CHANGES IN THE CONTENT OF POLYPHOSPHATES OF *SACCHAROMYCES* CELLS DURING GROWTH AND SPORULATION

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The abundance and molecular size of polyphosphates in yeast are known to be influenced by nutrient conditions, but there has been no previous study of changes in polyphosphate content during the growth and sporulation cycle of yeast.

Experiments were performed to determine the quantitative and qualitative changes which occur during the life cycle of a strain of *Saccharomyces chevalieri* with respect to polyphosphates. The concomitant changes in other phosphorus-containing substances were monitored in an attempt to assess the functional significance of polyphosphates in the overall (phosphorus-) metabolism of the yeast cell. The relationship of the changes in the polyphosphates to the concomitant successive stages of culture growth and spore development is discussed.

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TABLE OF ABBREVIATIONS USED IN THE TEXT

ADP - adenosine diphosphate

ATP - adenosine triphosphate

DNA - deoxyribonucleic acid

PCA - perchloric acid

PP - polyphosphate

TCA - trichloro-acetic acid

RNA - ribonucleic acid

UV - ultra-violet

Fraction 1,2,3,4 refers to the subdivision of the polyphosphates as explained under "Methods".

INTRODUCTION

The presence of polyphosphates in yeast was first reported by Liebermann (1890) and Ascoli (1899). Nucleic acid extracts were shown to contain a component which could be precipitated by addition of barium salts at acid pH. Liebermann identified it as a metaphosphate but assumed it to be a component of the nucleic acids. A more complete analysis which clearly established the separate identities of metaphosphate and the nucleic acids was performed by MacFarlane (1936). She was the first to distinguish two polyphosphate fractions on the basis of difference in extraction behaviour; (1) soluble in cold dilute acid, and (2) insoluble in cold acid but extractable with dilute NaOH (pH 9) at room temperature.

In 1944, Jeener and Brachet, in search of a method for the estimation of (yeast) nucleic acid based on its affinity for basic dyes discovered that yeast cells exposed to orthophosphate after phosphorus starvation showed greatly increased phosphate uptake concomitant with enhanced metachromasy upon staining with toluidine blue. Schmidt et al. (1946) and Wiame (1947a,1947b) found that the accumulated material responsible for the intense staining

reaction was polyphosphate and not, as Jeener and Brachet had originally assumed, RNA. Wiame's finding indicated that "volutin" or "metachromatic granules", long known to be present in a number of micro-organisms, were in fact deposits of polyphosphates. This fact, together with the just emerging recognition of the importance of phosphorus in cell metabolism stimulated research which in the span of about 15 years, demonstrated the presence of polyphosphates in a wide range of organisms. The very extensive literature which accumulated during this time was thoroughly reviewed by Kuhl (1960) and more recently by Harold (1966). Most micro-organisms investigated were found to contain polyphosphate; it has also been found in some mosses (Keck and Stich, 1957) and insects (Heller, et al. 1950; Pierpoint, 1957; Przelecka and Wroniszewska, 1958). Among the higher plants polyphosphates have been detected in spinach (Miyachi, 1961); tomato (Klein, 1952); corn (Vagabov and Kulaev, 1964) and the dodder *Cuscuta reflexa* (Tewari and Singh, 1964). More recently, Grossman and Lang (1962); Penniall and Griffin (1964); Griffin et al. (1965); and Griffin and Penniall (1966) have detected and characterized long chain polyphosphates from animal nuclei. In contrast to micro-organisms, in which over 50% of the total cell phosphorus may be present as polyphosphate, the amounts detected in higher plants and animals have been

minute. Whether this is indicative of a basic functional difference remains to be determined.

Extraction schemes designed to recover all of the polyphosphate present in cells were influenced by the discovery of MacFarlane (1936) mentioned above, which indicated that extraction with cold 5% TCA brought only part of the polyphosphate content into solution. This was later confirmed by Juni et al. (1947); Wiame (1949) and Schmidt (1951). On the basis of this difference in extraction behaviour the corresponding polyphosphate fractions were designated "acid soluble" and "acid insoluble" (notwithstanding the fact that all purified polyphosphates are acid soluble). The difference between the soluble and insoluble fraction appears to be one of average chain length. Estimates of 10-20 and 50-85 phosphate groups per molecule, respectively, for the soluble and insoluble fractions have been reported (Yoshida, 1955; Katchman and Fetty, 1955; Katchman and Smith, 1958).

A variety of methods were developed to recover the "insoluble" polyphosphates: extraction with NaOH (MacFarlane, 1936; Katchman and Fetty, 1955; Niemeyer and Richter, 1969) hot TCA or PCA (Wiame, 1949; Yoshida and Yamataka, 1953; Drews, 1958) boiling water (Lohmann and Langen, 1956) and hot 2N NaCl (Kaltwasser and Schlegel, 1959). Harold (1963) used a somewhat different approach by digesting the cells

first with hypochlorite. The polyphosphates which are insoluble and remain as a precipitate under these conditions can then be extracted from the residue with water. Liss and Langen (1960,1958) developed a procedure whereby they fractionated the polyphosphates into four groups which differed in average molecular weight. The details of their extraction scheme will be described under Methods since this was the fractionation used in the experiments to be described.

In all cases the extracts prepared contained not only polyphosphates but also nucleic acid and to a lesser extent protein. In order to estimate the polyphosphate content of specific extracts, two basic approaches have been used; (1) other phosphorus containing compounds are first removed either by precipitation of the polyphosphate with a barium salt as demonstrated some time ago by Liebermann (1890), or by adsorption of the nucleic acids with charcoal followed by filtration (Müller-Felter and Ebel, 1962). Precipitation with barium, however, is not complete and thus Liebermann's method is not suitable for quantitative determinations. In the foregoing procedures the polyphosphate content is estimated by orthophosphate determination following acid hydrolysis of the polymer. (2) the acid hydrolysis is performed without prior separation and allowance is made for the orthophosphate liberated

by the concomitant partial hydrolysis of the nucleic acids (Liss and Langen, 1960; Langen and Liss, 1958; Ehrenberg, 1961; Kaltwasser and Schlegel, 1959).

Methods for the separation of polyphosphates according to chain length using paper chromatography have been developed by Ebel and others and were reviewed by Hettler (1958). With the refinements introduced by Ohashi and van Wazer (1964) separation up to the octamer is possible and above that the polymers can be segregated into broad groups differing from one another in average chain length. Anion exchange chromatography allows separation up to a chain length of about 12 (Matsuhashi, 1963) and a broad group separation of higher molecular weight polyphosphates similar to that with paper chromatography is possible. Estimations of average chain length in these cases are based on end group titration (Matsuhashi, 1963; Schmidt, 1951; van Wazer, 1958; Yoshida, 1955; Liss and Langen, 1960) and viscometry (Harold, 1963; van Wazer and Campanella, 1950; Yoshida, 1955).

Much of the early work on polyphosphates was simply an attempt to establish their presence or absence in specific organisms. Wiame (1949) was the first to try to establish their physiological role. He investigated the changes in both the soluble and insoluble fraction of polyphosphates when bakers' yeast was exposed to different

conditions. His experiments, briefly, showed the following: Yeast cells grown overnight in the absence of phosphorus contained only traces of soluble and no insoluble polyphosphates. Addition of phosphate to the medium at this stage led to a rapid accumulation of both soluble and insoluble polyphosphate. Return of cells to a phosphate free medium was followed by a rapid decline of the insoluble fraction and a concomitant increase in orthophosphate, but had no effect on soluble fraction levels. Growth in a phosphorus free medium following polyphosphate accumulation also showed that the decrease in insoluble polyphosphate occurred concomitantly with an increase in nucleic acid phosphorus. If growth under these conditions was allowed to proceed for extended periods, a substantial decline in the soluble fraction was noticed and curiously, a small recovery of the insoluble fraction also occurred. Lastly, it was found that polyphosphate was accumulated to a greater extent if exposure of cells to phosphate followed a period of phosphorus starvation. All of this increase was in the soluble fraction. From this information Wiame concluded that there are two metabolically distinct fractions, one of which, the insoluble fraction, is more active, that is, it undergoes greater fluctuations than the soluble fraction.

Other reports (Kuhl, 1960; Harold, 1966; Katchman and Fetty, 1955) confirmed Wiame's findings and suggested

that the two fractions may have different biological functions. Langen, Liss and Lohmann (1956; 1958; 1962; 1962; 1960) separated the polyphosphates into four groups as mentioned above and traced the appearance of ^{32}P in the various fractions with time. Incorporation of ^{32}P was found to occur via ATP into the high molecular weight fraction 4 with the subsequent appearance of label in fractions 3,2 and 1. Addition of iodoacetate under anaerobic conditions following brief exposure to ^{32}P resulted in a very similar pattern, i.e. reduction in the amount of label in fraction 4 and a concomitant sequential increase in fractions 3,2 and 1. During this time the amount of orthophosphate increased substantially and the level of fraction 4 declined, while fractions 3,2 and 1 remained virtually unchanged.

From this evidence the authors concluded that incorporation of orthophosphate occurred only into the high molecular weight fraction 4 with subsequent cleavage to shorter chain lengths. It also indicated that the relative constancy of the soluble polyphosphate fraction of Wiame was not a consequence of metabolic inactivity since in fact both insoluble and soluble fractions had the same turnover rates and stand in a precursor-product relationship. The results of Matsuhashi (1963) who separated the extracted polyphosphates into seven groups of increasing average chain length by means of column chromatography throw some

doubt on this interpretation. While he too found phosphate incorporation primarily in the fraction of largest molecular weight, there was very little activity in two of the three acid soluble fractions while the third - that with the lowest molecular weight, having chain lengths between 3-6 phosphate moieties - showed considerably higher activity which was not due to ADP, ATP or pyrophosphate contamination. The question of the functional homogeneity of the polyphosphates is thus not yet established.

