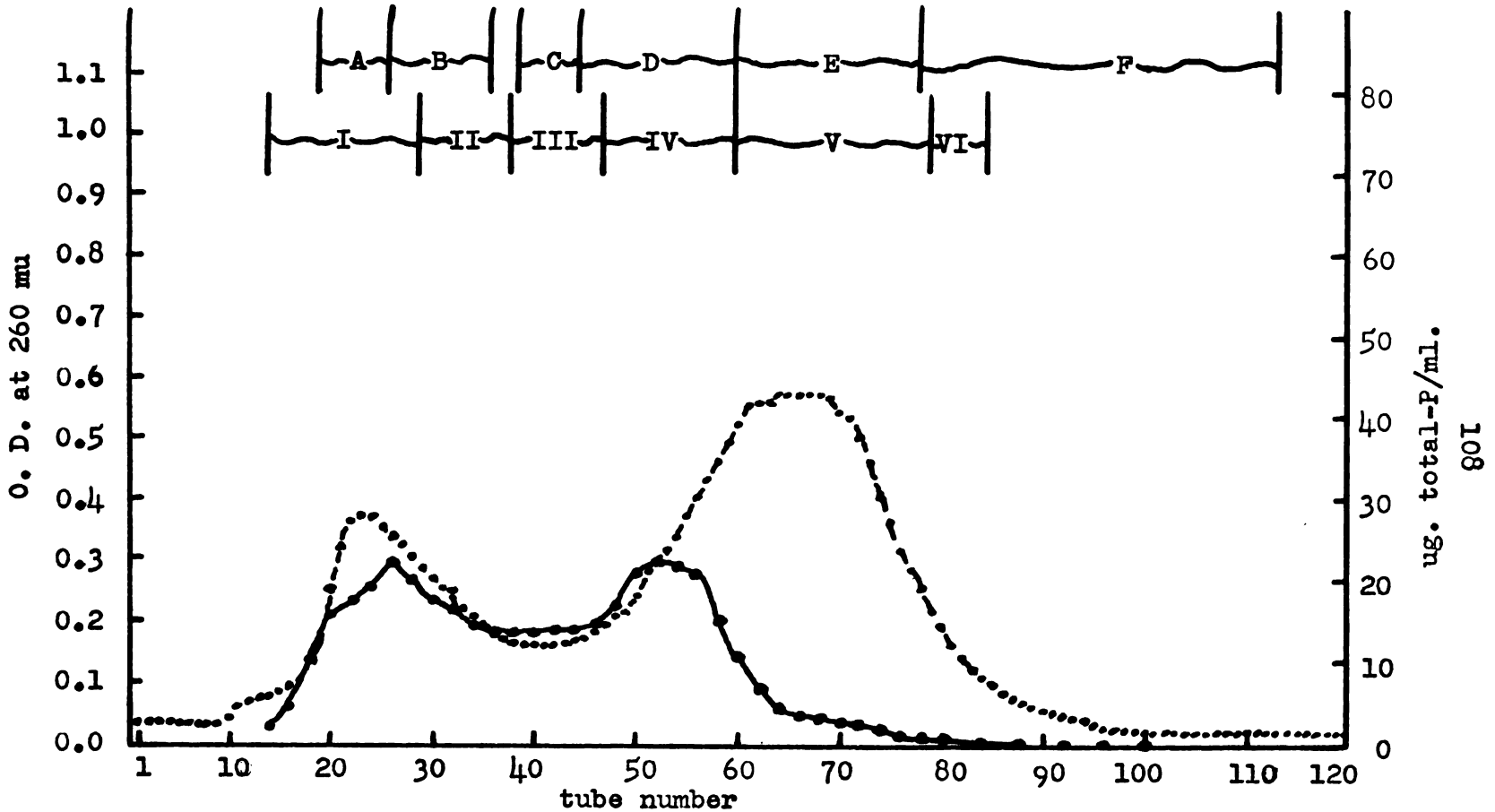

Figure 23, 3 hours in dark

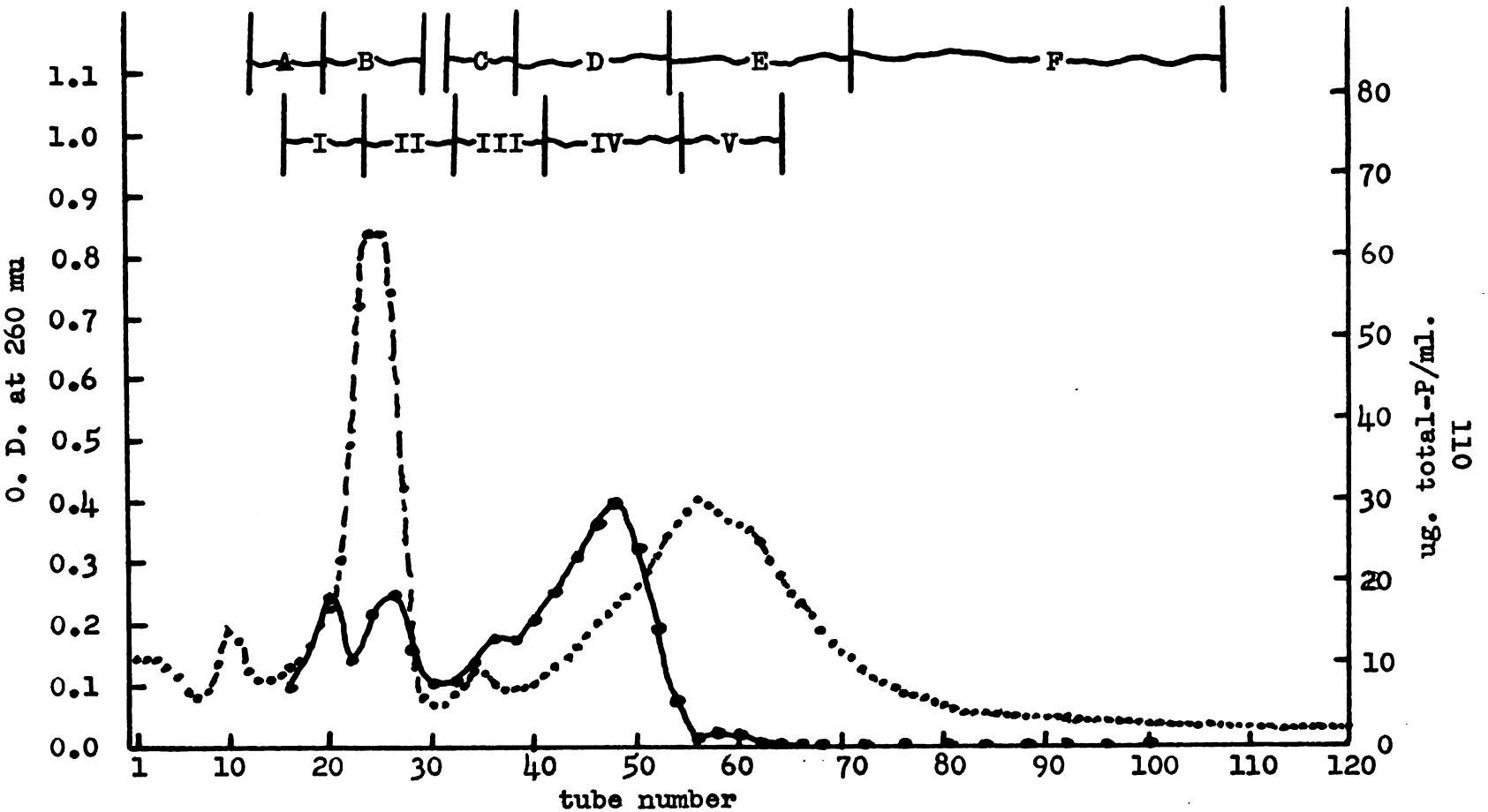

Figure 25, 9 hours in dark

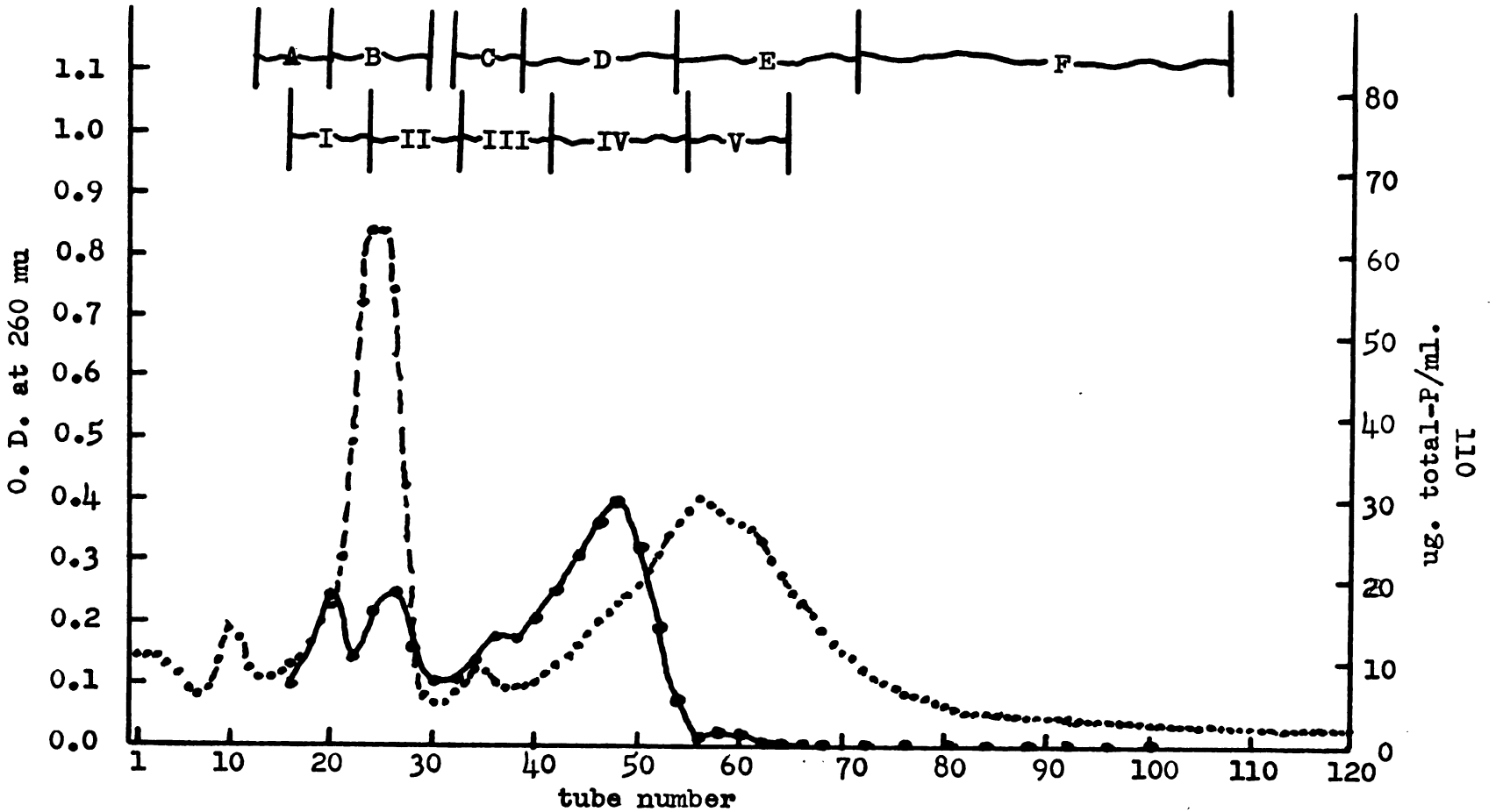

Figure 25, 9 hours in dark

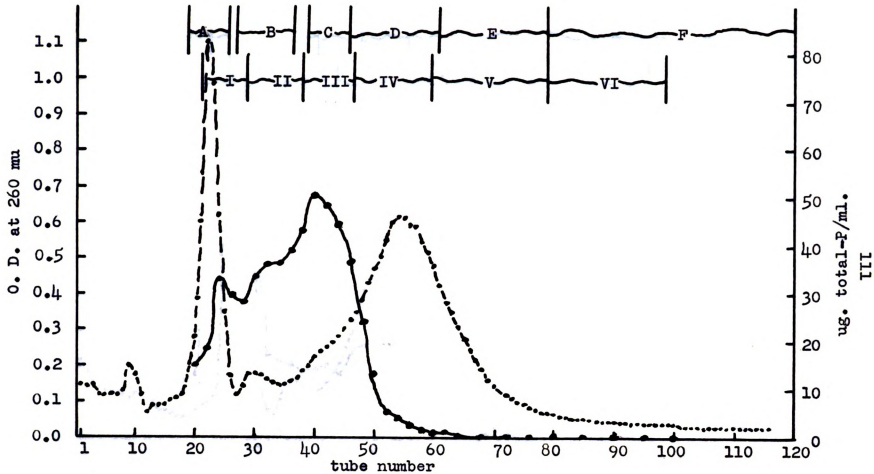

Figure 26, 21 hours in light

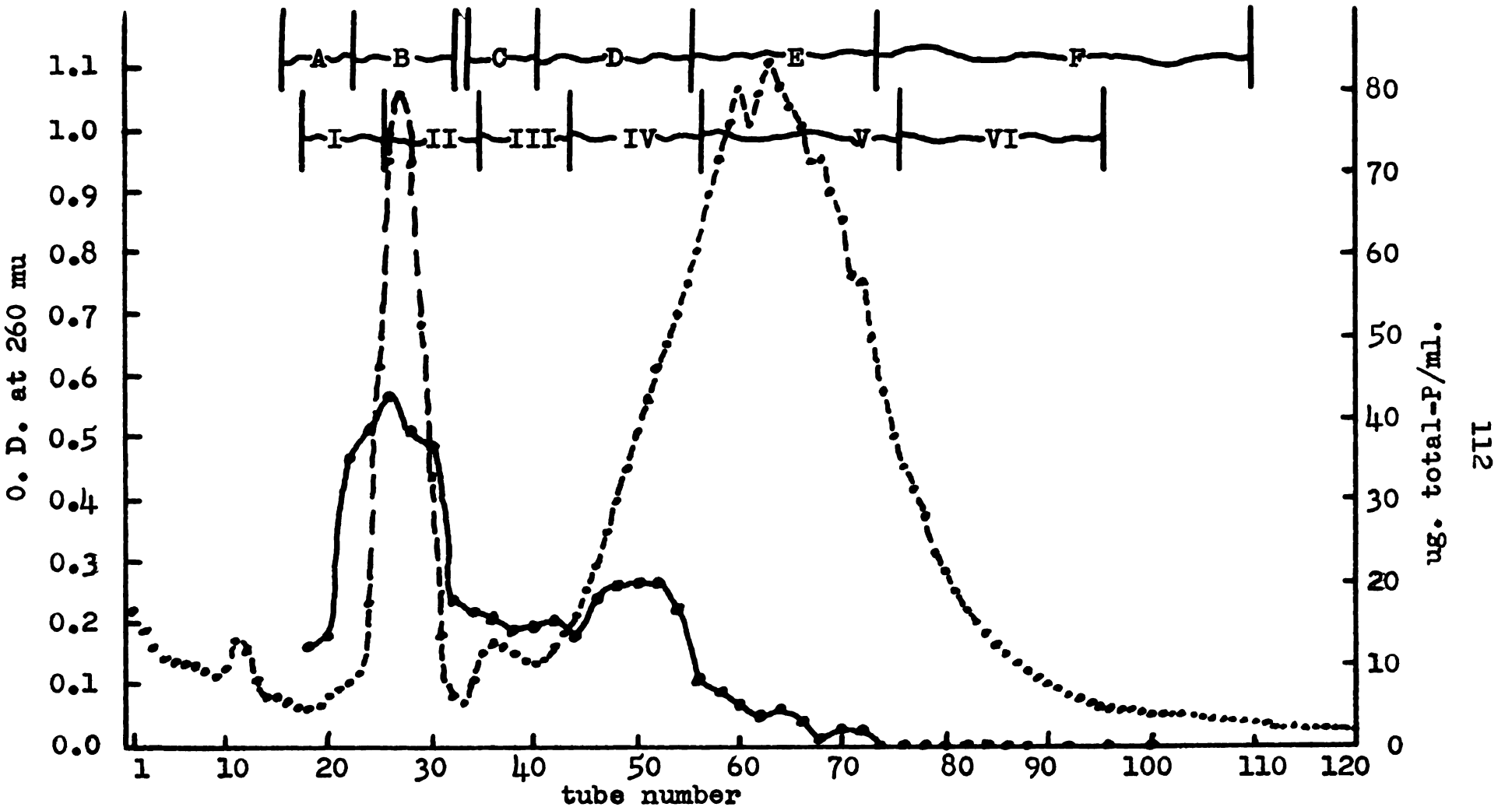

Figure 27, 9 hours in light and then 3 more hours in light after the addition of 40 p.p.m. chloramphenicol to the nutrient