The synthesis of long chain polyphosphate appears to be limited to a reaction which is mediated by polyphosphate kinase and involves the addition of the terminal phosphate group from ATP to a (long chain-) polyphosphate molecule. The enzyme was first detected by Yoshida and Yamataka (1953) in yeast and has since been found in a number of other organisms (Harold, 1966). The enzyme isolated from *Escherichia coli* and extensively purified by Kornberg et al. (1956) reversibly transferred the terminal phosphate group from ATP to polyphosphate and appeared to require the presence of a primer. Breakdown of polyphosphate on the other hand appears to involve a considerable number of enzymes, some of which are very specific e.g. the tripolyphosphatase and tetrapolyphosphatase, reported by Mattenheimer (1956a; 1956b; 1956c) to be acting only on tripolyphosphate and tetrapolyphosphate respectively. For

a full discussion of the various enzymes the review by Harold (1966) should be consulted. Of particular interest with regard to the function of polyphosphates is the presence of enzymes which mediate the phosphorylation of glucose and fructose by polyphosphates apparently without ATP participation.

The question concerning the role or roles which the polyphosphates play in cellular metabolism is still unanswered despite the efforts of a large number of investigators. At least in part this is due to the far reaching effect which any alteration in the polyphosphate pool has on other processes within the cell. Some broad relationships have been clarified, but in no case has a specific pathway of reactions involving polyphosphate been identified. The prime requirement other than presence of phosphate for polyphosphate synthesis is an oxidizable substrate to provide the necessary energy. During growth there appears to be a competitive relationship between nucleic acid and polyphosphate synthesis; if nucleic acid synthesis is blocked because of the lack of an essential metabolite, polyphosphate levels increase (Kuhl, 1960; Harold, 1966; Smillie and Krotkov, 1960; Winder and Denneny, 1957; Domanski-Kaden and Simonis, 1972). Conversely during periods of maximum growth polyphosphate levels tend to be low. The utilization of polyphosphates for phospholipid and nucleic acid biosynthesis during growth

in phosphorus free medium has been demonstrated in a number of organisms (Winder and Denneny, 1957; Baker and Schmidt, 1964; Stahl et al., 1964; Harold, 1962; Harold, 1963) but apparently this does not occur when orthophosphate is introduced into the medium. Under these conditions the endogenous pool of polyphosphate shows no turnover, and incorporation of phosphorus into nucleic acid is entirely at the expense of orthophosphate from the medium (Schmidt, 1966). Under normal conditions of growth polyphosphates thus appear to have no specific role. This conclusion was also drawn by Harold (1964) who found no difference between the growth rate of wild type *Aerobacter aerogenes* and that of a mutant strain lacking polyphosphate kinase (and which was therefore devoid of polyphosphate).

The evidence cited so far indicates that perhaps the sole function of polyphosphate is that of phosphorus storage in a form which does not constitute an osmotic stress. Early speculation that polyphosphate accumulation represents an energy storage system proposed by Hoffman-Ostenhof and Weigert (1952) and based at least partly on the recognition of the "energy rich" nature of the ester bond (Yoshida, 1955) has now been discounted. Harold (1965) has shown that at least part of the polyphosphate is broken down non-enzymatically with the dissipation of the transfer potential of the bond as heat. Furthermore, a number of

objections have been raised concerning the capacity of the limited energy pool represented by the polyphosphates to contribute to the cell requirements in any significant way (Kuhl, 1960). What seems more attractive at the present time is to ascribe to polyphosphates the role of a buffer, with synthesis and degradation being dependent on either the ATP/ADP ratio as in fact has been demonstrated *in vitro* by Kornberg (1956) and/or the intracellular orthophosphate level as described by Harold (1966).

From the foregoing it is clear that considerable attention has been paid to a variety of aspects of polyphosphate metabolism; this is particularly true for yeast, the organism in which polyphosphates were first detected. Probably because of the manner in which the topic was first introduced by Jeener and Brachet (1944) and subsequently explored by Wiame (1949; 1947a) most investigators following in their footsteps used stress conditions whereby growth of cultures was alternated between phosphorus-rich and phosphorus free media and the effect of such treatment on polyphosphates in particular and the phosphorus metabolism in general examined. With the exception of the work of Katchman and Fetty (1955) no attempt appears to have been made to follow the changes in polyphosphate content during "normal" growth of batch cultures of yeast. These authors resolved the extracted polyphosphates into "acid soluble"

and "acid insoluble" components according to the criteria of Wiame (1949) and found the former to remain at constant levels during growth, whereas the long chain "insoluble" fraction appeared to be absent during early log phase, accumulated rapidly to reach maximal values with the onset of deceleration and thereafter decreased in amount. No comparable work on yeast has since been undertaken in spite of the development of extraction procedures which offered increased resolution (Liss and Langen, 1960; Langen and Liss, 1958; Matsuhashi, 1963; Niemeyer and Richter, 1969; Kulaev, 1966). Particularly the method of Liss and Langen, (1960) seemed a promising tool for further investigation because the fractions successively extracted by different solvents may be of different origin or functional significance. In this connection it should be mentioned that despite these authors well-documented conclusion, namely that their long chain fraction 4 stands in a precursor-product relationship with fractions 3 → 2 → 1, there is some evidence that the relationship on a functional level may be more complex. One indication is the finding of Ehrenberg (1961) that at low levels of phosphate in the external medium polyphosphates of intermediate chain length (Liss and Langen's fraction 2) accumulate first, whereas at high levels of phosphate in the medium long chain polyphosphates are the predominant product. The work of Matsuhashi (1963) referred to earlier

(page 7) provides additional evidence that polyphosphates may not be all of common origin.

The physiological, biochemical and morphological changes occurring during sporulation in yeast have been the subject of investigation by a large number of laboratories. Summaries of the most recent findings have been provided by several review articles (Fowell, 1969; Haber and Halvorson, 1972; Tingle, Singh Klar, Henry and Halvorson, 1973).

Sporulation can be initiated by transfer of cells to a nitrogen-free medium containing a non-fermentable carbon-(and energy-) source (Miller, 1963). The gross changes which follow are an increase in size and dry weight of the cells, fragmentation and diminution of the vacuole, and duplication of DNA followed by meiosis (Croes, 1967; Keup, 1967; Pontefract and Miller, 1962; Sando and Miyake, 1971). The haploid nuclei become enclosed in a prospore envelope (Miller and Hoffman-Ostenhof, 1964; Moens, 1971; Moens and Rapport, 1971; Guth, Hashimoto and Conti, 1972) which gradually increases in size as the spores mature. At maturity the cell, which has now become an ascus, contains a maximum of four ascospores.

The focus of most of the recent work has been on aspects of the biochemistry of the sporulation process. Croes (1967a; 1967b), Esposito et al. (1969), and Sando and Miyake (1971) have investigated aspects of the protein and

nucleic acid metabolism. Kane and Roth (1974) have done likewise with carbohydrates, Illingworth et al. (1973) with lipids. The present work is the first to consider changes in polyphosphates and their possible role in yeast ascospore development.

The author has undertaken experiments in an attempt to follow the changes in content of the four polyphosphate fractions of Liss and Langen (1960) during growth and possibly to find a correlation between any or all of these fractions and that of the potential or actual polyphosphate "sinks" e.g. the phospholipids and nucleic acids. In addition the fate of the polyphosphates after transfer of stationary phase cells to sporulation medium and consequent ascospore formation was monitored. Since the developmental processes which are set in motion with the transfer of yeast cells to a sporulation environment are well known and the concomitant structural changes easily monitored, this was felt to be a good system for the exploration of the interrelationship between the various phosphorus pools. Sporulation medium contains no phosphorus, so all the changes which occur in the various pool levels will be the consequence of internal shifts. There are no reports in the literature concerning polyphosphate metabolism during ascospore development in fungi.

MATERIALS AND METHODS

MATERIALS

The following chemicals were supplied by Fisher Scientific, Fair Lawn, New Jersey:

Ammonium molybdate
1-Butanol
Calcium chloride
Chloroform
Cupric sulfate pentahydrate
Ferric chloride
Folin reagent
Glucose (dextrose)
Hydrochloric acid (conc.)
Orcinol
Potassium acetate
Potassium phosphate
Potassium tartrate
Sodium bisulfite
Sodium carbonate
Sodium hydroxide
Sodium perchlorate
Trichloro-acetic acid

Phosphate standards were supplied by the Hartman-Leddon, Co., Philadelphia, Pa.; anhydrous ethyl ether by Mallinckrodt Can. Ltd., Toronto, Ont.; perchloric acid by the McArthur Chemical Co., Montreal, P.Q. and Allied Chemical Co., Morristown, N.J.

Yeast RNA was obtained from Schwarz Bioresearch, Orangeburg, N.Y.; bovine serum albumin from Sigma Chemical Co., St. Louis, Mo.; ribose from the California Corporation for Biochemical Research, Los Angeles, Cal., and amidol from British Drug Houses Ltd., Poole, England.

METHODS

Organism and Cultivation

The strain of yeast used in the experiments was EVE46, obtained in 1965 from Professor U. Santa Maria, Instituto Nacional de Investigaciónes Agronómicas (Ministerio de Agricultura) Madrid, Spain.

It was chosen for its consistently high sporulation; 90 - 95% of the cells contained spores after twenty hrs in sporulation medium.

Stock cultures were maintained on slants of Malt extract-Yeast extract-Peptone-Glucose agar (see below) by weekly transfers. Following each transfer, cells were allowed to grow for one day at room temperature and then stored at 4°C.

Media

Growth medium

Malt extract-Yeast extract-Peptone-Glucose medium (MYPG):

Difco malt extract	3	g
Difco yeast extract	3	g
Difco peptone	5	g
Glucose	10	g
Distilled water	1	litre

pH after autoclaving 5.7 - 5.8.

Sporulation medium

Potassium acetate	10	g
Distilled water	1	litre

pH after autoclaving 7.6 - 7.8.

Vegetative Growth

Liquid cultures were started by transferring a loop of cells from a stock culture slant to a 500 ml Erlenmeyer flask containing 125 ml of MYPG medium supplemented with 0.1% potassium dihydrogen phosphate (KH_2PO_4). The cells were allowed to grow overnight with shaking (approximately 100 strokes per min., stroke length 1 1/2 inches) at 27°C to a density of 5 - 7 x 10^7 cells per ml. This culture was then used to inoculate fresh medium to a density of 1 x 10^6 cells per ml. The fresh cultures were then grown in the same manner. At intervals samples were removed for determinations of cell density, dry weight and for the extraction of specific cell components.