Figure 28. Total RNA and total phosphorus per 10 ml. p.c.v. of Chlorella during synchronous growth (▲ = 21 hours in light instead of darkening at 18 hours, □ = 40 p.p.m. chloramphenicol added at nine hours in light)

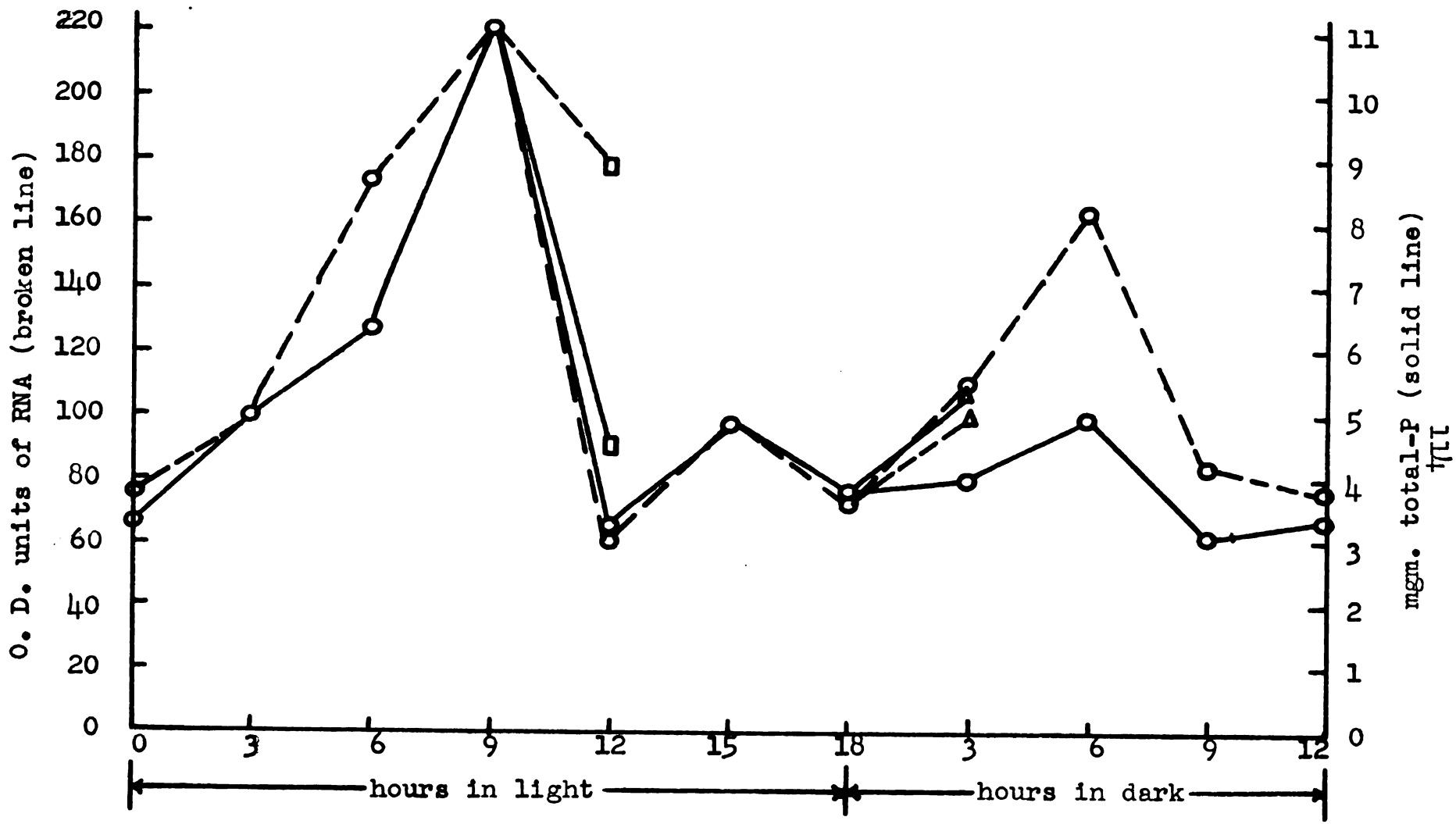
O. D. units of RNA (broken line)
220
200
180
160
140
120
100
80
60
40
20
0
11
10
9
8
7
6
5
4
3
2
1
0
mgm. total-P (solid line)
0
3
6
9
12
15
18
3
6
9
12
hours in light
hours in dark

Figure 28

are also interesting. At six hours of light the synthesis of RNA was somewhat ahead of polyphosphate synthesis. This fact would support the hypothesis, discussed earlier (6, 20, 31), that polyphosphate is synthesized on the surface of RNA and then both are utilized. Whether the chloramphenicol induced delay in RNA loss after 9 hours of light was due to synthesis of RNA with different properties or to the specific utilization of polyphosphate but not RNA, is not known. Takeda (132) reported that the RNA, which was synthesized in the presence of chloramphenicol, exhibited different physical properties than normal RNA.

In the dark part of the life cycle a rapid build-up of RNA occurred in the first six hours with very little increase in polyphosphate. This was then reversed in the last six hours of darkness. If the lights were left on for three hours longer than usual, this divergence was not noted. This effect of light on RNA synthesis was already noted in Figure 15 in the work with Anabaena. Further evidence that light influences the quantity and nature of RNA, independent of the life cycle, is shown in Figures 29 and 30. These algae were not synchronized. One culture from which the RNA-polyphosphate was isolated had been exposed to a high level of continuous light and almost all of the RNA eluted at a low salt concentration, (Figure 29). In another case the algae were grown under a low level of continuous light and the RNA eluted at a higher salt concentration, (Figure 30).

In the operation of the Chlorella synchronization

Figure 29. Elution pattern of total RNA and phosphorus from a random culture of Chlorella grown under continuous high light intensity (solid line = ug. total-P/ml./10 ml. p.c.v.; dotted line = optical density at 260 mu/10 ml. p.c.v.; salt gradient in this case was the same as normally used for Anabaena elutions.)

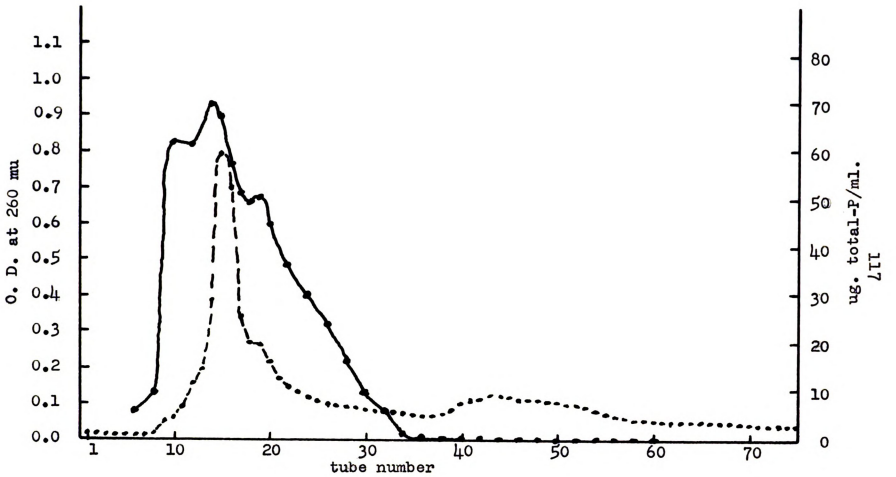
O. D. at 260 mu
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
10
20
30
40
50
60
70
tube number
80
70
60
50
40
30
20
10
0
ug. total-P/ml.
117

Figure 29

Figure 30. Elution pattern of total RNA and phosphorus from a random Chlorella culture grown under continuous low light intensity (solid line = ug. total-P/ml./10 ml. p.c.v.; dotted line = optical density at 260 mu/10 ml. p.c.v.)

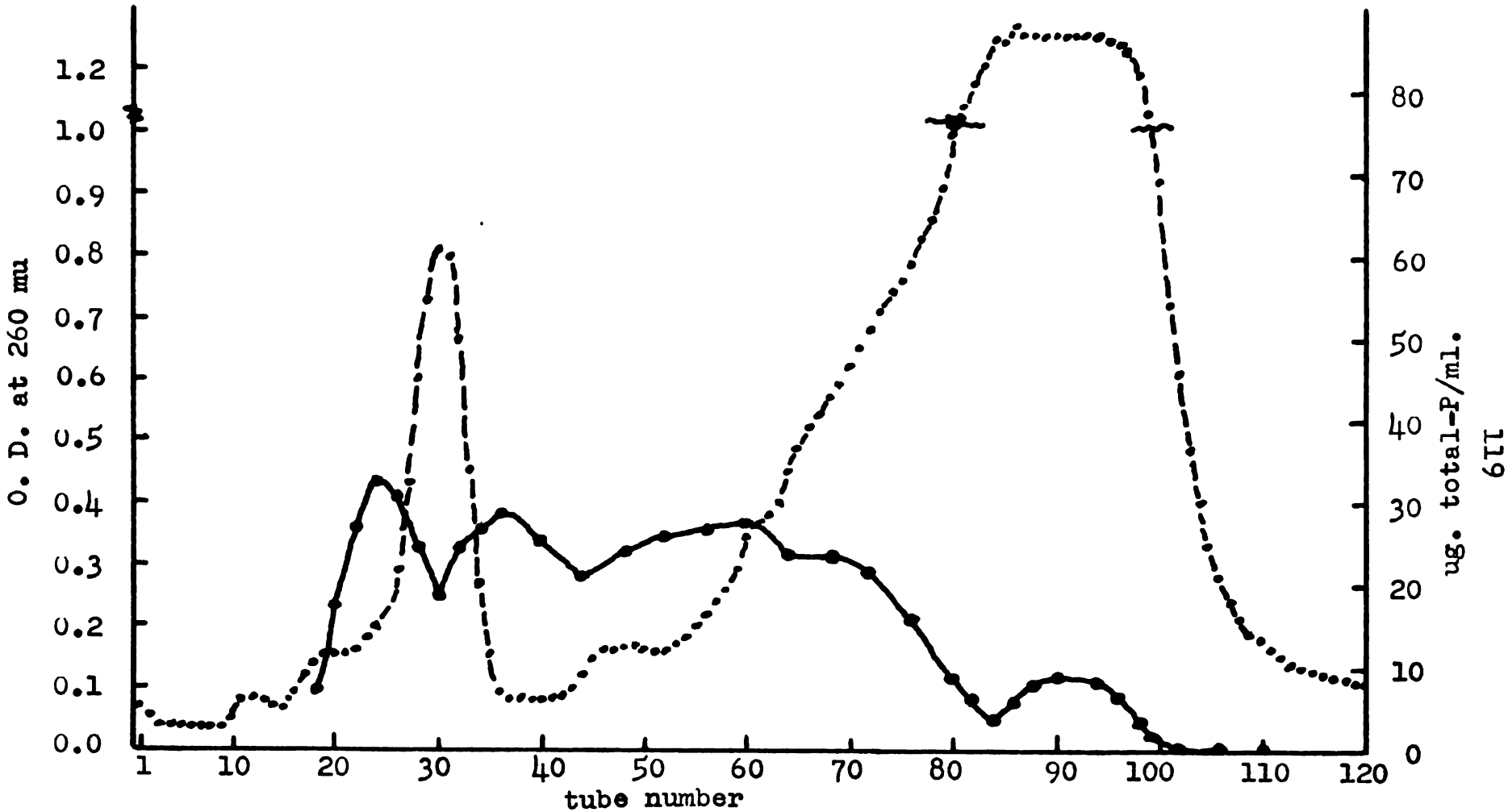
O. D. at 260 mu
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
10
20
30
40
50
60
70
80
90
100
110
120
tube number
ug. total-P/ml.
80
70
60
50
40
30
20
10
0

Figure 30

apparatus it was noted that a culture had to be quite dense to withstand continuous high illumination. In several cases the use of high illumination too early in the establishment of a culture resulted in clumping and loss of the culture. Part of the cause of the observed effect of high light on RNA synthesis might be photo-oxidation. Sager (114) and Anderson (5) have obtained evidence that plants deficient in carotenoids can not withstand prolonged high illumination due to the photo-oxidation of their chlorophyll. Anderson suggested that carotenoids might also protect such porphyrin-containing substances as catalase and the cytochromes.

In Table XII the amounts of total RNA and total phosphorus are recorded and summarized from the fractionations illustrated in Figures 8-14, 16-27, 29 and 30. It is interesting to note that the ratio of total RNA to total phosphorus in the middle part of the Anabaena growth curve was about the same as in synchronized Chlorella in the light phase of their life cycle. The very low ratios observed in continuous high light are compatible with the fact that no shortage of ATP would have existed under these conditions and, consequently, an accumulation of polyphosphate could occur.

Changes Within RNA-Polyphosphate Areas During the Life Cycle

Figures 31 and 32 illustrate the changes in amount of RNA in areas A-F during the life cycle. Figures 33 and 34 give the parallel changes in percent distribution of RNA among these areas.

All of the areas reached a peak magnitude at the nine hour light stage and areas B, D, and E also reached a second peak at the six hour dark stage. Area F, which required the highest salt concentration for elution, showed the greatest changes. The main effect of chloramphenicol was a large increase in areas B, D, and E with almost no effect upon the rapid utilization of area F.

Changes in the magnitude of the phosphorus areas are shown in Figures 35 and 36. Changes in percent distribution of these areas are shown in Figures 37 and 38. It is interesting to note, that at the six and nine hour light stages IV and V were the dominant phosphorus areas, whereas in the case of RNA area F was dominant. It is possible that as the RNA of area F became phosphorylated it was eluted at a lower ionic strength and appeared in areas D and E. Such a hypothesis is supported by the fact that area F of the RNA reached its greatest percent of the total RNA at the six hour light stage although there was slightly more area F RNA at the nine hour light stage.