Sporulation

The large cell populations required for studying changes in cell constituents during the sporulation process were conveniently obtained with a Microferm bench top fermentor (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey) with 14 litre fermentor jars. Fermentor jars containing 4 - 6 litres of fresh MYPG medium supplemented

with 0.1% KH_2PO_4 were inoculated from the overnight culture to a density of 1×10^6 cells per ml followed by the addition of sterilized antifoaming agent (Dow Corning Antifoam 'A' compound). The cultures were then grown at 27°C with agitation at 200 rpm and aeration at the rate of 4 litres per min for 24 hrs. The cells were harvested by centrifugation in a Sorvall RC2-B centrifuge (5,000 rpm; 10 min) at 4°C, washed twice with sterile distilled water and resuspended in 10 - 12 litres of sporulation medium to a density of 5×10^7 cells per ml. The sporulation cultures were incubated at 27°C with vigorous agitation and aeration (250 rpm; airflow of 10 litres per min). Samples were withdrawn at intervals, starting at T_o and continuing for up to 45 hrs after inoculation, and treated in the manner described later.

Preparation of Cell Extracts

Extractions of polyphosphates

All extractions were carried out with samples of 1×10^{10} cells or approximately 1.0 g fresh weight, according to the method of Liss and Langen (1960) with some modifications. Their procedure, which allows the separation of polyphosphates into four fractions which differ in average chain length, involves the following steps:

1. Cells were separated by centrifugation and washed twice with distilled water. The pellet was resuspended in 6 ml of 4% TCA and left for 15 minutes (min) at 0°C. The cells were centrifuged and washed with 4 ml of 1% TCA, the supernatants combined and neutralized with NaOH. This fraction contains the free pools of all acid soluble compounds and the low molecular weight polyphosphates (3 - 9 phosphate groups per molecule).

2. The cells were then extracted twice for 5 min each at room temperature with 5 ml of perchlorate-TCA solution (10 ml saturated $NaClO_4$ plus 6.6 ml of 1 N TCA in 100 ml aqueous solution). The supernatant contains polyphosphates with an average chain length of 20 phosphate groups and small amounts of nucleic acids and proteins.

3. The residue was taken up in 1.6 ml of distilled water and sufficient 0.2 N NaOH added to bring the pH to 9 - 10 (this requires between 1.1 - 1.4 ml of base, depending on the age of the culture). After 15 min at 0°C the cells were centrifuged and re-extracted with 3.0 ml of distilled water for an additional 5 min. The combined extracts contain polyphosphate with an average chain length of 55 phosphate groups per molecule together with some nucleic acids and proteins.

4. The pellet left after the previous extraction was suspended in 4 ml of distilled water and 0.8 ml of 1/50 M $CaCl_2$

was added. After 20 min at room temperature the cell residue was collected by centrifugation and washed once with 5 ml of distilled water. The combined supernatant contains the high molecular weight fraction of the polyphosphates with an average chain length of 260 phosphate moieties per molecule in the native state.

All extracts and the cell residue were neutralized with NaOH or HCl, made up to 25 ml with distilled water and stored in 4 oz screw cap bottles at 4°C after addition of several drops of chloroform to prevent microbial growth.

All extractions performed on growth culture samples were performed immediately after harvesting. In contrast, sporulating cell samples were frozen in liquid nitrogen after two washes with distilled water and subsequently stored at -50°C. Extraction procedures followed the foregoing scheme.

Extraction of nucleic acids

Two methods of extracting ribonucleic acid were employed. The method of Herbert, Phipps and Strange (1971), which is a modification of the procedure of Trevelyan and Harrison (1956), was used to extract growth culture samples. It has the advantage of being relatively rapid with greater than 95% recovery of RNA in one extraction step and avoids the problem of excessive solubilization of carbohydrates which occurs during extraction at higher temperatures. The

presence of large quantities of carbohydrates renders analysis by the orcinol method (Herbert, Phipps and Strange, 1971) virtually impossible. The method, as applied in the present study, is as follows:

1. Extraction of acid soluble pools: Washed cells were extracted twice with 4 ml of 0.25 N PCA at 0°C for 15 min each, followed by a distilled water rinse and neutralization of the extract with NaOH.

2. Extraction of lipid components: The pellet from the previous extraction step was extracted twice with 5 ml of 95% ethyl alcohol (30 min each at room temperature). This was followed by three extractions of 3 min each with 5 ml of a 95% ethanol: ethyl ether mixture (3:1) in an 80°C water bath. The supernatants of all five extraction steps were combined and the cell residue was dried to remove all solvents.

3. Extraction of nucleic acids: The dried pellet was taken up in 10 ml of 0.5 N PCA and left, with occasional stirring, in a 37°C water bath for 2 hrs. Following centrifugation the pellet was washed once with 5 ml of 0.5 N PCA and the supernatants were combined and neutralized.

The residue was taken up in distilled water and all fractions made up to 25 ml with the appropriate solvent. If deemed necessary chloroform was added to prevent microbial growth and the extracts were stored in 4 oz screw cap bottles at 4°C.

Extracts prepared by the method of Herbert et al. (1971) could not be used for estimation of RNA content by the orcinol method in sporulating cells because of their much higher carbohydrate content and the consequent hexose interference with ribose determinations. This difficulty was less pronounced with the method of Ogur and Rosen (1950) and their scheme was adopted for all sporulation cultures:

1. Extraction of alcohol-soluble compounds with 5 ml of 70% ethanol was followed by a wash with 5 ml of acidified ethanol (0.1% PCA in 70% ethanol) all at 0°C. The residue was then extracted twice with 5 ml of a 3:1 mixture of ethanol: ethyl ether at 65°C (boiling point).

2. Extraction of acid soluble compounds was carried out by resuspending the pellet of the previous step in 5 ml of cold (0°C) 0.2 N PCA. The cells were pelleted and re-extracted with a second aliquot of acid.

3. The residue of the preceding extraction was taken up in 5 ml of cold 1 N PCA and the suspension stored at 4°C for 18 hrs. After centrifugation the residue was washed once with 5 ml of 1 N PCA.

This extraction step was repeated once or twice to effect complete removal of RNA.

4. Extraction of deoxyribonucleic acid (DNA) was accomplished by suspending the pellet of the previous step in 5 ml of 0.5 N PCA and leaving the suspension for 20 min at 70°C.

This process was repeated at least once; in late spore stages a third extraction was found necessary.

5. The last step involved suspending the residue in 5 ml of 2N NaOH and heating the suspension in a boiling water bath with frequent stirring for 10 min. This process was then repeated using 2 N HCl instead of base and the supernatants were combined.

All fractions were neutralized, made up to 25 ml and stored as described above.

Phospholipids

Lipids were extracted by the method of Herbert et al. (1971). Washed PCA-extracted cells were twice extracted with 5 ml of 95% ethanol at room temperature for 30 min each, followed by three extractions with 5 ml of boiling ethanol: ether (95% ethanol: ethyl ether = 3:1) for 3 min each. The combined supernatants were found to contain at least 97% of the phospholipids.

Chemical Analyses

Phosphorus was determined according to the method of Allen (1940).

For orthophosphate determinations a sample of up to 20 ml containing maximally 0.4 mg of phosphorus was placed in a 25 ml volumetric flask to which the following were added in the order given: 2.0 ml of 60% w/v PCA;

2.0 ml of amidol reagent (1 g of 2,4-diaminophenol hydrochloride and 20 g of sodium bisulfite dissolved in glass-distilled water and made up to 100 ml) and 1.0 ml of 8.3% ammonium molybdate solution. The volume of sample plus reagents was made up to 25 ml with distilled water and the absorption was read after 5 min at 720 nm using a Bausch and Lomb Spectromic 20 and 1/2 inch cuvettes. The colour remains stable for 30 min. The amount of phosphorus present was read from a calibration curve obtained by applying the same procedure to aliquots of standard phosphate solutions containing from 0.01 to 0.4 mg of phosphorus.

Total phosphorus (ash phosphorus) was determined by placing a sample in a micro-Kjeldahl flask, adding a small quantity of distilled water and 2.2 ml of 70% w/v PCA. The flask was then heated with a bunsen burner until the content had become colourless. After cooling, the content of the flask was transferred to a 25 ml volumetric flask. Two ml of amidol solution, one ml of 8.3% ammonium molybdate and water to 25 ml were added and the absorption at 720 nm read after a 5 min interval against a blank prepared in the same way except that distilled water was used instead of an aliquot of sample.

Protein was assayed according to Lowry et al. (1951). To 1.0 ml samples were added 5 ml of a solution composed of 50 ml of 2% Na_2CO_3 in 0.1 M NaOH and 1 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate. After 10 min 0.3 ml of 2N Folin

reagent was added and mixed vigourously. The absorbance was read at 500 nm after 30 min using bovine serum albumin as a standard.

RNA assays with the orcinol reagent were performed according to Herbert et al. (1971). To 1.0 ml samples were added 3 ml of freshly mixed orcinol reagent (1 volume of 1% w/v orcinol in distilled water plus 4 volumes of 0.090% w/v $FeCl_3 \cdot 6H_2O$ in concentrated AnalaR grade HCl sp. gr. 1.186) in glass stoppered test tubes which were heated in a boiling water bath for 20 min. After cooling, n-butanol was added to 15 ml and the absorbance read at 670 nm against a reagent blank treated in the same manner. Ribose and purified yeast RNA were used as standards.

Nucleic acids were also estimated by measuring the absorption of UV light at 260 nm of sample extracts by comparison with the absorption of a standard yeast RNA solution treated in the same manner.

Polyphosphate

The method employed makes use of the fact that polyphosphates are completely hydrolysed in 7 min by 1 N HCl at 100°C (Wiame, 1949; Lohmann and Langen, 1956; Kaltwasser and Schlegel, 1959) and can then be determined colourimetrically as orthophosphate as described above. To allow for the partial hydrolysis of nucleic acids present in the extracts a correction was made by measuring

orthophosphate (P_i) after 7 min and 30 min hydrolysis. The kinetics of nucleic acid hydrolysis (i.e. RNA) are such that the amount of orthophosphate liberated during the first 7 min equals that liberated during the following 23 min under the conditions described (Langen and Liss, 1958; Lohmann and Langen, 1956; Ehrenberg, 1961). To calculate the polyphosphate content of the fractions analysed, the following formula was used:

$$PP = P_{i\ 7\ min} - (P_{i\ 30\ min} - P_{i\ 7\ min})$$

$$\text{i.e.}\quad PP = 2P_{i\ 7\ min} - P_{i\ 30\ min}$$

A standard curve obtained by hydrolyzing purified yeast RNA in 1 N HCl at 100°C is shown in the appendix which confirms the validity of the above correction factor.

Estimation of fraction 1 polyphosphate is complicated by the presence of a large number of other phosphorus containing compounds in the TCA extract. The main component, apart from polyphosphate, is orthophosphate (OP) which was determined directly with aliquots of the extract according to Allen (1940). For polyphosphate determinations the formula shown above is then modified as follows:

$$PP = 2P_{i\ 7\ min} - P_{i\ 30\ min} - OP$$

The correction factor in the formula in this instance is designed to allow for the hydrolysis of glucose-6-phosphate and fructose 1,6-diphosphate (Lohmann, 1928).

Phospholipids

Samples of lipid extracts were ashed with 70% PCA as described for total phosphorus determination by the method of Allen (1940).

RESULTS

Vegetative Growth

The growth pattern of *Saccharomyces chevalieri* Guillermond strain EVE46 under the conditions described in Materials and Methods was investigated. Increase in cell numbers was monitored initially by direct counting using a Spencer AO haemocytometer counting chamber and appropriately diluted aliquots of culture. At the same time the increase in dry weight was followed by filtering aliquots of the culture through a pre-weighed millipore filter of 0.45 µm pore size. Some difficulty was experienced with both methods, particularly with the latter. In attempting to make the dry weight samples as reliable a growth indicator as possible a minimum of 1×10^8 cells per sample were used. This required relatively large volumes of culture in the early stages of growth i.e. immediately following inoculation of fresh medium. It was found that under these circumstances the chance collection of debris out of the medium resulted in substantial variability in calculated dry weights per cell. Prefiltration of the medium before autoclaving did not eliminate this problem, instead it resulted in substantially slower growth rates which was interpreted as indicating the

removal of one or more medium components during filtration and was thus deemed an unsuitable practice. More consistent results were obtained using only freshly prepared medium and this practice was henceforth maintained. Despite the variability of the results obtained it was nevertheless clear that the dry weight per cell was not constant but depended on the physiological age of the culture. Average dry weight per cell during mid log phase was 2.6×10^{-5} µg $\pm$ 0.4×10^{-5} µg and declined to 1.9×10^{-5} µg $\pm$ 0.2×10^{-5} µg for stationary phase cells.

This was unexpected for two reasons, a) casual examination indicates that average cell diameters are greater at stationary phase and b) when making cell counts, every bud, no matter how small, was counted as a cell. This ought to result in lower cell weight estimates during log phase when cells with small buds represent a much greater proportion of the total than in stationary phase. This seeming contradiction may be accounted for by the size of the cell vacuole which is either not seen or small during log phase and very prominent in stationary phase.

Direct cell counts presented a problem in that from early log phase to the beginning of deceleration flocculation was extensive, giving rise to cell agglomerates of up to 150 cells. These were quite resistant to separation by mechanical shaking action such as is provided by a vortex

shaker. To compensate for this uncertainty a large number of counts were made; the results are presented as a semilog plot (Fig. 1). The average generation time calculated from the graph is 86 ± 3 minutes. The end of log phase was reached after approximately 8 hrs at which time the cell density was approximately 5×10^7 cells per ml. Average stationary titre was found to be 1.6×10^8 cells per ml under the condition employed.

Changes in Polyphosphate Levels During Growth

That the polyphosphate content of cells during growth does not remain constant has been recognized for some time (Wiame, 1949; Schmidt et al. 1946; Katchman and Fetty, 1955; Smith et al. 1954). Figure 2 shows that polyphosphate content increases during log phase and continues to accumulate into early stationary phase although at a constantly diminishing rate. Maximal values are reached at T_{16}; at this stage the accumulated polyphosphates constitute approximately 50% of the total phosphorus content of the cells. The early stages of growth (i.e. before T_5) were not monitored because of the extremely large volume of culture which would be required at these low cell densities. It was also felt that the greater time required for harvesting would tend to distort the results since even at 0°C significant polyphosphate breakdown as well as changes within the various fractions may occur (Liss and Langen, 1962).

Figure 1 : Growth curve of EVE46.

One hundred and twenty five ml of fresh MYPG medium in a 500 ml Erlenmeyer flask was inoculated to a density of 1×10^6 cells per ml with yeast cells from an overnight culture. The culture was grown at 27°C with shaking at approximately 100 cycles per minute and a stroke length of 1 1/2 inches in an Eberbach shaking water bath.

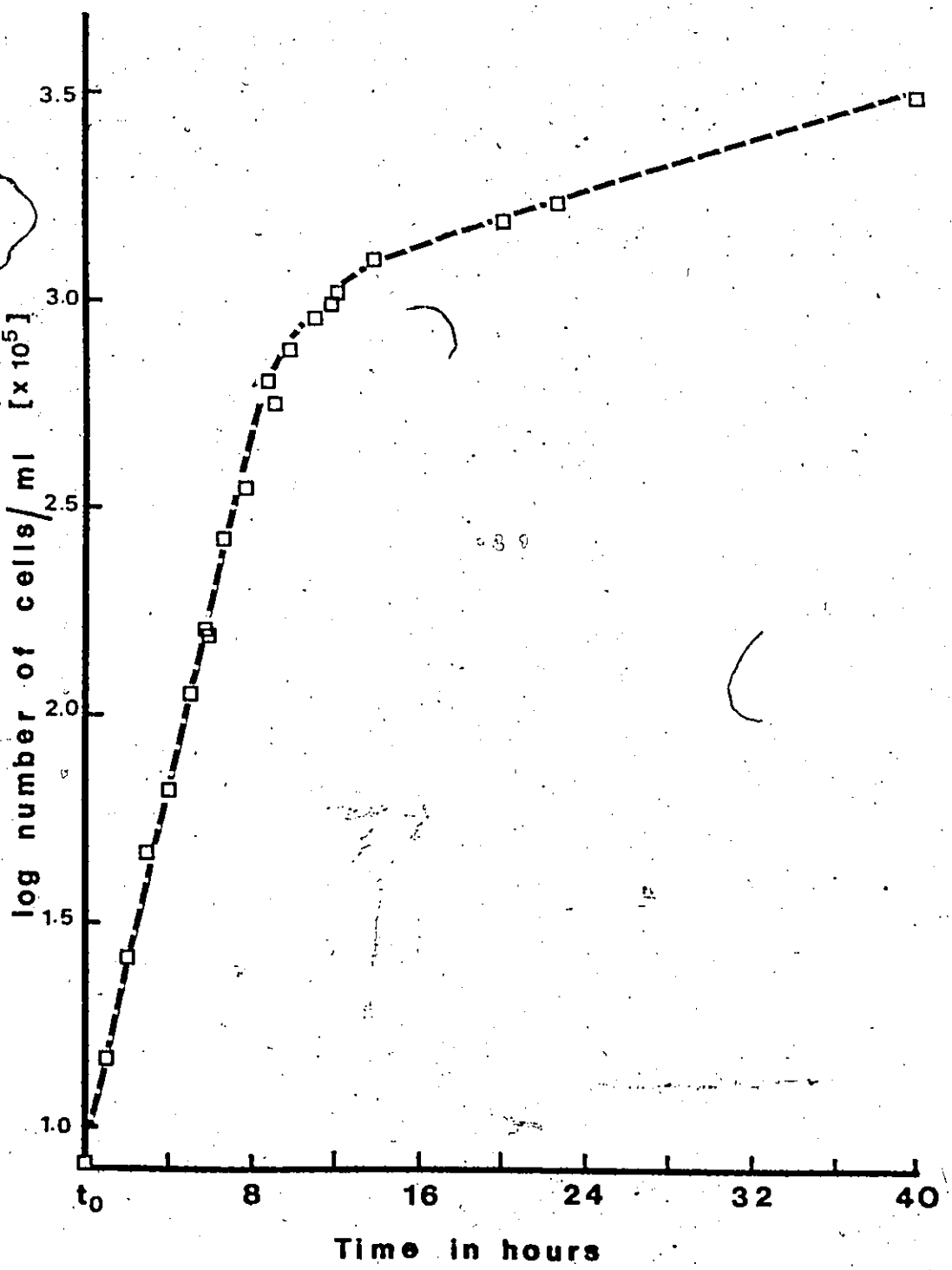
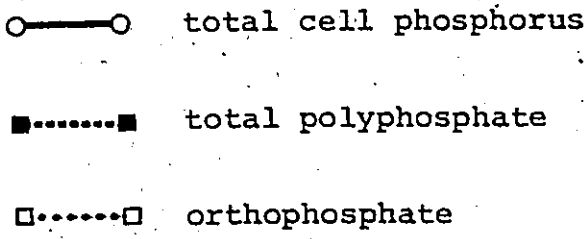
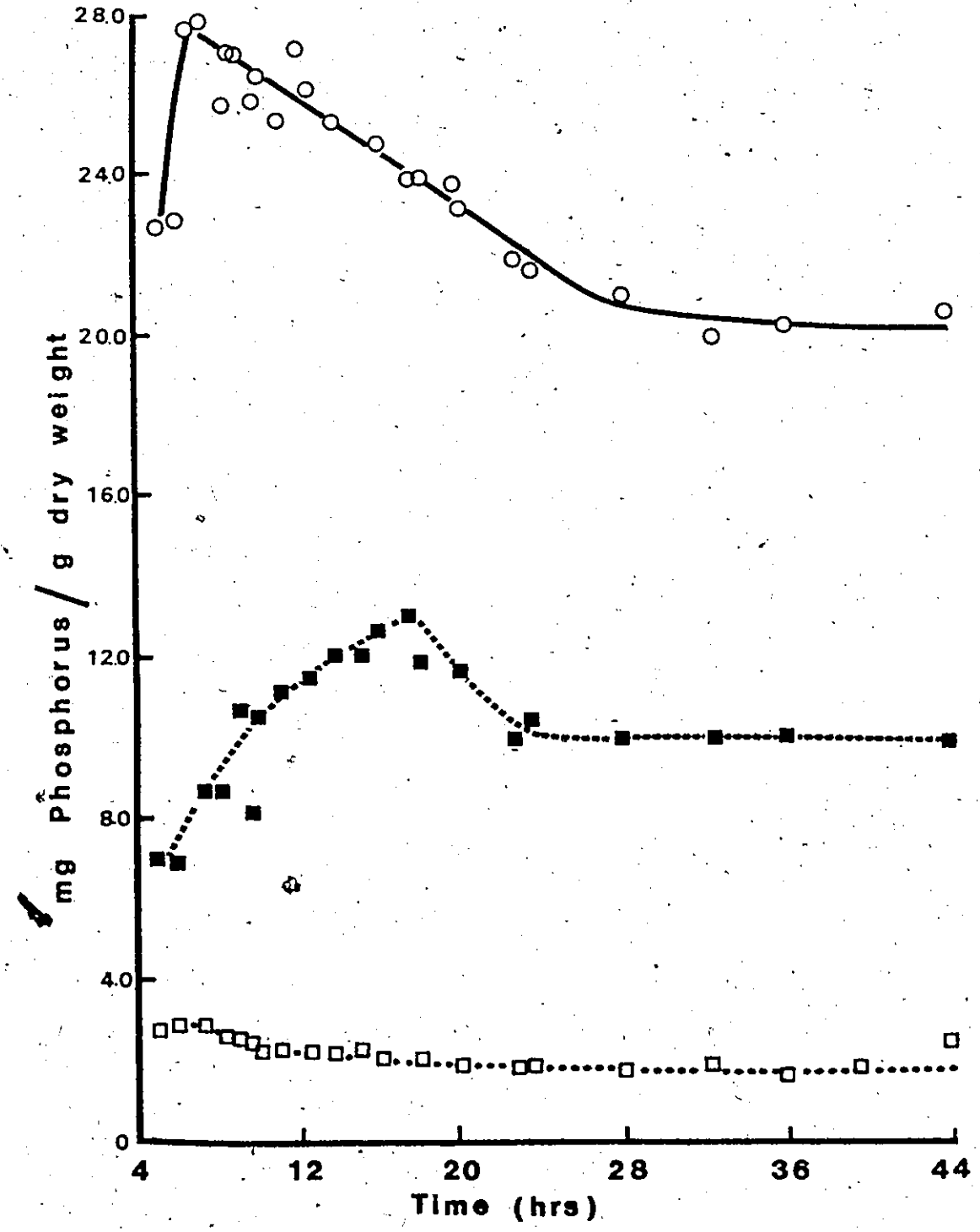