Areas roughly corresponding to the six RNA and polyphosphate areas were pooled and analyzed for total-phosphorus, Δ_7-phosphorus, and metachromasy. Aliquots were then dialyzed 24 hours against distilled water and analyzed in the same manner. Ultraviolet spectra were taken of both the dialyzed and non-dialyzed samples. With these data an idea of the dialysis rates and relative amounts of undenatured complex can be obtained. In Figure 39 the specific metachromasy of

Table XII. Influence of light upon the ratio of total RNA O. D. units to mgm. total phosphorus (one RNA O. D. unit = one ml. of a solution with an O. D. @ 260 mu of 1.0)

Fig.	organism	conditions	RNA units/10 ml. p.c.v.	mgm. tot.-P/ 10 ml. p.c.v.	RNA units / mgm. tot. -P
8	Anabaena	3.6 ml. p.c.v./l.	13.9	1.82	7.7
9	"	7.2 ml. p.c.v./l.	10.4	0.51	20.4
10	"	8.0 ml. p.c.v./l.	10.3	0.59	17.6
11	"	11.2 ml. p.c.v./l.	5.1	0.31	16.5
12	"	13.0 ml. p.c.v./l.	4.6	0.23	20.4
13	"	25.0 ml. p.c.v./l.	3.0	0.14	21.0
14	"	29.6 ml. p.c.v./l.	7.7	0.13	58.0
16	Chlorella	synchron. 0 hr.light	75.3	3.29	22.9
17	"	synchron. 3 hr.light	98.0	5.11	19.2
18	"	synchron. 6 hr.light	172.3	6.32	27.3
19	"	synchron. 9 hr.light	220.5	11.12	19.8
20	"	synchron. 12 hr.light	59.9	3.25	18.4
21	"	synchron. 15 hr.light	97.0	4.82	20.1
22	"	synchron. 18 hr.light	71.4	3.73	19.2
26	"	synchron. 21 hr.light	99.0	5.32	18.6
23	"	synchron. 3 hr.dark	109.3	3.95	27.7
24	"	synchron. 6 hr.dark	163.2	4.94	33.0
25	"	synchron. 9 hr.dark	82.5	3.05	27.0
27	"	synchron. + chloramphenicol	177.0	4.48	39.5
29	"	random,cont. high light	37.6	3.96	9.5
30	"	random,cont. low light	268.0	7.89	34.0

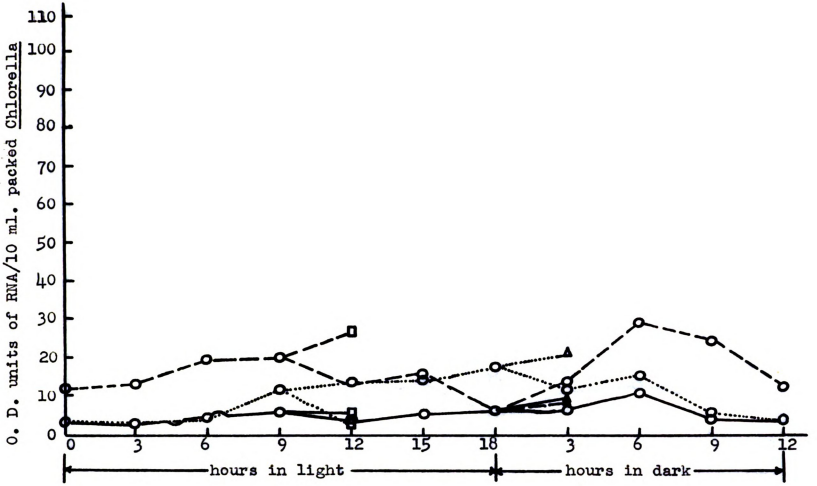

Figure 31, changes in Chlorella RNA during synchronous growth (area A = dotted line, area B = broken line, area C = solid line, ◻ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

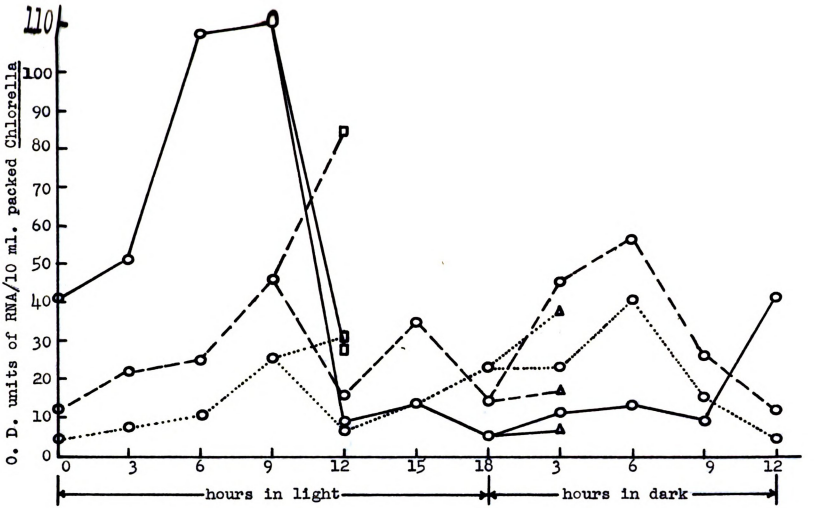

Figure 32, changes in Chlorella RNA during synchronous growth (area D = dotted line, area E = broken line, area F = solid line, ▯ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

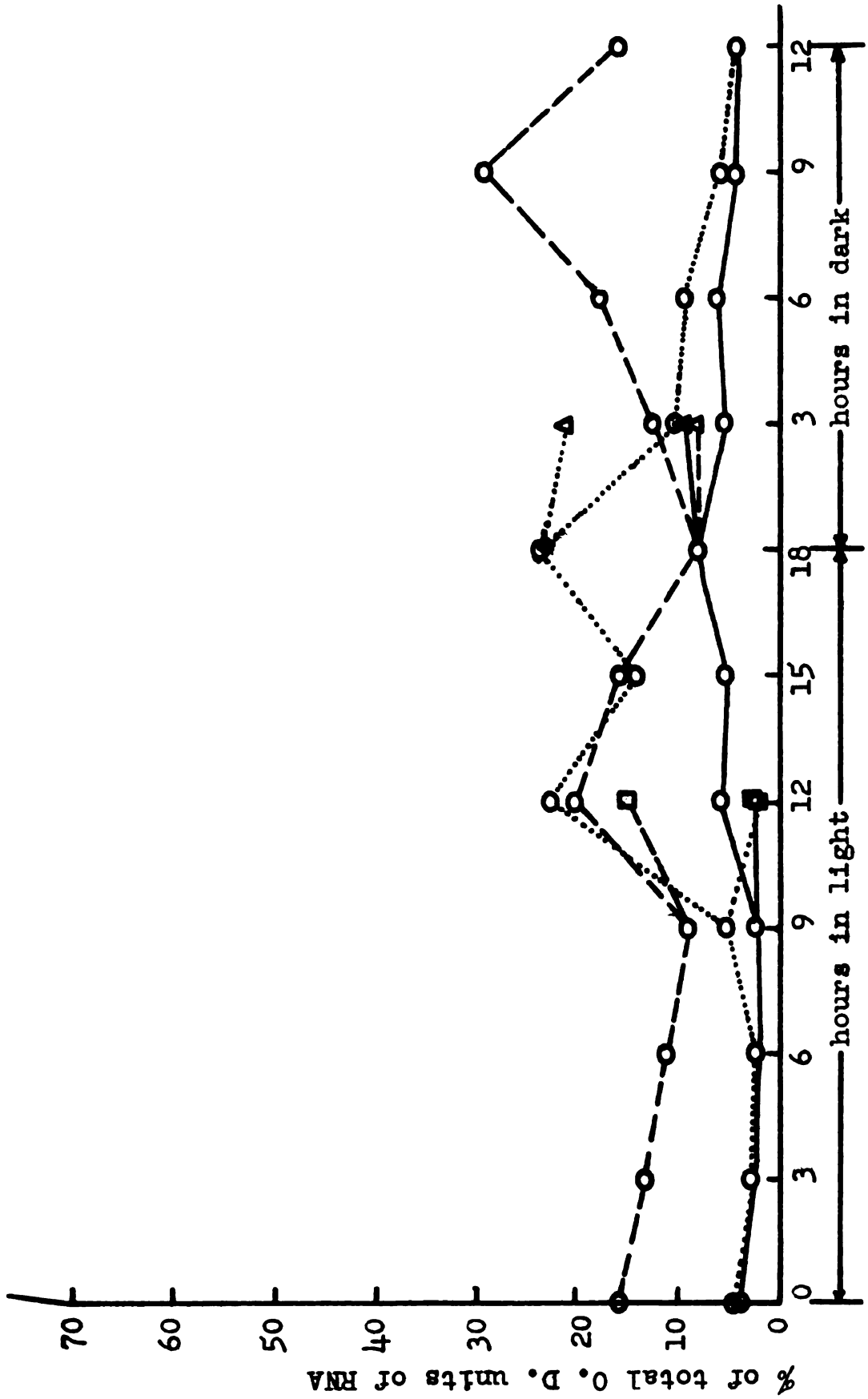

Figure 33, changes in Chlorella RNA during the synchronous growth (area A = dotted line, area B = broken line, area C = solid line, □ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

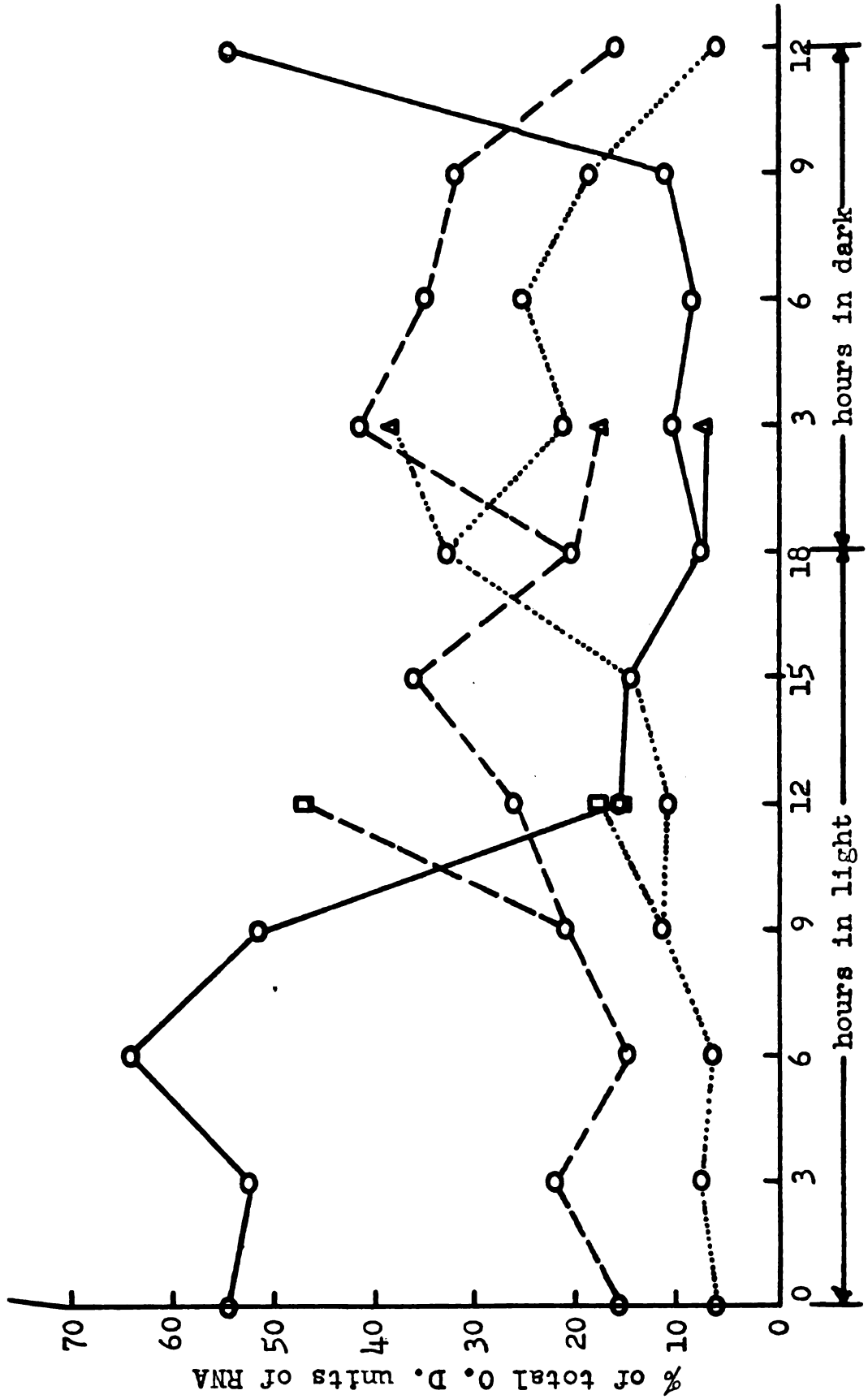

Figure 34, changes in Chlorella RNA during synchronous growth (area D = dotted line, area E = broken line, area F = solid line, ◘ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

areas C, D, E, and F, after dialysis, are given. Areas A and B never exhibited significant metachromatic reactions. The very high specific metachromasies of areas D, E, and F will be discussed in a later section.

In Figure 40 the changes in specific metachromasy upon dialysis are given. In this work specific metachromasy was calculated by dividing the metachromasy by the $\Delta 7$-phosphorus value. A change of 0 percent should be interpreted as a sample which was either all free polyphosphate, denatured complex, or a mixture of these. An example is area E at the 12 hour light stage. A 100 percent change represents a sample which was originally almost entirely composed of undenatured complex. Areas D and E are especially interesting. Both of these areas were primarily composed of undenatured complex between 0 and 9 hours light. Between 9 and 12 hours light they became denatured or released the polyphosphate as free polyphosphate, but when chloramphenicol was added at the 9 hour light stage they remained undenatured at 12 hours of light. It should be remembered that the amount of RNA in areas D and E also increased under the influence of chloramphenicol (Figures 32 and 34), but that the amount of phosphorus in these two areas was decreased at the same rate whether chloramphenicol was added or not (Figure 36).

Figures 41 and 42 show the changes in optical density at 260 mu as percent loss upon dialysis. These data must be interpreted only with the greatest of care, since these

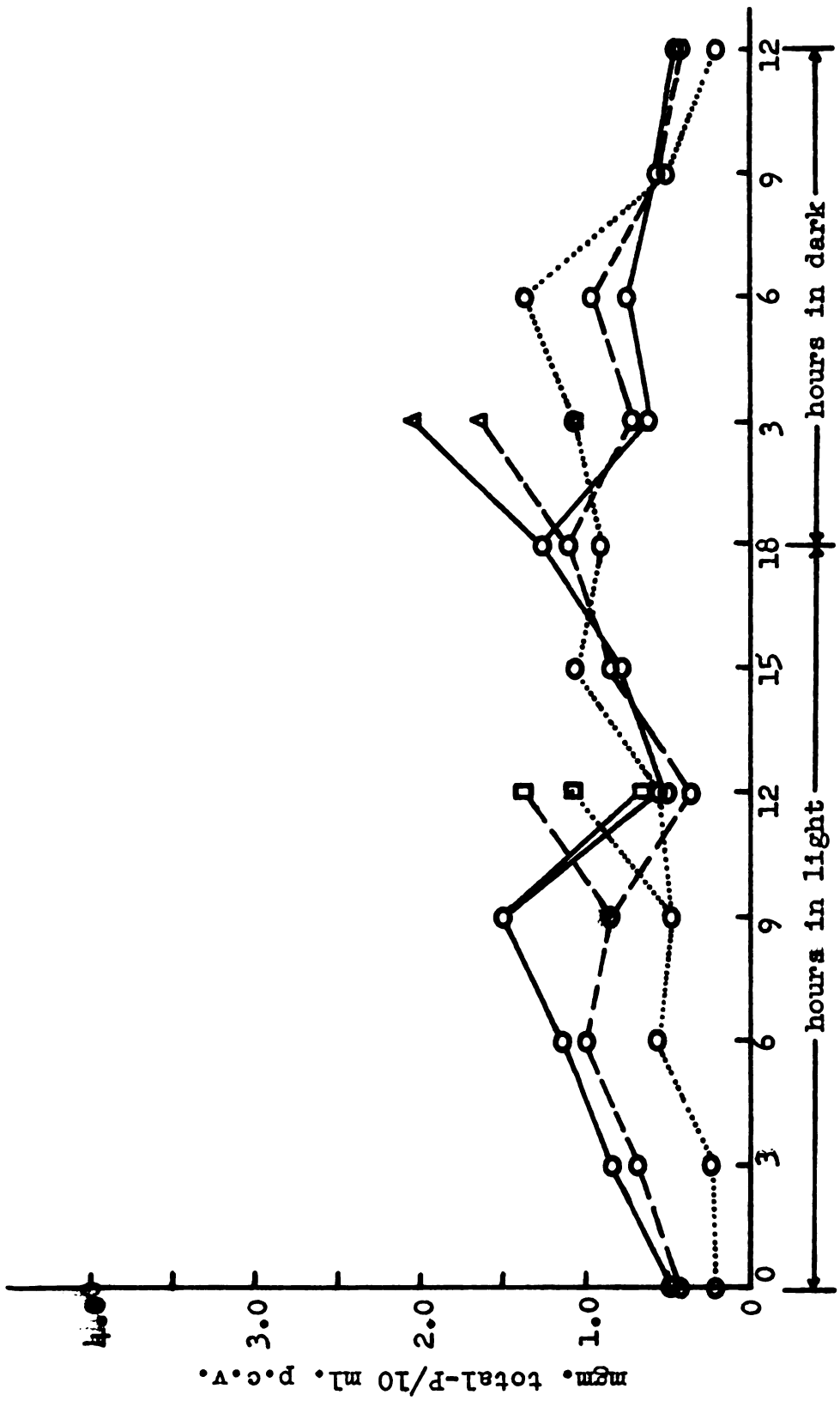

Figure 35, changes in Chlorella polyphosphate during synchronous growth (area I = dotted line, area II = broken line, area III = solid line, □ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

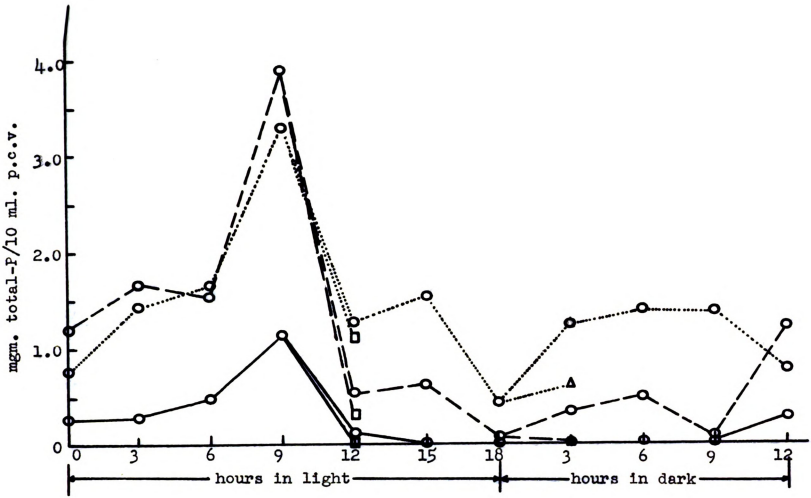

Figure 36, changes in Chlorella polyphosphate during synchronous growth (area IV = dotted line, area V = broken line, area VI = solid line, ▯ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

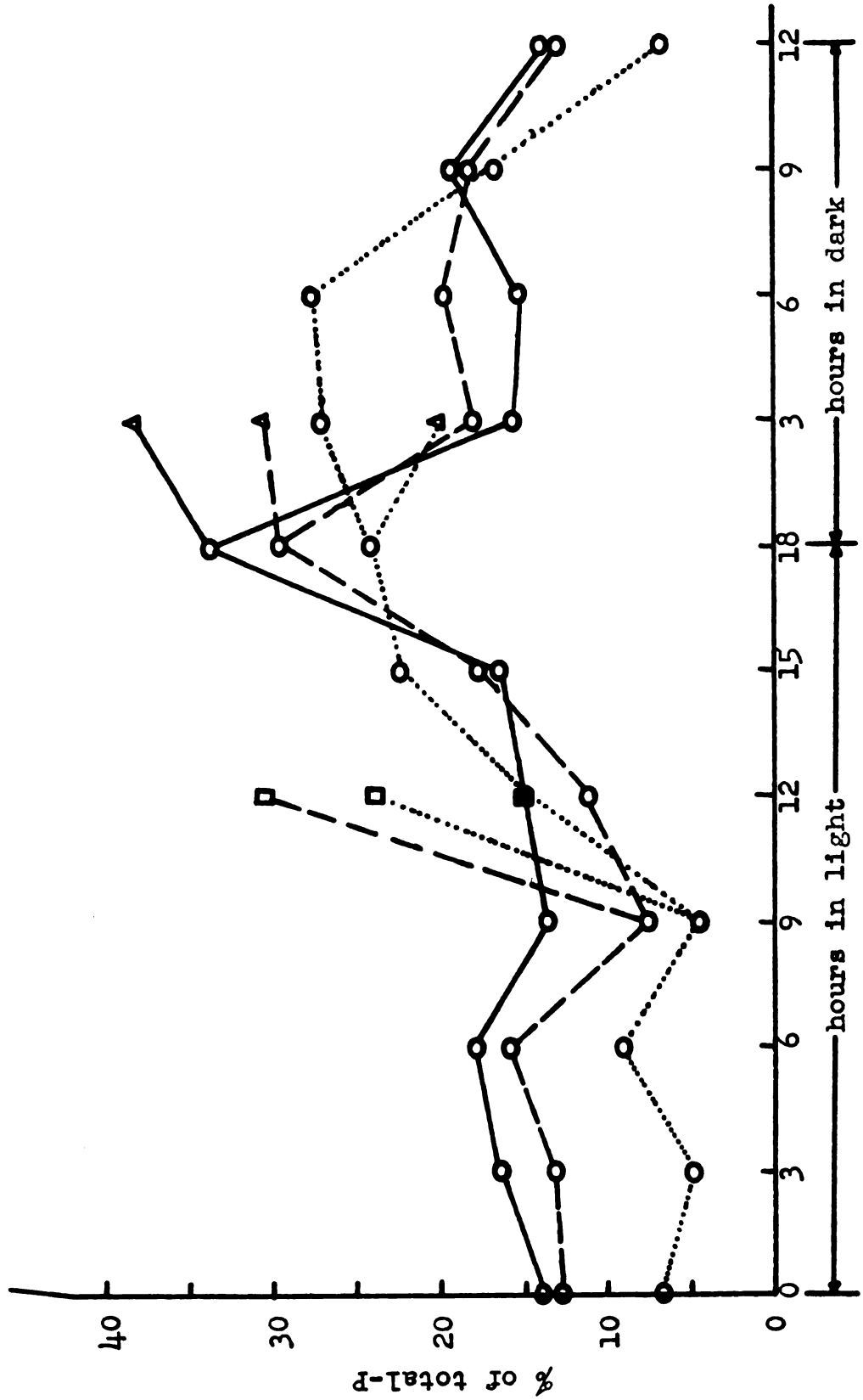

Figure 37, changes in Chlorella polyphosphate during synchronous growth (area I = dotted line, area II = broken line, area III = solid line, ▯ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

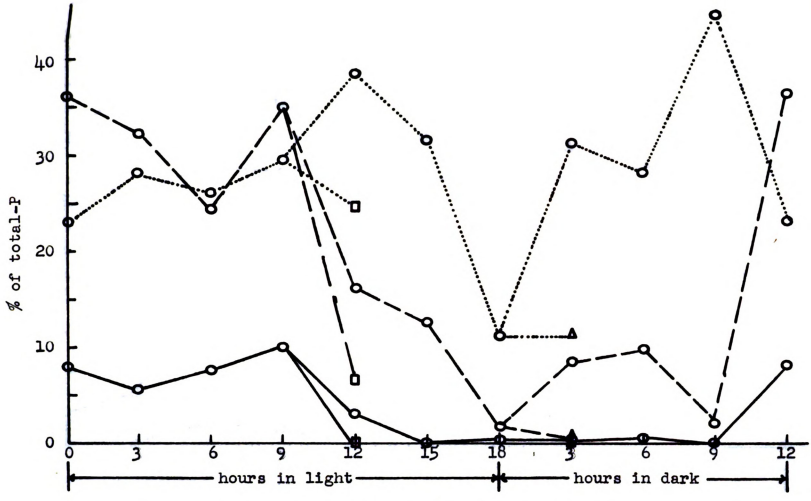

Figure 38, changes in Chlorella polyphosphate during synchronous growth (area IV = dotted line, area V = broken line, area VI = solid line, ▫ = chloramphenicol added at 9 hours in light, ▲ = 21 hours in light)

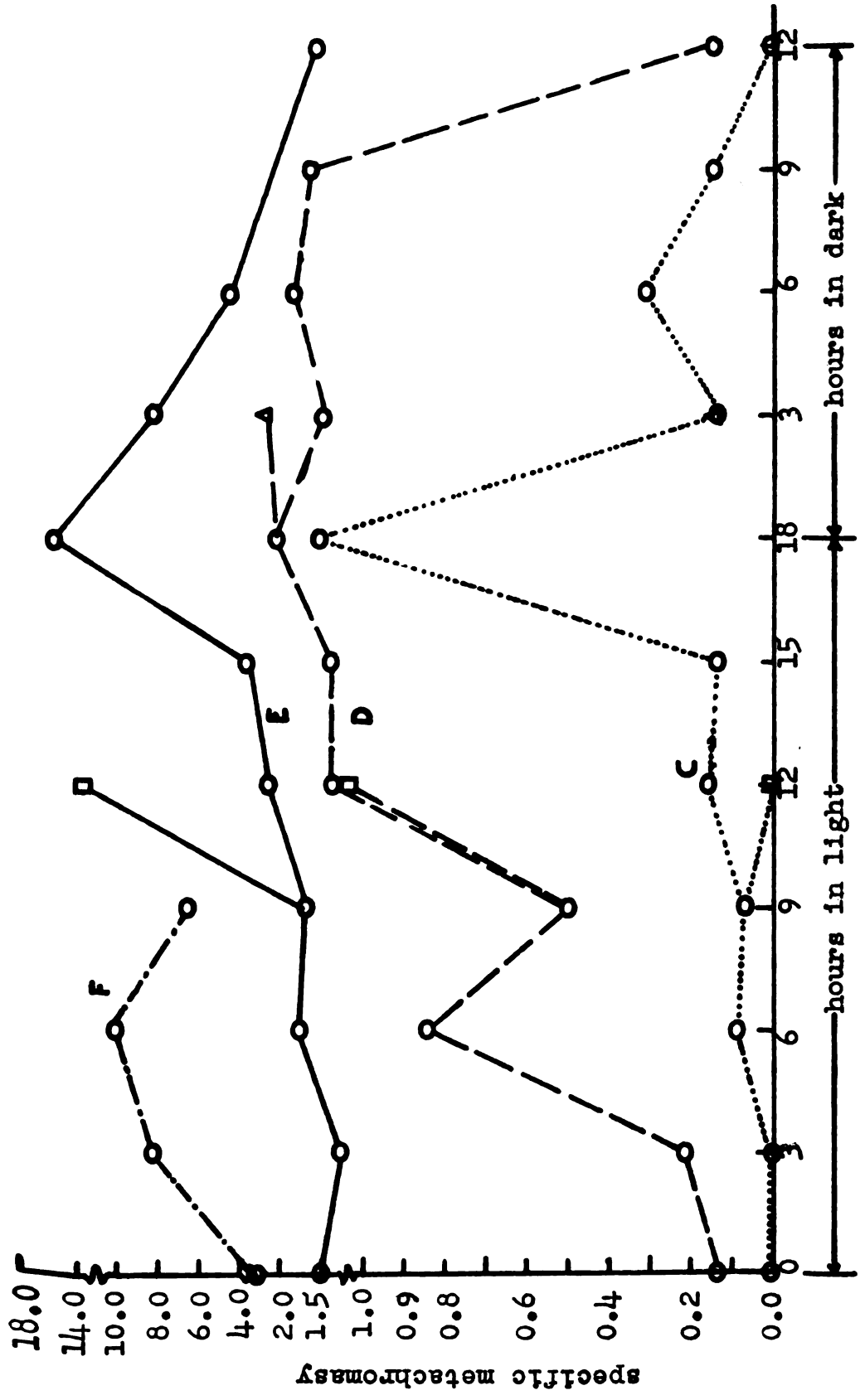

Figure 39, specific metachromasy of Chlorella RNA-polyphosphate complexes after dialysis against distilled water (area C = dotted line, area D = broken line, area E = solid line, area F = broken-dotted line, ▫ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

changes may be the reflection of the hyperchromic effects of dialysis, particularly for those points that show negative losses. They may also be a reflection of the degree of denaturation rather than the molecular size as will be demonstrated in a later section. Although care was taken in filling and draining the dialysis tubing, a small amount of dilution undoubtedly took place in the process. It is interesting to note, however, that some dialysis of RNA took place at some stage of the life cycle in each area with the possible exception of area F. Likewise some hyperchromicity was also demonstrated for all areas except D and perhaps F. If the maximum change during dialysis is noted for each area, the percent of dialysis will be found to decrease from area A to area F in sequence. This is in agreement with the literature reports that the molarity of elution of the nucleic acids are roughly correlated with their molecular size in modified-cellulose ion-exchange chromatography (12, 134).

In Figures 43 and 44 the percent loss in acid-labile phosphorus upon dialysis is given for the six areas during the life cycle. Some omissions were due to an insufficient amount of polyphosphate to carry out accurate experiments. One of the interesting facets of these data is the fact that, although neither area A nor area B ever showed significant metachromasy before or after dialysis, they never lost all of their Δ_7-phosphorus upon dialysis and at times they did not lose any. These facts are contradictory at first sight, since short polyphosphate chains dialyze completely

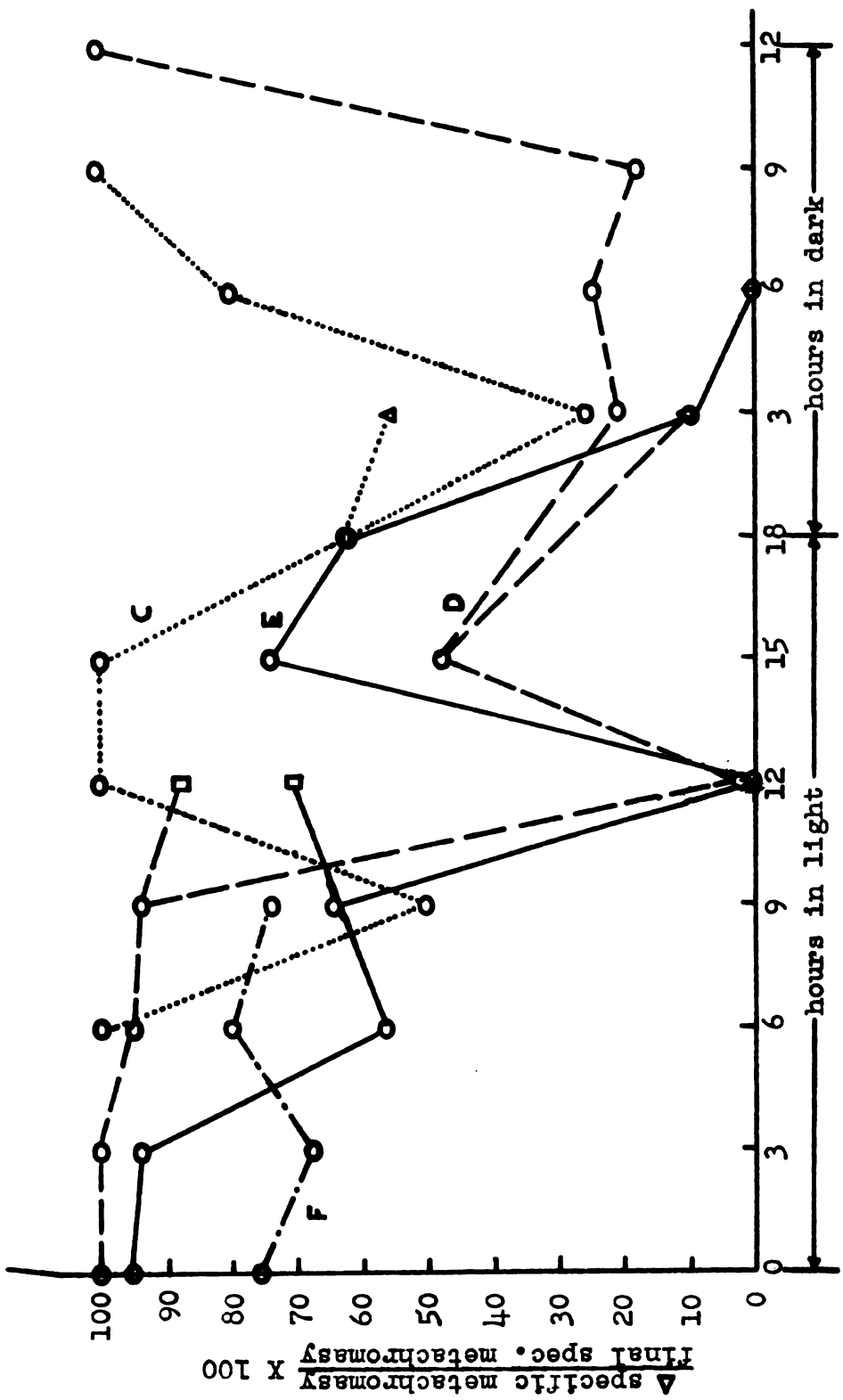

Figure 40, hypermetachromasity of Chlorella RNA-polyphosphate complexes upon dialysis against distilled water (area C = dotted line, area D = broken line, area E = solid line, area F = broken-dotted line, ■ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