3.5
3.0
2.5
2.0
1.5
1.0
log number of cells/ml [x 10^5]
t0
8
16
24
32
40
Time in hours

Figure 2 : Changes in total cell phosphorus, total polyphosphate and orthophosphate during growth.




28.0
24.0
20.0
16.0
12.0
8.0
4.0
0
mg Phosphorus / g dry weight
4
12
20
28
36
44
Time (hrs)

However, since the cells used to inoculate fresh media come from cultures with a density of between 5-7 x 10^7 cells per ml, which is equivalent to an 8-10 hr culture, and thus have presumably the corresponding polyphosphate content, it would seem that the initial consequence of the transfer is at least a partial breakdown of the polyphosphates. This would have to occur to explain the lower levels found at T_5.

Changes Occurring Within the Four Polyphosphate Fractions

Polyphosphate fraction 1 consists of molecules containing from three to eight phosphate groups [(Langen and Liss, 1958) as determined by paper chromatography carried out according to the procedure of Ebel (1953a; 1953b)] with an average chain length of four as determined by end group titration (Langen, Liss and Lohmann, 1962). As can be seen in Fig. 3 the low molecular weight polyphosphate fraction is at a low level during log phase and increases rapidly with the approach of stationary phase to a new constant level at which point it is the largest polyphosphate component.

Fraction 2 of the polyphosphates consists of molecules with an average chain length of 20 phosphate groups (Liss and Langen, 1960). Figure 4 shows this fraction to undergo very pronounced changes during growth. The sharp drop in fraction 2 levels beginning at T_8 coincides with

Figure 3 : Changes in polyphosphate fraction 1 during growth.

following
Pg. 74

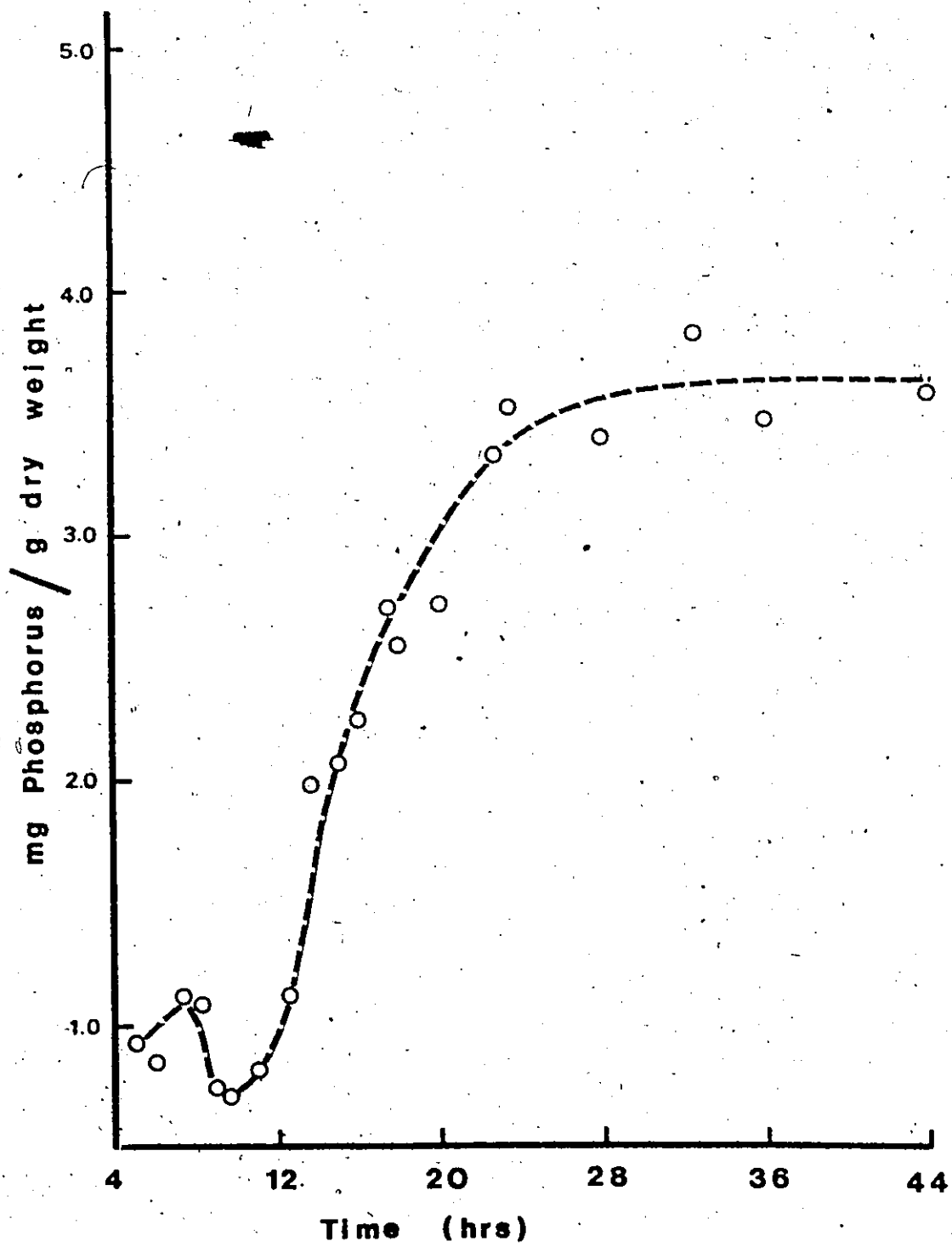


Figure 4 : Changes in polyphosphate fraction 2 during growth.

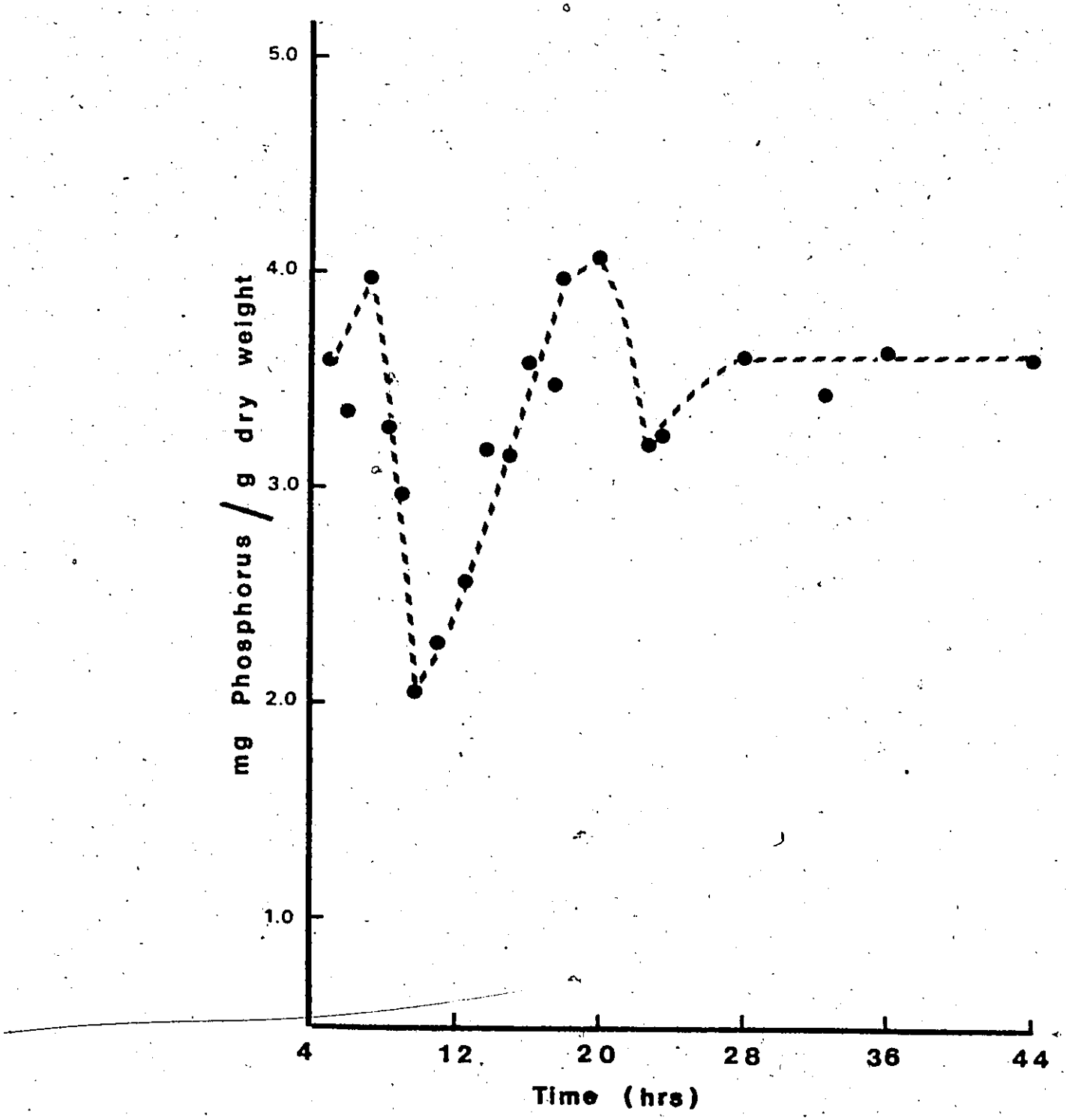
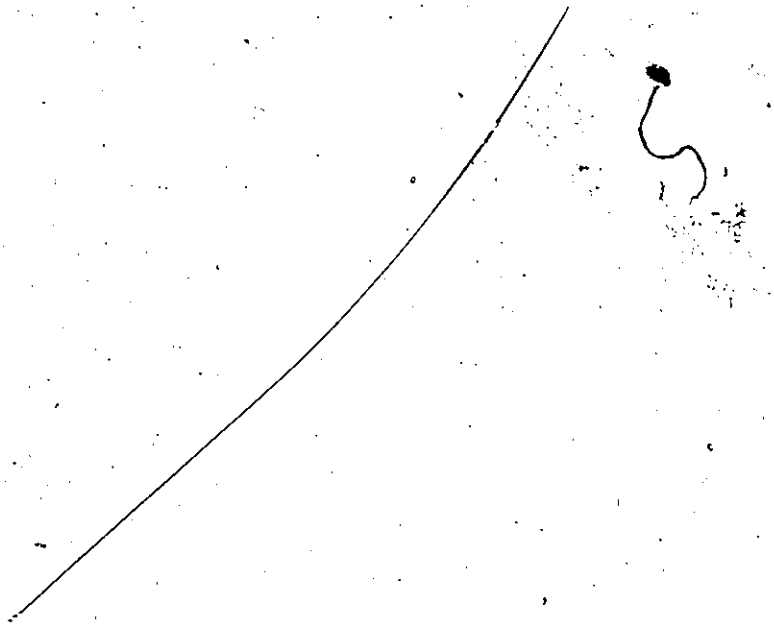

5.0
4.0
3.0
2.0
1.0
mg Phosphorus / g dry weight
4
12
20
28
36
44
Time (hrs)

Figure 5 : Changes in polyphosphate fraction 3 during growth.



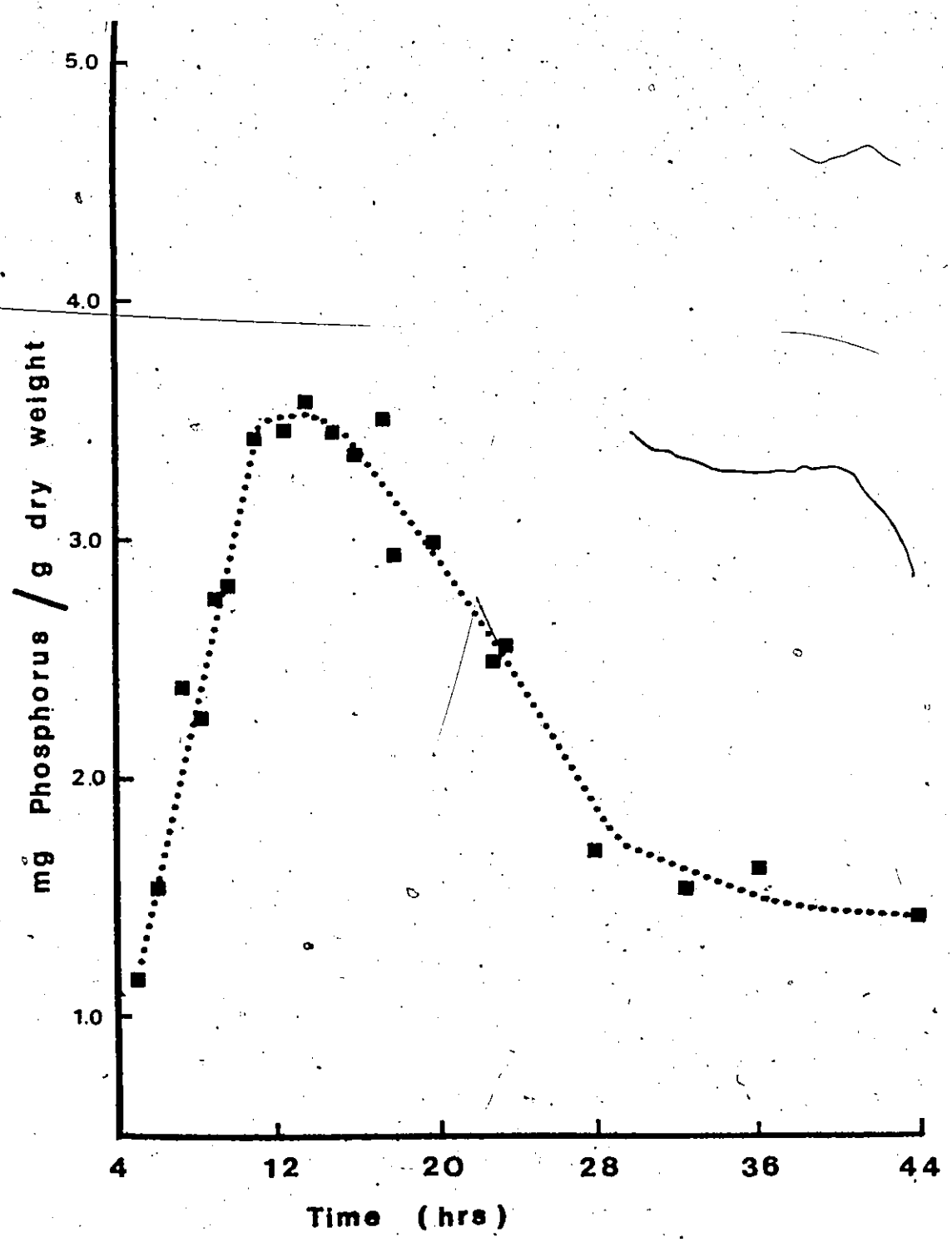
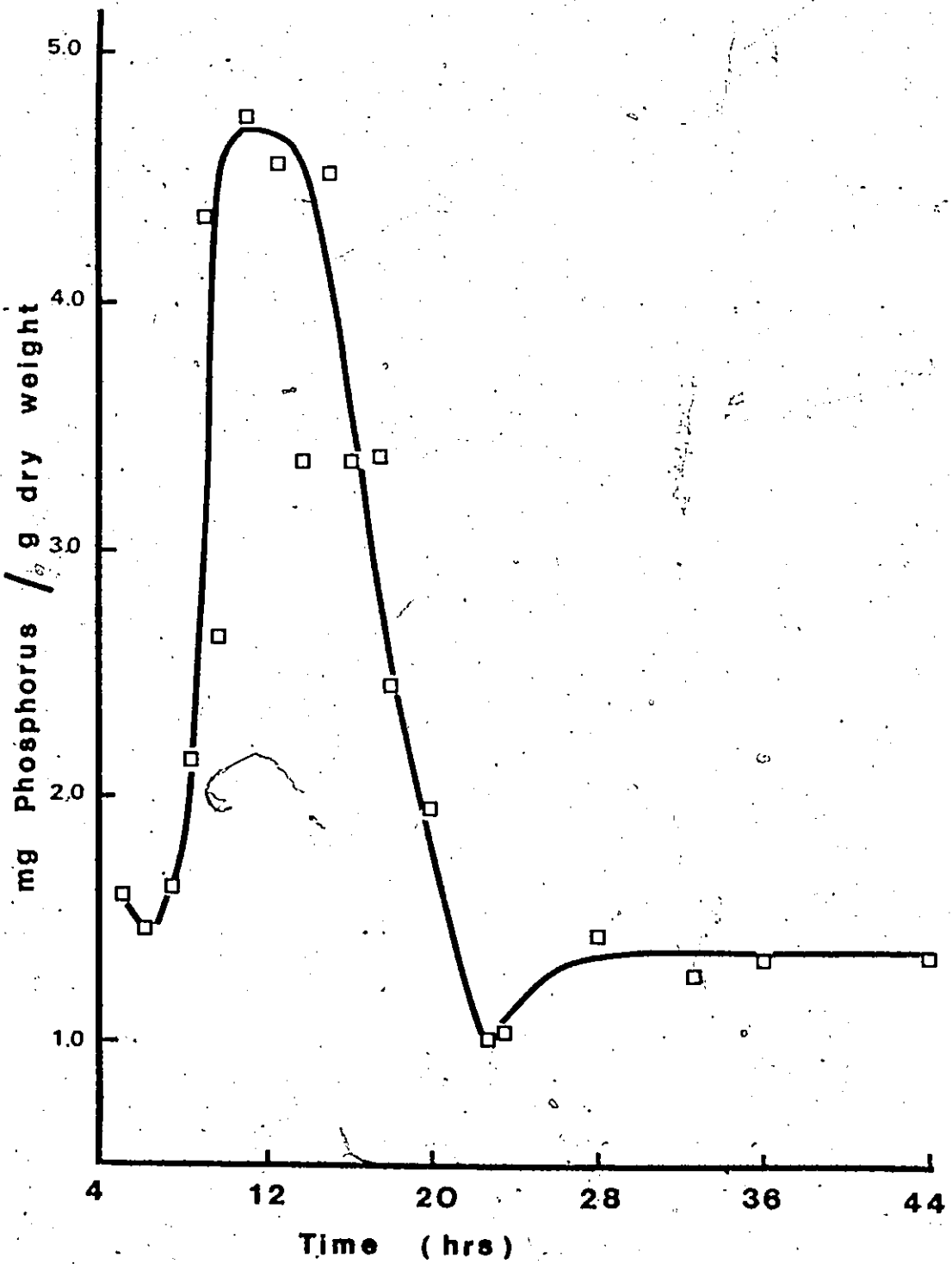