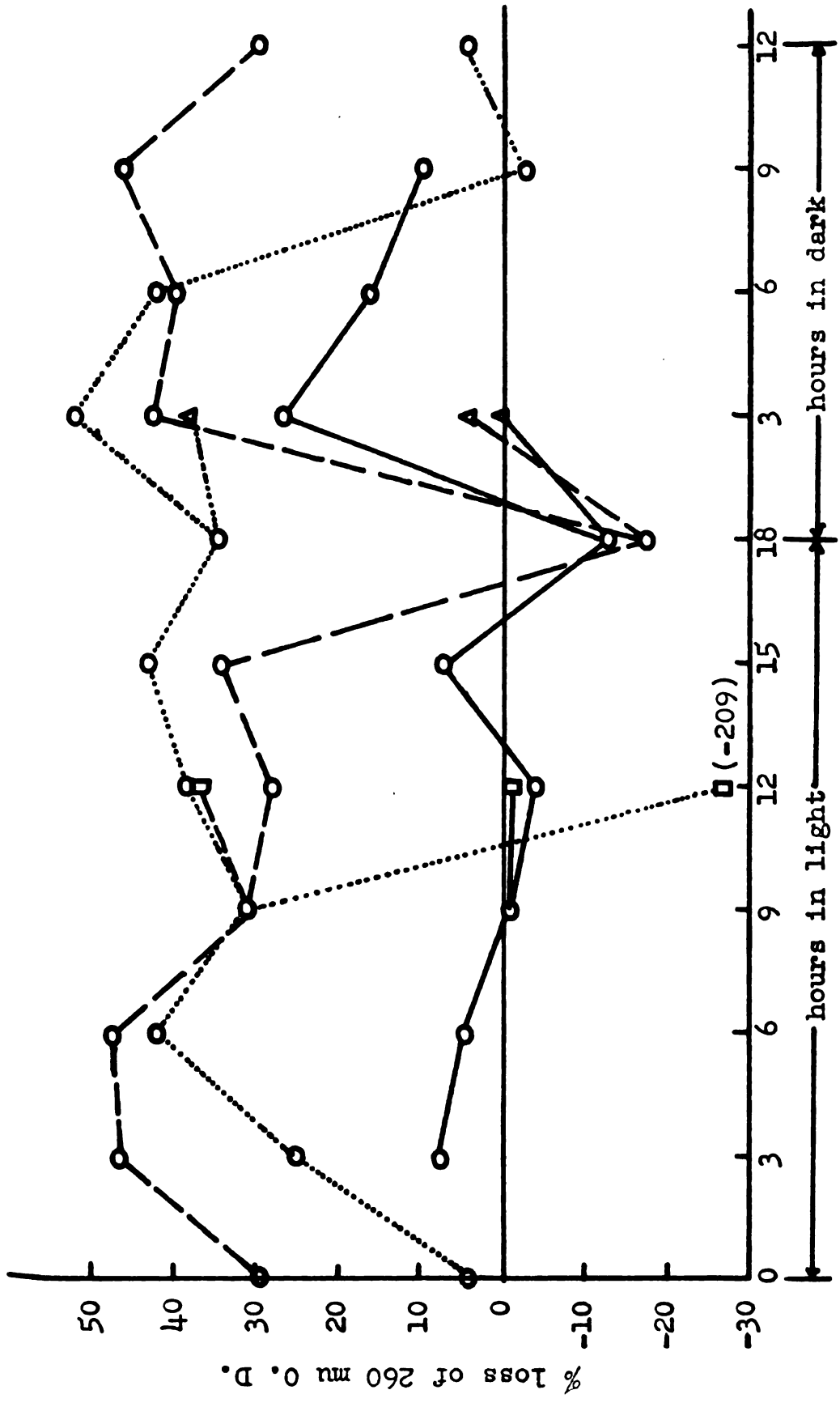

Figure 41, loss of Chlorella RNA upon dialysis against distilled water (area A = dotted line, area B = broken line, area C = solid line, □ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

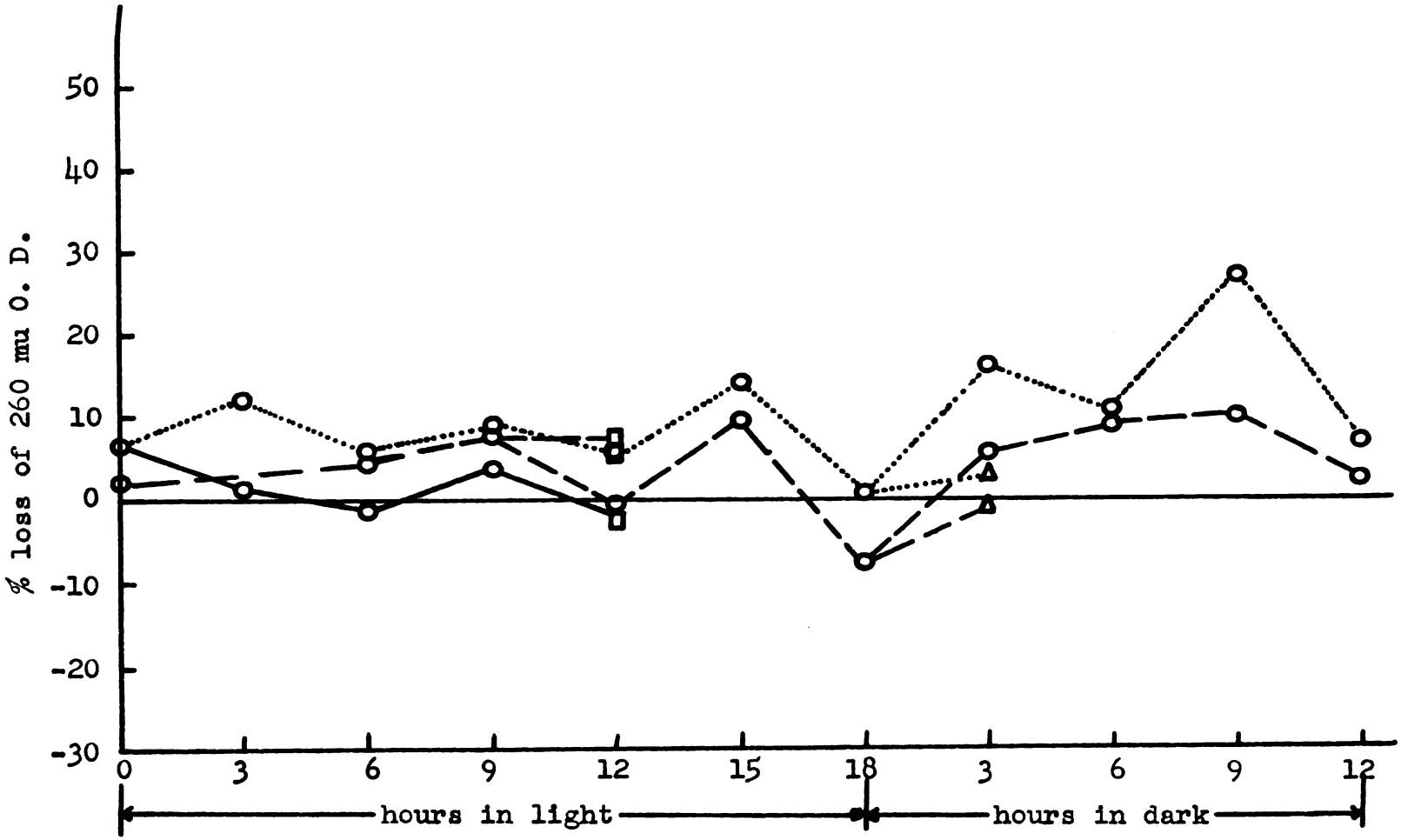

Figure 42, loss of Chlorella RNA upon dialysis against distilled water (area D = dotted line, area E = broken line, area F = solid line, ▯ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

under these conditions (23) and long chains give a strong metachromatic reaction. The most logical solution to this problem, it seems, is to assume that the polyphosphate was in the form of short chains, which give no metachromatic reaction, and that these chains were covalently bonded to RNA so that they would not dialyze. No previous reports of such complexes involving low molecular weight polyphosphates exist.

Some Properties of Various Isolated Fractions of RNA-Polyphosphate

changes in chromatographic patterns after dialysis

Figure 45 shows the elution pattern obtained when a sample of the complexes which elute at low ionic strength was dialyzed and then rechromatographed on a DEAE-cellulose column. The peaks were not shifted very far and, although there was a loss of 30 percent of the total phosphorus and 40 percent of the 260 mu O. D. upon dialysis, the distribution of Δ7-phosphorus and RNA-phosphorus was about the same in the pooled areas of the original column (Figure 29) and the column in Figure 45. Other than the losses due to dialysis there seemed to be little effect.

An aliquot of the pooled complex from the original column (Figure 29) was tested for an increase in metachromasy after freezing, five minutes of boiling, or 60 minutes of incubation with RNase. Although a very small amount of metachromasy could be detected in the original complex, no

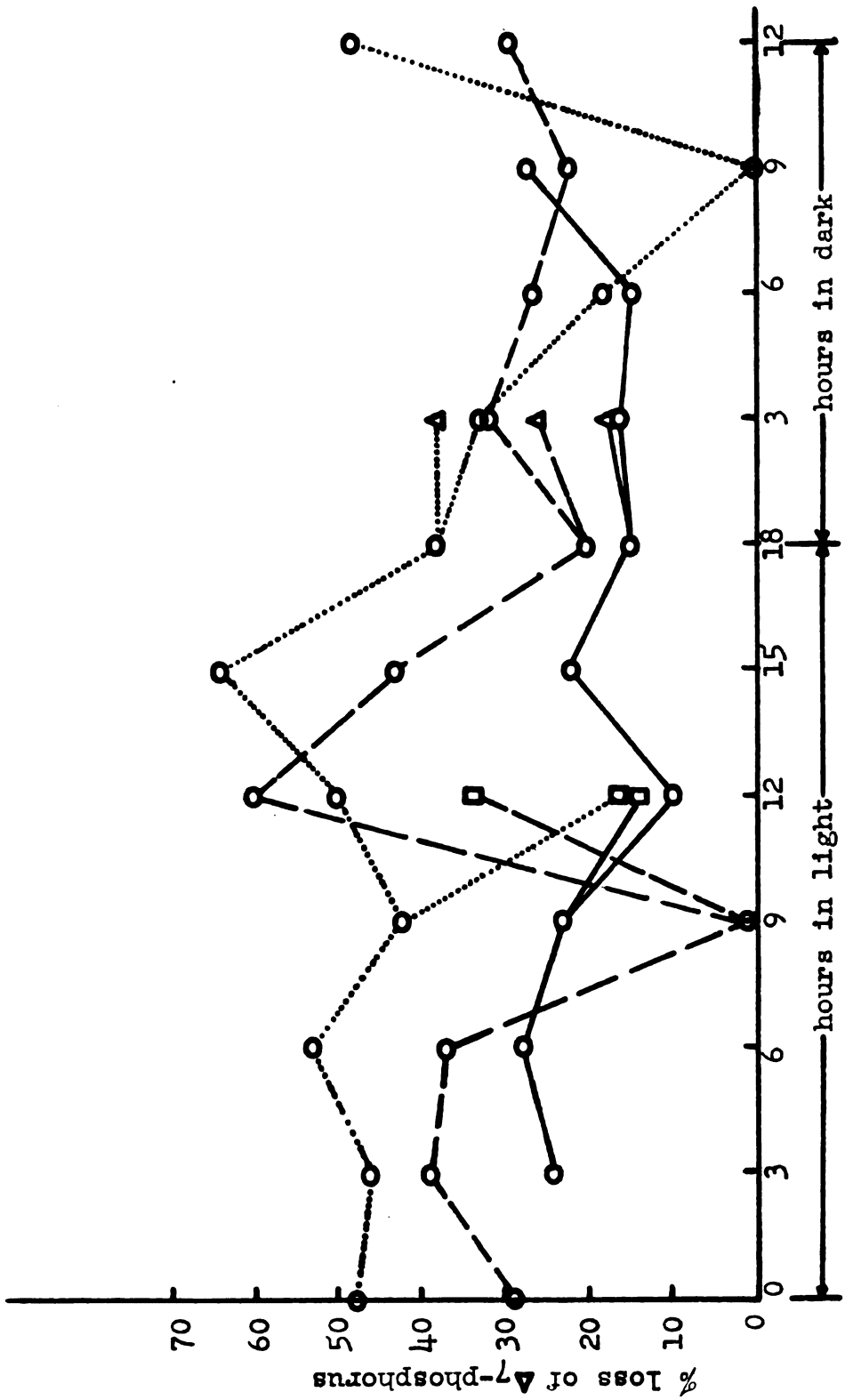

Figure 43, loss of Chlorella polyphosphate upon dialysis against distilled water (area A = dotted line, area B = broken line, area C = solid line, □ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