5.0
4.0
3.0
2.0
1.0
mg Phosphorus / g dry weight
4
12
20
28
36
44
Time (hrs)

Figure 6 : Changes in polyphosphate fraction 4 during growth.


5.0
4.0
3.0
2.0
1.0
mg Phosphorus / g dry weight
4
12
20
28
36
44
Time (hrs)

the beginning of deceleration. Following a re-accumulation between T_{10} and T_{20} another transient decrease was found which is not correlated with any overt culture changes. Croes (1967b) found that the end of log phase growth in yeast coincided with the complete disappearance of glucose from the culture medium and that the new, slower growth rate established thereafter was at the expense of the accumulated ethanol present in the medium. In other words, the end of log phase was a reflection of a changeover from fermentation to respiration. The drop in fraction 2 levels occurring during this time may in turn be a consequence of the altered metabolism of the culture. It is tempting to carry the comparison one step farther and ascribe the second dip in fraction 2 levels starting at T_{20} to another change in the metabolism of the culture, namely, the exhaustion of ethanol in the medium which, by analogy with Croes data ought to occur about this time.

Fraction 3, composed of molecules with an average chain length of 55 (Liss and Langen, 1960) increases linearly during logarithmic growth of the culture (Fig. 5). As the average generation time of the culture becomes longer during deceleration, the rate of accumulation slows likewise. After T_{14} a gradual reduction in fraction 3 polyphosphates occurs.

Fraction 4 which consists of molecules having an average of 260 phosphate groups (Liss and Langen, 1960),

undergoes the largest fluctuations (Fig. 6). The rapid increase, beginning after approximately 7 hrs of growth coincides with the end of log phase and the decline of fraction 2. It also occurs at a time when RNA levels decline sharply (Fig. 8). Peak levels are reached at approximately T_{11} and are followed by an equally rapid decline.

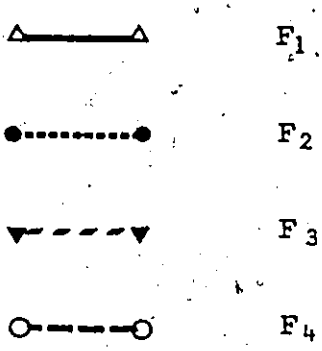
If the four polyphosphate fractions are expressed as percent of the total polyphosphate (Fig. 7), the fluctuations observed are very similar to the absolute changes represented in Figures 3-6.

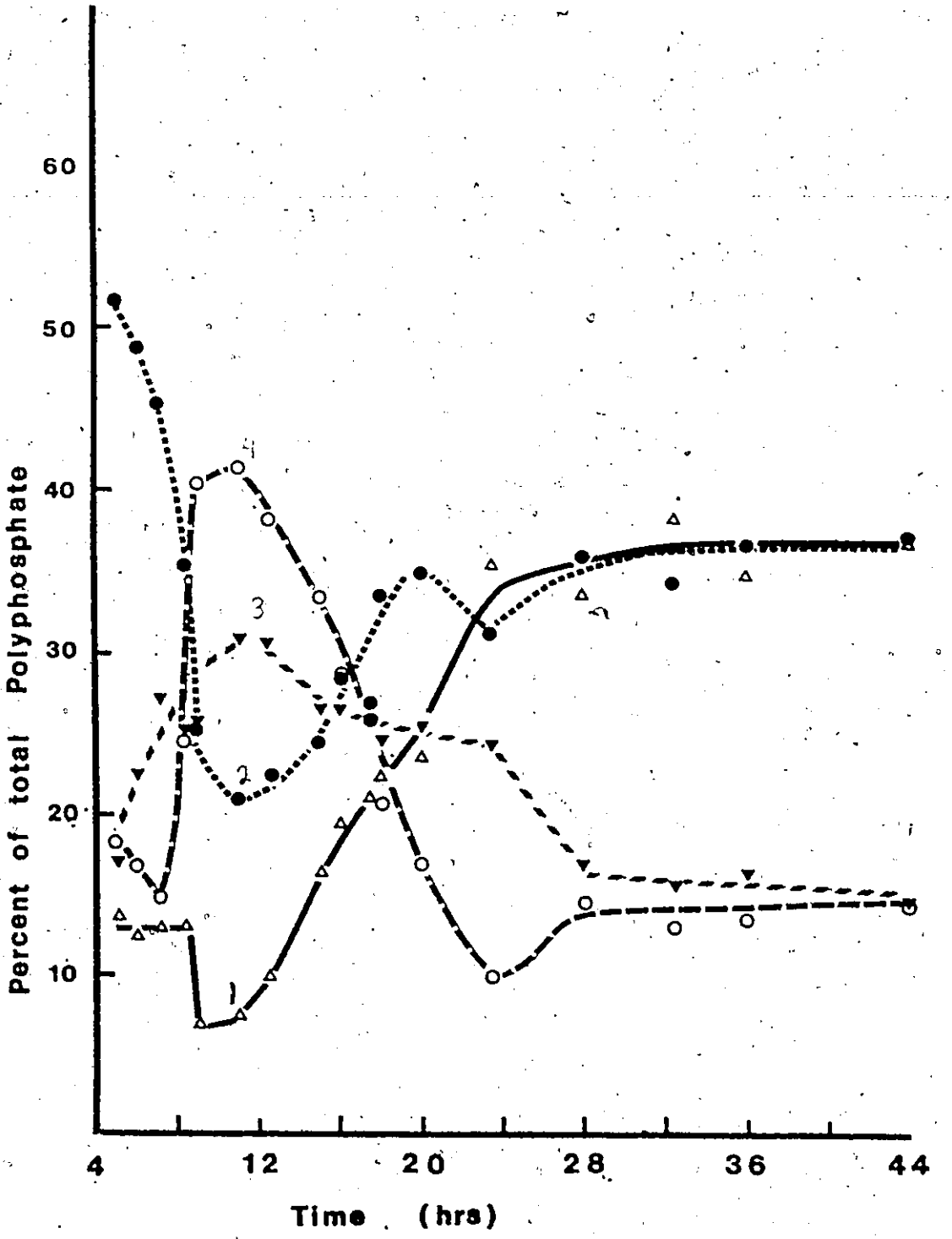
Changes in Other Phosphorus Containing Fractions During Growth.

Ribonucleic acid

Cells were extracted by the method of Herbert et al. (1971) and RNA content estimated by UV absorption at 260 nm and the orcinol method (Herbert et al., 1971) using yeast RNA as a standard. Nucleic acid phosphorus could not be determined directly because of the presence of polyphosphates in all nucleic acid extracts. Instead, it was calculated in the following way: known quantities of yeast RNA were ashed and the total phosphorus content determined. One mg of RNA was found to contain 74 µg of phosphorus, which corresponds to a ratio of 13.5. The phosphorus content of samples of extracts was then calculated based on the OD_{260} as follows:

Figure 7 : Percentage composition of the polyphosphates during growth.
The fluctuations in the individual fraction levels with time are expressed as percent of the total polyphosphate content.




60
50
40
30
20
10
Percent of total Polyphosphate
1
2
3
4
12
20
28
36
44
Time (hrs)

$$\text{phosphorus content} = OD_{260} \times \frac{\text{mg RNA}}{OD_{260}\,\text{unit}} \times \frac{.074\ \text{mg P}}{\text{mg RNA}} \qquad (1)$$

similarly, readings taken using the orcinol method of RNA determinations were converted to the corresponding phosphorus values.

For comparison the RNA phosphorus content was calculated using the results of the analysis of samples extracted by the method of Liss and Langen (1960) according to the following consideration:

$$\text{RNA phosphorus} = RP + (TP-PP)_{F_{2,3,4}} - LP \qquad (2)$$

where

RP = residue phosphorus i.e. the phosphorus determined after ashing of the cell residue following polyphosphate extraction.

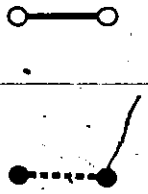
TP = total phosphorus content of fractions 2,3 and 4.

PP = polyphosphate content of fractions 2,3 and 4.

LP = lipid phosphorus which was taken as 1.15 mg/g dry weight (see Table 2).