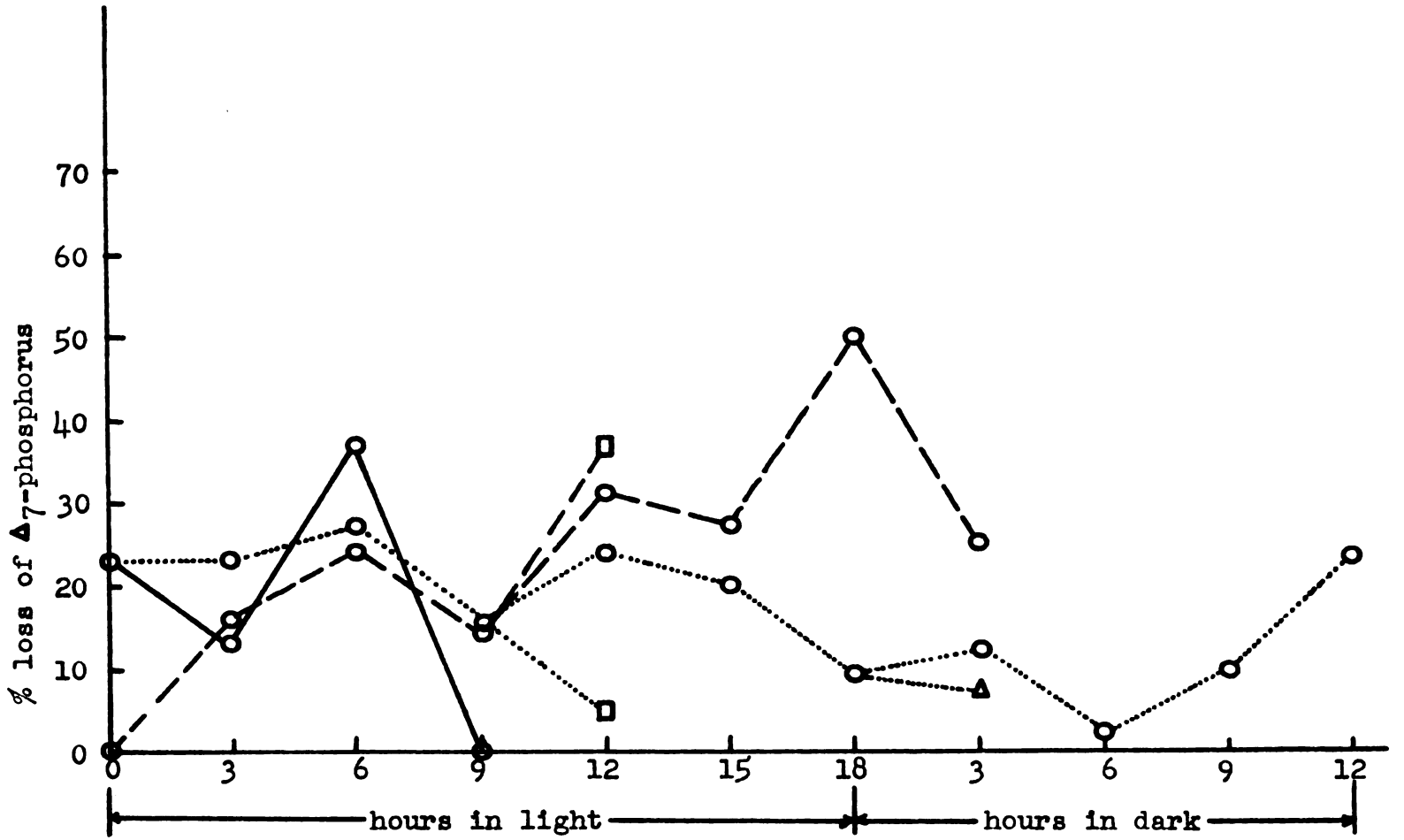

Figure 44, loss of Chlorella polyphosphate upon dialysis against distilled water (area D = dotted line, area E = broken line, area F = solid line, ◘ = chloramphenicol added at 9 hours in light, Δ = 21 hours in light)

increases could be detected after these treatments.

When area E complex of the relatively undenatured type characteristic of the six and nine hour light stages was dialyzed and rechromatographed on DEAE-cellulose, a considerable change was found. Figure 46 illustrates the new elution pattern and the centers of the original areas occupied by the pooled RNA and polyphosphate are indicated at the top of the figure. A new small peak of free RNA was located at about tube 12. The major changes were a shift of the centers of RNA and polyphosphate to a position 16 tubes earlier in the elution and a considerable sharpening of the "peaks." The fact that the RNA and polyphosphate peaks do not correspond in Figure 46 suggests that the material was not homogeneous and that the complexes which eluted first had a higher percentage of polyphosphate. Metachromasy was tested on tubes 48, 56, and 62. All three had significant values. The shift in elution position of the polyphosphate in Figure 46 after dialysis suggests that the polyphosphate was connected with RNA either before dialysis, after dialysis or both, since free polyphosphate should have been uneffected by dialysis.

base ratios

An effort was made to determine base ratios in the different elution areas as an independent verification that the various RNA peaks were actually different and not merely polymers of each other. Table XIII gives the base ratios

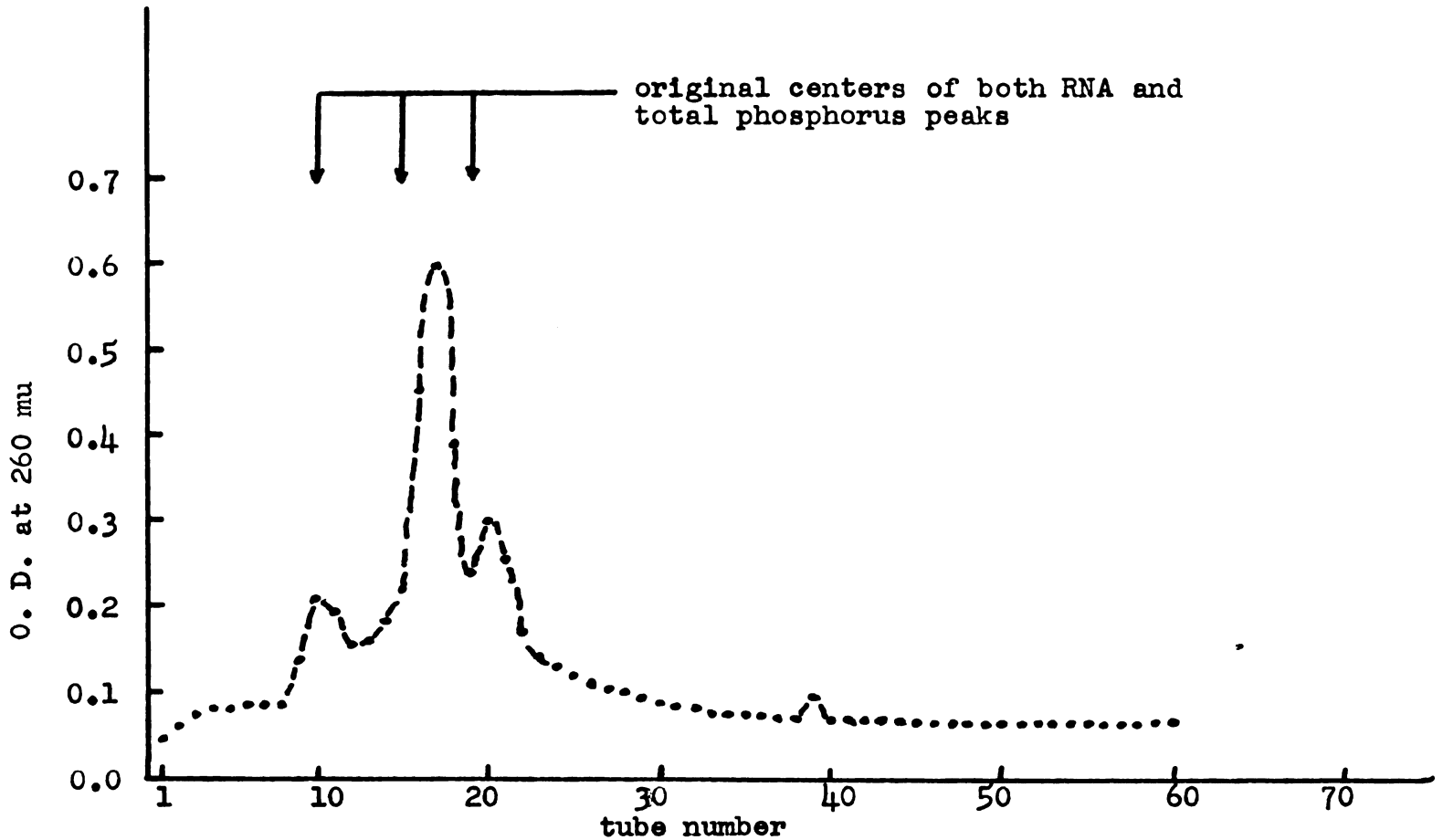

Figure 45, RNA elution curve of dialyzed low molecular weight complexes from Chlorella (a portion of combined tubes 10 through 26 from Fig. 29 after dialysis against distilled water; salt gradient = 0 to 2 M NaCl)

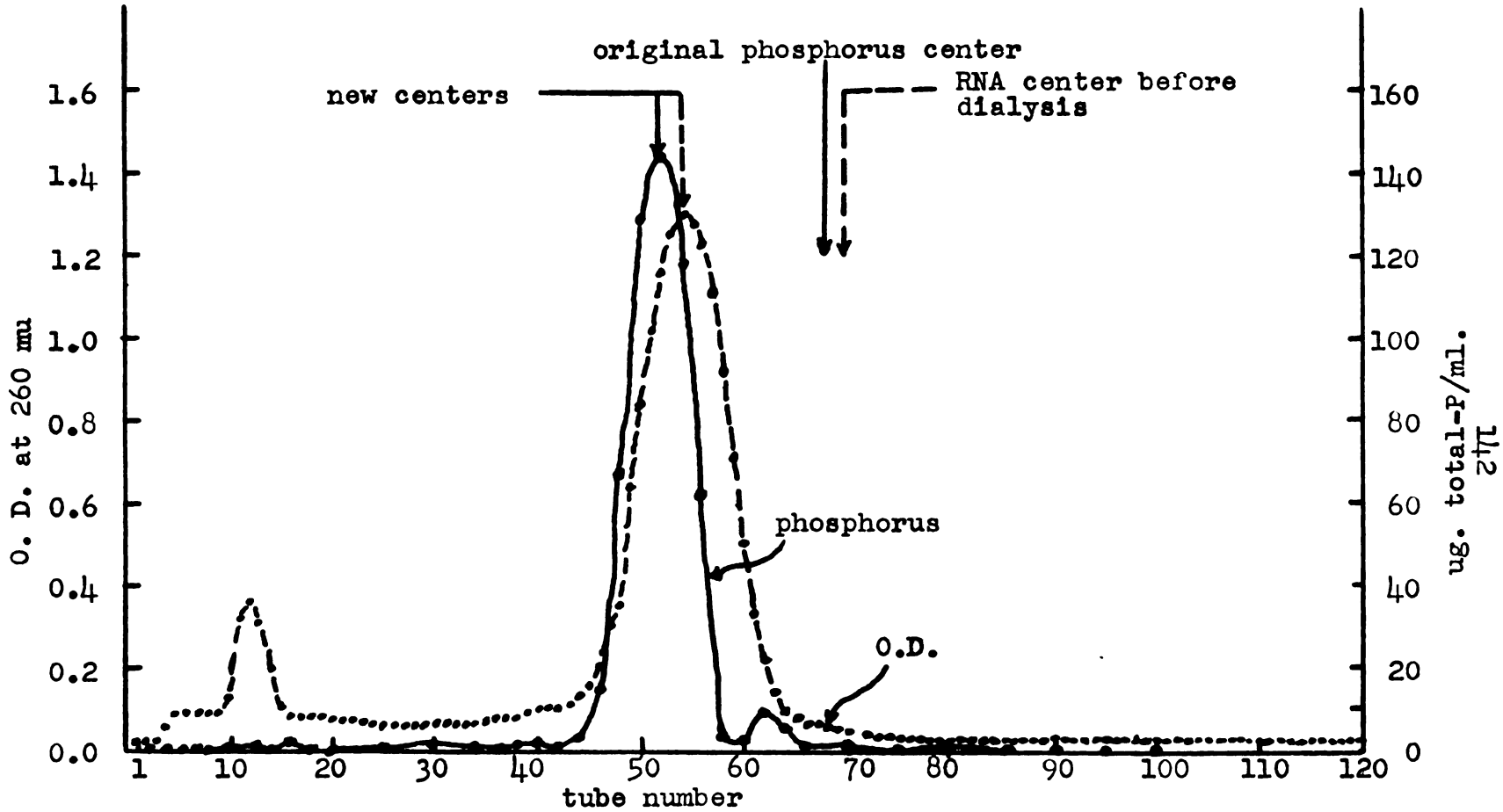

Figure 46, elution pattern of total RNA and phosphorus of Chlorella area E complex after dialysis against distilled water (a portion of area E from Figs. 18 and 19 after dialysis against distilled water)

found in the RNA areas from the nine hour light stage. This stage was ideal for this type experiment since all six areas were clearly present at the same time. It was not determined whether the base ratio was the same in these areas at other stages of the life cycle. It is obvious that the base ratios were different in the various areas at this stage of the life cycle.

It would be interesting to determine the base ratios at other stages of the life cycle and perhaps, thereby construct some interconversion sequences. Similarly it would be interesting, but laborious, to follow the uptake of P^{32}-phosphate into the various complexes in this system.

ribose and deoxyribose

The pooled areas from the randomly grown Chlorella, (Figure 30), were each analyzed for ribose and deoxyribose. No deoxyribose could be found. The ribose values are given in Table XIV. Calculations were made of the ug. ribose per RNA O. D. unit in areas D and E. These came out 12.9 and 16.9 ug. respectively, and a value of 15 ug. ribose per RNA O. D. unit was arbitrarily taken for approximate calculations. The value of 20 RNA O. D. units per mgm. total-phosphorus has been derived (Table XII). It can then be calculated that for every micromole of ribose in the total system of complexes there were about 16 micromoles of phosphorus of which 15 micromoles would be polyphosphate-phosphorus. This value was about the same for Anabaena in the middle part of

its growth curve and for synchronized Chlorella in the light phase of the life cycle.

Further Characterization of the Complexes

Figure 47 illustrates the infrared spectrum of material from area D of the nine hour light stage, before and after dialysis against distilled water. The band at 7.7 to 8.0 u is characteristic of linear polyphosphates (26). The only qualitative changes evident upon dialysis were in the region from 12.2 to 13.0 u. A small peak appeared at about 12.6 u in the dialyzed material. This region is characteristic of the molecule as a whole rather than of its bonds.

The area E complex from the random Chlorella shown in Figure 30 was used to study the effects of a number of treatments on its metachromasy. The results are given in Table XV.

Since dialysis against distilled water seemed to be the most effective method of releasing metachromasy, a study was carried out to see what was responsible for this change. Two possibilities seemed likely. One was the possibility that the pH of the distilled water was low enough to cause a selective hydrolysis and the other possibility was that the low ionic strength, which is known to cause the cleavage of such hydrogen-bond systems as involved in the DNA double helix, might be causing the change. By dialyzing samples of areas D and E against buffers of known pH and NaCl concentration these two possibilities were tested. Figure 48

Table XIII. Base composition of the RNA in the pooled areas from the nine hour light stage of Chlorella

area	mole percent			
	adenine	guanine	CMP	UMP
A	35.3	14.1	10.8	39.9
B	45.6	22.4	7.9	24.1
C	18.3	27.8	9.9	44.0
D	26.8	32.9	16.8	23.6
E	26.7	25.9	16.1	31.3
F	34.2	19.8	20.8	25.2

Table XIV. Concentration of ribose in the pooled areas from Figure 30 (random Chlorella)

area	ribose (ug./ml.)
A	4.0
B	22.2
C	19.9
D	8.3
E	14.7
F	63.2

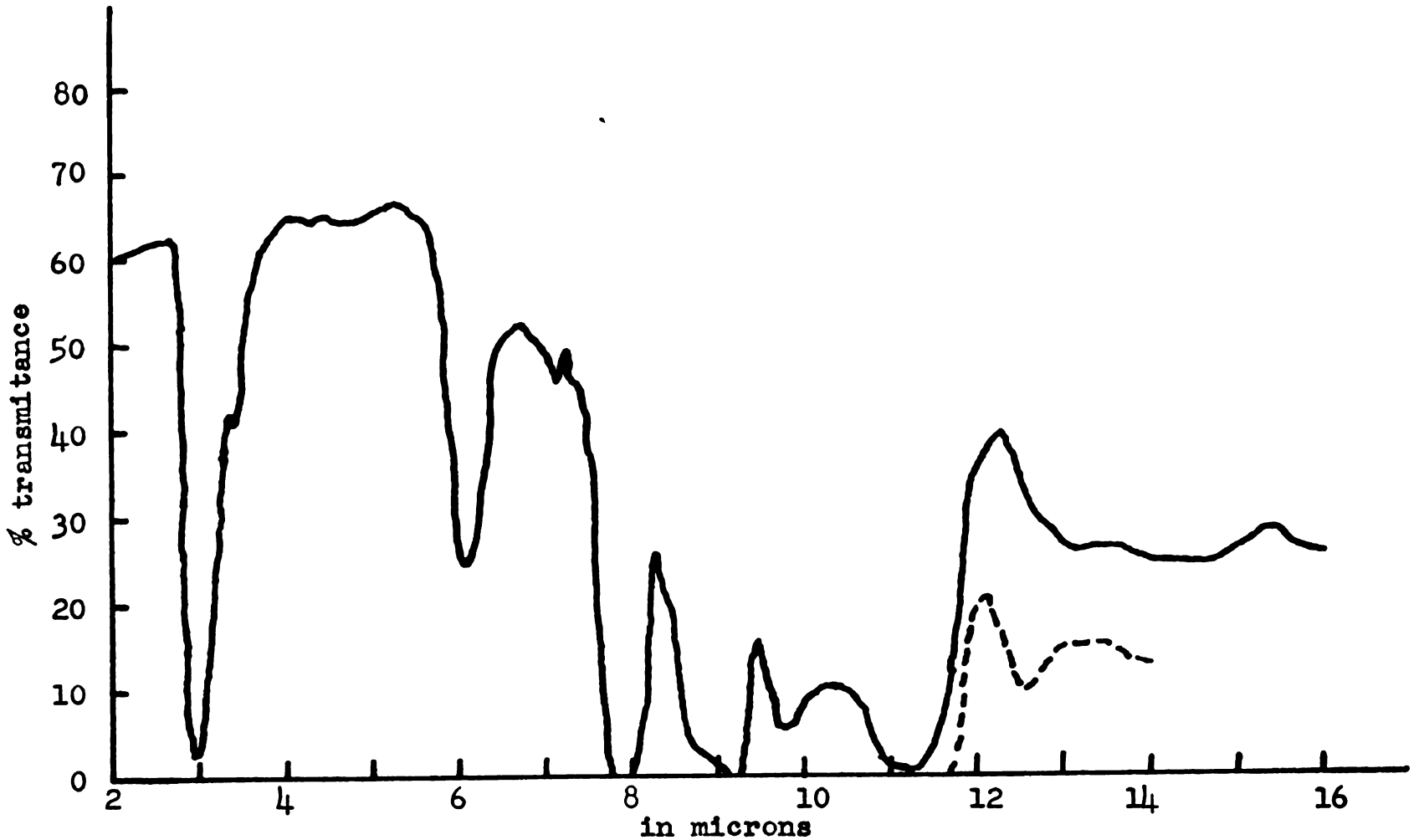

Figure 47, infrared spectrum of area D, 9 hours light stage, synchronized Chlorella (broken line = qualitative spectrum after dialysis against distilled water)

shows the effect of pH at two concentrations of NaCl, and Figure 49 shows the effect of NaCl molarity at pH 8.0. The specific metachromasies are accurate, since Δ_7-phosphorus was determined on each sample after the experiment.

There seemed to be a pH effect at a low salt molarity, but not in 0.1 M NaCl. It was fortunate that the pH used in both the phenol extraction and the DEAE-cellulose chromatography was in the pH region of greatest stability. This complex was apparently stabile in NaCl solutions above 0.2 M if the pH was between 6 and 8. In Figure 49 the percent loss of Δ_7-phosphorus is also plotted. It was inversely related to the amount of denaturation of the complex. The interpretation of these data could be that the dialysis denatured some tertiary structural system in the RNA, resulting in hyperchromicity and exposure of the polyphosphate. The fact that boiling and the alkaline pH utilized in the isolation failed to release metachromasy argues against this interpretation. Another possibility is that the polyphosphate chains were involved in a hydrogen-bond or a salt-bridge system with the nitrogen bases of the RNA at intervals along the polyphosphate chain. Such a system might exhibit the observed properties.

Effects of Adsorption on Charcoal

On the basis of a charcoal separation procedure, Liss (89) believed that polyphosphate and RNA were separate entities. When charcoal, prepared by the method of Liss, was

Table XV. Effects of various treatments on the metachromasy of area E RNA-polyphosphate from random Chlorella (Fig. 30)

treatment	specific metachromasy
none	0.07
24 hrs. dial. against distilled water	1.7
freezing and thawing	0.07
10 min. boiling	0.04
12 hrs. treatment with Na•EDTA	0.07
18 hrs. dial. against 0.01 M acetate, pH 5.5 at 2° C.	1.4
18 hrs. dial. against 0.01 M acetate, pH 6.0 at 2° C.	1.2

Figure 48. Changes in RNA-polyphosphate upon 10 hours dialysis at room temperature (Solid line = 0 hour light sample, area D; specific metachromasy before dial. was 0.07 and after dial. was 1.50; dialyzed against 1 l. 0.01 M tris or acetate buffer. Broken line = 3 hour light sample, area E; specific metachromasy before dial. was 0.09 and after dial. was 1.27; dialyzed against 1 l. 0.01 M tris or acetate buffer with 0.1 M NaCl added.)

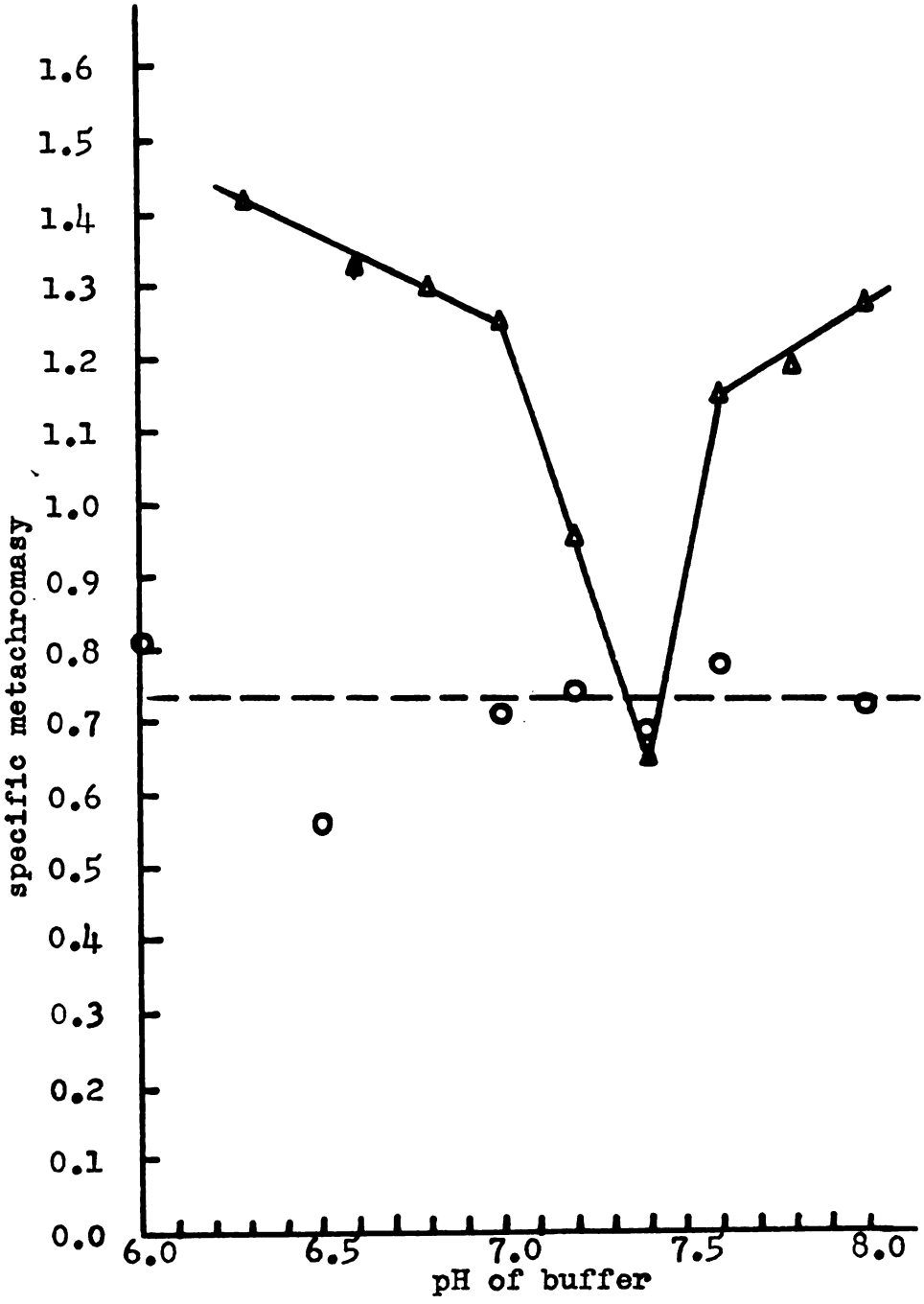
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
specific metachromasy
6.0
6.5
7.0
7.5
8.0
pH of buffer

Figure 48

Figure 49. Changes in RNA-polyphosphate upon dialysis for 16 hours at room temperature against one liter of 0.01 M buffer, pH 8.0 (6 hour light sample, area D; specific metachromasy before dial. was 0.025)

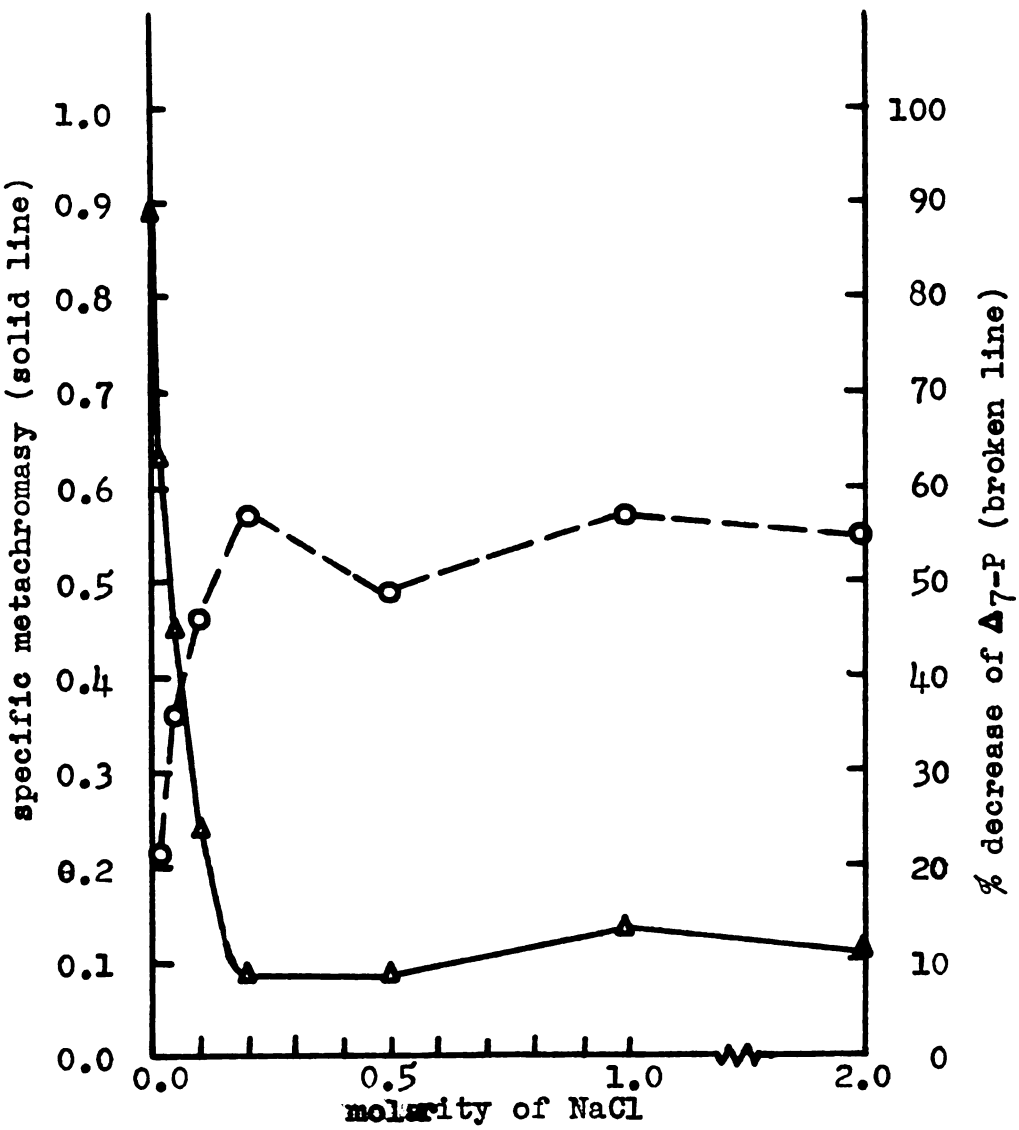
specific metachromasy (solid line)
% decrease of Δ7-P (broken line)
molarity of NaCl
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
2.0

Figure 49

used to attempt the separation of the polyphosphate and the RNA in various complexes the results of Liss could not be duplicated. When ribose was determined before and after adsorption it was found that all but traces of ribose were removed. However, relatively large amounts of Δ_7-phosphorus were sometimes also removed. This loss varied from six to 44 percent of the Δ_7-phosphorus.

Erratic, but large, effects upon the specific metachromasy of the polyphosphate remaining after charcoal adsorption were also noted. In order to resolve this problem two experiments were run. In one, samples of complex from various areas B and D were used, and the results are shown in Table XVI. In the other, synthetic polyphosphate was used and the results are shown in Table XVII. Because the charcoal destroyed the metachromasy, it was apparently catalyzing a slow and probably random cleavage of the free polyphosphate chains. Also a significant percent of the free polyphosphate was adsorbed by the charcoal, thus making any attempts to separate naturally occurring RNA-polyphosphate with charcoal of doubtful value. These facts are important in the interpretation of the data in Table XVI. The data on area D are the most important. The fact that Δ_7-phosphorus was released by the action of charcoal and was then able to dialyze, even though the samples had been dialyzed previously, indicates that the charcoal also catalyzed the cleavage of the polyphosphate in naturally occurring material. This was also indicated by the fact that

the specific metachromasy of the charcoal treated complexes after dialysis was still below that of the dialyzed complex which hadn't been treated with charcoal. In the case of the three and six hour dark samples of area D, (Figures 23 and 24), no specific metachromasy changes were obtained. These samples also showed very little increase in specific metachromasy when dialyzed (Figure 40). Thus they were probably composed primarily of free polyphosphate of considerable length. Quite a few random cleavages of a long polymer would be needed to lower the specific metachromasy very much.

Hyperchromicity of RNA-Polyphosphate

Some experiments were carried out to determine whether a difference could be detected between the hyperchromicity of native complexes and denatured complexes, when they were boiled and then cooled rapidly, (Table XVIII). Areas D and E from the 6 hour dark Chlorella showed very little hyperchromicity. This corresponds with the fact that these areas showed very little increased metachromasy upon dialysis, (Figure 40). In the case of areas A, C, and D from Figure 27, significant amounts of hyperchromicity were observed. Dialyzed aliquots of these areas showed consistently lower but still significant amounts of hyperchromicity.

Enzymatic Studies

characteristics of the yeast polyphosphatase complex

This enzyme complex was remarkably stabile at room temperature and seemed to be made up mostly of pyrophosphatase

Table XVI. Effects of charcoal and dialysis upon dialyzed and undialyzed areas B and D of Chlorella RNA-polyphosphate

material	charcoal treatment before dialysis			dialysis after charcoal treatment	
		spec. metachromasy			
	% loss Δ7-P	before charcoal	after charcoal	total % loss of Δ7-P	specific metachromasy
Fig. 27, area B	27	0.06	0.00	49	0.00
Fig. 27, dial. area B + 1 % NaCl	31	0.00	0.00	62	0.00
Fig. 27, area D	18	0.14	0.15	32	0.96
Fig. 27, dial. area D + 1 % NaCl	37	1.16	0.20	47	0.89
Fig. 23, area D	18	1.14	1.17	-	-
Fig. 24, area D	13	1.35	1.38	-	-

Table XVII. The effects of charcoal upon synthetic polyphosphate in a one percent NaCl solution

percent loss of Δ_7-P	35.0
specific metachromasy before treatment	1.28
specific metachromasy after treatment	0.26

and a highmolecular weight random polyphosphatase. That other polyphosphatases existed in the complex was illustrated by the activity on tripolyphosphate, but this activity was relatively low. When measured by orthophosphate release the action of the enzyme complex was very slow on synthetic polymers with an average length of 16 phosphates and not very much faster on tripolyphosphate, (Table XIX). This low activity was not increased when the enzyme was added in portions during the incubation and the activity was inhibited by orthophosphate. The fact that a very active, random cleavage polyphosphatase was present is clear, when the data in Table XX are considered. Decrease in metachromasy is a function of polymer length and three to six minutes was sufficient to reduce the metachromasy of the synthetic polyphosphate to 30 percent of the original level. This enzyme complex had no activity against AMP or the triphosphate chain in ATP.

action of yeast polyphosphatase on RNA-polyphosphate

When the same yeast polyphosphatase preparation was incubated with RNA-polyphosphate from area D of Figure 27 for ten minutes, the specific metachromasy decreased from 0.14 to 0.09. Then an aliquot of the enzyme treated material was dialyzed against distilled water for 24 hours. A decrease of 23 percent in Δ_7-phosphorus was observed and the specific metachromasy increased to 0.87. These values are to be compared to a loss of only four percent of the Δ_7-phosphorus and a specific metachromasy of 1.16 upon dialysis

Table XVIII. The hyperchromic effects of boiling Chlorella RNA-polyphosphate for ten minutes, followed by rapid cooling

sample	260 mu O. D.		percent hyperchromicity
	before ▲	after ▲	
6 hr. dark, area D	0.377	0.380	0.8
6 hr. dark, area E	0.506	0.516	2.0
Fig. 27, area A	0.127	0.147	15.8
Fig. 27, dial. area A	0.236	0.252	6.8
Fig. 27, area C	0.237	0.262	10.5
Fig. 27, dial. area C	0.236	0.253	7.2
Fig. 27, area D	0.673	0.759	12.8
Fig. 27, dial. area D	0.639	0.678	6.1

Table XIX. Release of orthophosphate by the action of the yeast polyphosphatase complex on known substrates

assay: 2 ml. substrate, 0.1 ml. of 0.015 M $MgCl_2$, and 0.4 ml. of enzyme preparation; incubate at 30° C. Final concentrations of total-P and ortho-P were determined; all substrates adjusted to pH 7.6.

substrate	incubation time	ug. ortho-P/ml. minus endogenous	ug. total-P/ml.
pyro-P	30 min.	426	770
tripoly-P	30 min.	74	1100
"	10 hours	297	"
"	20 hours	400	"
"	36 hours	440	"
"	60 hours	480	"
synthetic poly-P	1 min.	23	1025
" "	5 min.	44	"
" "	15 min.	45	"
" "	30 min.	52	"
" "	1 hour	54	"
" "	1 hour *	47	"
" "	10 hours	80	"
" "	20 hours	95	"
" "	36 hours	105	"
" "	60 hours	130	"
" "	84 hours	140	"
synthetic poly-P **	30 min.	0	560
ATP	30 min.	0	550
AMP	30 min.	1	650

* 0.1 ml. enzyme added at 0, 15, 30, and 45 minutes
** 50 ug. ortho-P/ml. added to substrate before the enzyme

of untreated area D. When the enzyme-treated and dialyzed area D sample was then subjected to polyphosphatase action again, the specific metachromasy declined rapidly to 0.37 after three minutes and 0.31 after six minutes. This indicates that the random cleavage polyphosphatases had low activity on "native" RNA-polyphosphate, but were effective on dialyzed RNA-polyphosphate. In the latter case the polyphosphate chains would have been released from their hydrogen-bonding to RNA and would have been subject to the attack of the enzyme.

action of RNase and snake venom phosphodiesterase on RNA-polyphosphate

As discussed in previous sections RNase failed to release the metachromatic reaction of undialyzed RNA-polyphosphate complexes and in some cases decreased the metachromasy. It was also observed that neither RNase, isolated snake venom phosphodiesterase, nor lyophilized snake venom had any effect on the metachromasy of synthetic polyphosphate, thus eliminating the possibility that they contain polyphosphatases.

These enzyme preparations were then tested on several RNA-polyphosphate complexes, (Table XXI). In the case of dialyzed area D from Figure 27, after it was incubated with RNase for 20 minutes, and then with snake venom phosphodiesterase for another 20 minutes, an aliquot was dialyzed. After dialysis the specific metachromasy was 0.38 and there was only about a 15 percent loss of Δ_7-phosphorus. Areas D and E from a random Chlorella culture were tested in the same way except

Table XX. Effects of yeast polyphosphatase on the metachromasy of synthetic polyphosphate

assay: 2 ml. substrate, adjusted to pH 7.6, plus 0.2 ml. enzyme preparation; incubated at room temperature

minutes incubation	metachromasy (ug. hexameta-P/ml.)	specific metachromasy
0	13.9	1.0
3	5.1	0.37
6	4.2	0.30

that a solution of lyophylized rattlesnake venom was used instead of purified snake venom phosphodiesterase. In this case very little effect on the metachromasy of area D was found, but the metachromasy of area E was found to be effected in about the same way as area D, Figure 27.

These effects of RNase and snake venom phosphodiesterase on the metachromasy of RNA-polyphosphates are another piece of evidence that a connection exists between the RNA and the polyphosphate. Since after the action of the RNase and snake venom phosphodiesterase or the action of polyphosphatase, dialysis fails to remove very much of the polyphosphate, it seems likely that the polyphosphate chains are covalently bonded to the RNA. One way in which this could occur would be by means of a phosphate ester involving the secondary hydroxyl at the end of the polyphosphate chain and a hydroxyl of the ribose in RNA. Such a structure would be in accord with the observed properties of RNA-polyphosphate complexes. It would account for the very high specific metachromasy observed in dialyzed complexes since the secondary end hydroxyl is inhibitory to the metachromatic reaction. The effects of RNase and snake venom phosphodiesterase could be due to removal of RNA obscuring the surface of the polyphosphate or to cleavage of the RNA chain in such a way that it was more random in arrangement and thus interfered with the metachromatic reaction on the surface of the polyphosphate.

Another possibility is that the snake venom phospho-

Table XXI. Effects of RNase and snake venom phosphodiesterase on the metachromasy of Chlorella RNA-polyphosphate

assay: 2 ml. RNA-polyphosphate solution, ± a few crystals of RNase and a 20 min. incubation, ± 0.2 ml. snake venom phosphodiesterase for a variable number of additional minutes of incubation

substrate	RNase	snake venom P-diesterase	incub. time (min.)	specific metachromasy
6 hr. dark, area C	-	-	0	0.00
6 hr. dark, area C	+	+	20	0.00
area D, Fig. 27	-	-	0	0.14
area D, Fig. 27	+	+	20	0.00
dial. area D, Fig. 27	-	-	0	1.16
" " "	+	+	20	0.42
" " "	-	+	5	0.88
" " "	-	+	10	0.84
" " "	-	+	20	0.76
" " "	+	+	0	0.94
" " "	+	+	5	0.82
" " "	+	+	10	0.75
" " "	+	+	20	0.33
dial. area D, from a random culture	-	-	0	0.63
"	+	+	0	0.55
"	+	+	5	0.66
"	+	+	10	0.61
"	+	+	20	0.66
area D, from a random culture	-	-	0	0.19
"	+	+	20	0.22
area E, from a random culture	-	-	0	0.56
"	+	+	20	0.39
dial. area E, from a random culture	-	-	0	3.1
"	+	+	0	1.6
"	+	+	5	1.1
"	+	+	10	1.2
"	+	+	20	1.1

diesterase cleaved an ester bond between the polyphosphate and the 5' hydroxyl of ribose in the RNA. Such activity is possible, since Razzell (109) found that this enzyme attacks ATP slowly, releasing pyrophosphate, but not orthophosphate. It also attacks di(thymidine-5')-pyrophosphate to yield thymidylic acid at a rate ten times faster than it attacks pTpT.

In a similar manner RNase might hydrolyze an ester between a pyrimidine 3'-hydroxyl and the terminal secondary hydroxyl of a polyphosphate chain, since it is also a phosphodiesterase with specificity only for the pyrimidine 3'-phosphate portion. An example of this type of activity is the action of RNase on the synthetic methyl ester of 3' UMP as reported by Brown (17).

No matter what the mechanism of the effects of RNase and snake venom phosphodiesterase on the metachromasy, the possibility exists that some or all of the polyphosphate involved in the complexes was relatively short in length and gave a high specific metachromasy after dialysis by virtue of the fact that the two ends were esterified to RNA. This possibility is supported by the fact that mild alkaline hydrolysis, which does not hydrolyze polyphosphates, drastically reduces the specific metachromasy of RNA polyphosphates, (Table V). It is also supported by the fact that yeast polyphosphatase reduced the metachromasy of the complex rapidly, but did not release the polyphosphate to dialyze. For example, the metachromasy of dialyzed area D, from

Figure 27 was reduced to 17 percent of its original value within five minutes by the action of yeast polyphosphatase.

CONCLUDING REMARKS

In this section an attempt will be made to postulate an overall picture of polyphosphate metabolism. This picture will be based upon the author's knowledge of the literature and an extrapolation of the data presented in this thesis. As discussed earlier many microorganisms incorporate orthophosphate into high-molecular-weight, organically bound polyphosphate very rapidly. This incorporation apparently takes place by way of ATP. The result is a complex which involves RNA, polyphosphate, and probably other components as well.

This incorporation of orthophosphate into polyphosphate only occurs at one stage of the cell-division cycle. The resulting RNA-polyphosphate has a very high molecular weight and gives no metachromatic reaction. It seems likely that the RNA portion of this complex is synthesized first, and that the polyphosphate is then elaborated by an enzymatic process involving ATP. As this complex is phosphorylated its point of elution from DEAE-cellulose is moved to a lower ionic strength, probably due to changes in the folding and number of exposed charges on the molecule.

The structure, which the author considers most likely to be present in this newly synthesized RNA-polyphosphate complex, is one in which polyphosphate chains of various

lengths form bridges between the ribose moieties within RNA chains. Such bridges could be terminated by an ester bond at each end. This ester bond could be formed by splitting out water between a ribose hydroxyl and the secondary hydroxyl of the terminal phosphate of a polyphosphate chain. These polyphosphate bridges might connect separate RNA chains or form links between nucleotides on the same RNA chain.

In the original "undenatured" RNA-polyphosphate complex these polyphosphate bridges seem to be involved in a hydrogen-bond system with the RNA. This system could be composed of a series of hydrogen-bonds between the phosphoryl-oxygens in the polyphosphate chain and either the amino nitrogens of guanine and cytosine or the nitrogens in the purine and pyrimidine ring systems of the RNA nitrogen bases. Such a hydrogen-bond system might have the unusually stabile properties observed in the isolated RNA-polyphosphate complexes.

No reports have been published which demonstrated a definite metabolic function of the RNA-polyphosphates. The author feels that the most probable metabolic function of the RNA-polyphosphates is protein synthesis. This function has been indicated by several factors. As was discussed earlier, the physiology of the volutin granule plastids in several microorganisms points to their utilization in energy-requiring processes at sites of active growth. Also the kinetics of high-molecular-weight polyphosphate utilization in unicellular organisms has

indicated that they are utilized most rapidly at the time of maximum protein synthesis. In this thesis the author has demonstrated that RNA-polyphosphate complexes are utilized very rapidly by synchronized Chlorella at a time shortly preceding nuclear division.

On theoretical grounds a complex of RNA, which is known to be involved in protein synthesis, and polyphosphate, which contains "high-energy" phosphate anhydride bonds, should be an ideal molecule for protein synthesis. Cytochemical evidence has indicated that the RNA-polyphosphate complex, in vivo, has protein attached. This protein may have a structural function, but it could also have an enzymatic role.

As a result of the metabolic utilization of the "undenatured" RNA-polyphosphate two types of material were produced. One seemed to be composed of RNA units with many short polyphosphate chains covalently attached to each RNA molecule. The other material was composed of an assortment of free polyphosphate molecules of various lengths. The polyphosphates which seemed to be attached to RNA at this time apparently have one end free and are probably esterified to ribose moieties at the other end. It is possible that both this RNA-attached polyphosphate and the free polyphosphate are next converted to ATP by an ADP-polyphosphate-phosphotransferase type of enzyme.

SUMMARY

Evidence was obtained to indicate that polyphosphates constitute about one-third of the total phosphorus in the actively growing Anabaena cell. The incorporation of radioactive phosphate into the polyphosphate of Anabaena was rapid.

A material containing polyphosphate and RNA could be isolated by relatively mild techniques. This material was free from serious contamination by DNA, protein, amino acids, carbohydrates, or other phosphorus-containing compounds. Chromatography on DEAE-cellulose columns resulted in the separation of RNA-polyphosphate fractions which had different base ratios. Changes in the ratio of RNA to polyphosphate were found to occur as Anabaena cultures aged. These changes were interpreted on the basis of increased shading in older cultures and the accumulation of cells at one stage of the life cycle in dense cultures.

In the case of synchronized Chlorella cultures a definite sequence of changes in the fractions of RNA-polyphosphate took place. During the first nine hours of light a build-up of RNA-polyphosphate complexes occurred and these complexes exhibited only trace amounts of metachromasy. Then between nine and 12 hours of light a sudden decrease in the amount of RNA and polyphosphate took place

accompanied by a change in the metachromatic characteristics and dialysis properties of the remaining material. The different fractions of RNA-polyphosphate changed independently during the life cycle. When chloramphenicol was added at the crucial nine hour light stage, the decrease in RNA was less pronounced and several of the remaining RNA-polyphosphate fractions maintained their previous characteristics.

In the dark phase of the synchronization cycle a build-up and decline of RNA took place without a corresponding change in polyphosphate.

The ratio of RNA to polyphosphate of Anabaena in the middle part of the growth curve and of Chlorella in the light phase of the synchronization cycle was constant. Calculations based on this ratio and the micromoles of ribose per unit of RNA resulted in a figure of 15 polyphosphate-phosphate units per RNA-nucleotide unit. This ratio was interpreted as being significant since it was essentially the same during synthesis and utilization of the complexes in both organisms.

The larger complexes found in the 0 to 9 hour light period of the Chlorella synchronization cycle exhibited the following properties:

1. large change in metachromasy upon dialysis against distilled water
2. large change in column chromatography properties upon dialysis against distilled water
3. no release of metachromasy after freezing, boiling, dialysis against 0.2 M NaCl at pHs between 6 and 8, or incubation with EDTA.

4. hyperchromicity upon boiling

5. effects upon the metachromasy of the dialyzed complex by polyphosphatase, RNase, and snake venom phosphodiesterase

The data obtained were interpreted to indicate that these complexes involve a system of hydrogen-bonds or salt-bridges and also a system of covalent bonds.

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