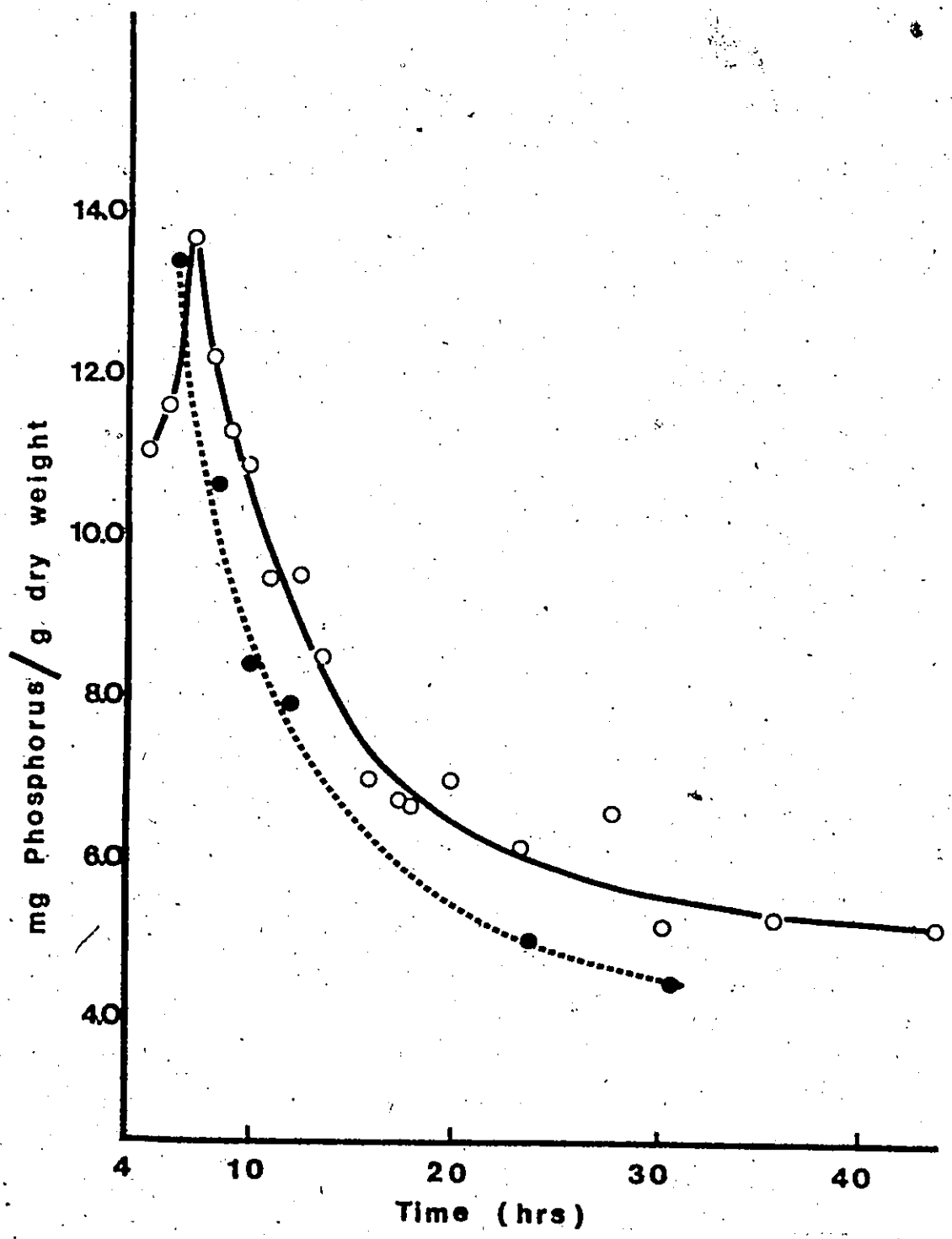
The values thus calculated were plotted and are shown on Fig. 8 as two separate curves. The systematic difference which is apparent may be indicative of an underestimation of the polyphosphate content of fractions 2,3 and 4 inherent in the assay method. It can also be ascribed to the inclusion of phosphoprotein phosphorus and to a much smaller

Figure 8 : Changes in RNA phosphorus levels during growth.



RNA phosphorus levels calculated as described on page 41.

RNA phosphorus levels determined after extraction of cells for 2 hrs at 37°C with 0.5 M PCA. RNA was determined from the OD_{260} of the extracts with yeast RNA as standard.


14.0
12.0
10.0
8.0
6.0
4.0
mg Phosphorus / g dry weight
4
10
20
30
40
Time (hrs)

extent, the inclusion of DNA phosphorus, none of which are allowed for in calculations using formula (2).

The difference is not due to incomplete extraction of RNA since repeat extractions performed yielded only an additional 2% of the amount of RNA extracted in the first step. The importance of Fig. 8 is not so much the magnitude of the fraction, but rather in that it shows the rapidity with which the RNA content of cells decreases during late log phase.

Orthophosphate

Inorganic phosphate was determined in extracts prepared using three different solvent systems as part of a larger extraction scheme:

1. Extraction with cold 4% TCA (polyphosphate extraction)
2. Extraction with cold 70% ethanol (Ogur and Rosen)
3. Extraction with cold 0.25 N PCA (Herbert et al.).

The orthophosphate levels measured after extraction of equivalent samples were found to be very similar regardless of the method used. The results obtained (Fig. 2) were essentially in agreement with the findings of Katchman and Fetty (1955).

Phospholipids

The extraction of lipids was carried out in five steps as described under Methods. In order to see whether

the extraction resulted in the recovery of all phospholipids present, each fraction was ashed with PCA and the phosphorus content determined. The results are shown in Table 1. From the extraction behaviour it was concluded that approximately 97% of all phospholipids were recovered with the method described.

Table 2 shows the amount of phosphorus present in the lipid fraction in different cell samples. The results are in agreement with the finding of Ehrenberg (1961) that phospholipid levels are constant throughout the growth phase.

Residue phosphorus

The cell residue which remains after samples of cells are extracted invariably contains phosphorus, the amount present depends on the extraction procedure employed. After polyphosphate extraction the majority of the nucleic acids and virtually all the phospholipids and phosphoproteins remain in the cell residue. The method of Ogur and Rosen (1950) is designed to remove all phosphorus containing fractions but in fact, as will be shown later, does not do so when used with yeast spores. Both with spores and with vegetative cells the amount of phosphorus remaining in the residue is related to the age of the culture (see Table 2). The nature of the phosphate containing material which remains in the residue following extraction by the method of Ogur and Rosen is unknown but it seems possible that at least part of it is polyphosphate as suggested by Langen and Liss (1958).

Table 1

Extraction of Phospholipids

Extraction #	Solvent used	Treatment	Ash phosphorus (µg/sample)	% phosphorus of total recovered
1	95% ethanol	30 min room temp.	98	50
2	95% ethanol	30 min room temp.	39	20
3	ethanol: ethyl ether (3:1)	3 min 80°C	34	17
4	ethanol: ethyl ether (3:1)	3 min 80°C	17	8
5	ethanol: ethyl ether (3:1)	3 min 80°C	9	5

Table 2

Phospholipid and Cell Residue Phosphorus Levels During Growth

Age of culture*	Lipid phosphorus** mg/g dry weight	Residue phosphorus*** mg/g dry weight
6.5	1.18	1.14
8.5	1.14	1.33
10	1.18	1.72
12	1.21	1.94
31	1.08	2.11

* Age of culture was considered as the number of hrs after inoculation.

** Extractions were made using the method of Herbert et al. (1971).

*** Cells were previously extracted using the method of Ogur and Rosen (1950).

Total phosphorus

Extraction of samples according to the method of Liss and Langen (1960) yields five fractions, four of which contain polyphosphates of varying average chain length as well as other phosphorus containing compounds. The remaining fraction is the cell residue which contains most of the nucleic acids and phosphoproteins. Aliquots of all fractions were routinely ashed and analysed for their phosphorus content according to the method described (Allen, 1940) and the total phosphorus content of the samples determined.

Parallel with the removal of cell samples from the culture for extractive purposes, samples were also withdrawn for dry weight determinations. Aliquots of culture were filtered through 0.45 µm pore size millipore filters, washed twice with distilled water and dried. A weighed portion of the dried cake was then ashed and the phosphorus content determined. The values obtained by the two methods were always within 5% of one another. The results of these determinations are shown in Fig. 2. The considerable increase in total phosphorus content during the first 8 hours is primarily due to the accumulation of polyphosphates during this period. The reduction after T_8 on the other hand may be attributed to a decline in nucleic acid levels (see Fig. 8) and only later on, i.e. after T_{16} to lowered polyphosphate levels.

Changes Occurring During Sporulation

Sporulation of strain EVE46

Cells grown in the Microferm fermentor as described in Methods showed a slower growth rate than flask-grown cultures. The average generation time was 103 ± 5 min and the titre of the culture after 24 hrs of growth was found to be 1.6 - 1.7 x 10^8 cells per ml. The growth curve (Fig. 9) shows that stationary titre is achieved at approximately 20 hrs which is comparable to the situation with flask-grown cultures. The stationary titre was also very similar and thus the pre-condition for assuming that cells grown in fermentor batches are comparable to cells grown in flasks in the shaking water bath appears to be satisfied. This equivalence was later extended to two more parameters, the size of the polyphosphate pool and the content of RNA which were both found to be the same at stationary phase, regardless of the preceding growth regime.

All sporulation cultures were started by collecting 24 hr stationary phase cells and resuspending them to a density of 5 x 10^7 cells per ml in 1% potassium acetate as described. In order to establish the time course of sporulation and relate it to other events later on, the parameters described before were monitored. Dry weight changes expressed as percent of T_o values are shown in Figure 10 and agree with those reported elsewhere (Banerjee, 1970; Esposito et al., 1969; Illingworth et al., 1973). The

Figure 9 : Growth of EVE46 in Microferm fermentor. Initial cell density - 1×10^6 cells per ml. Final (stationary phase) titre - 1.6×10^8 cells per ml. Airflow of 4 l/min and agitation of 200 rpm. Calculated generation time from graph 103 ± 5 min.

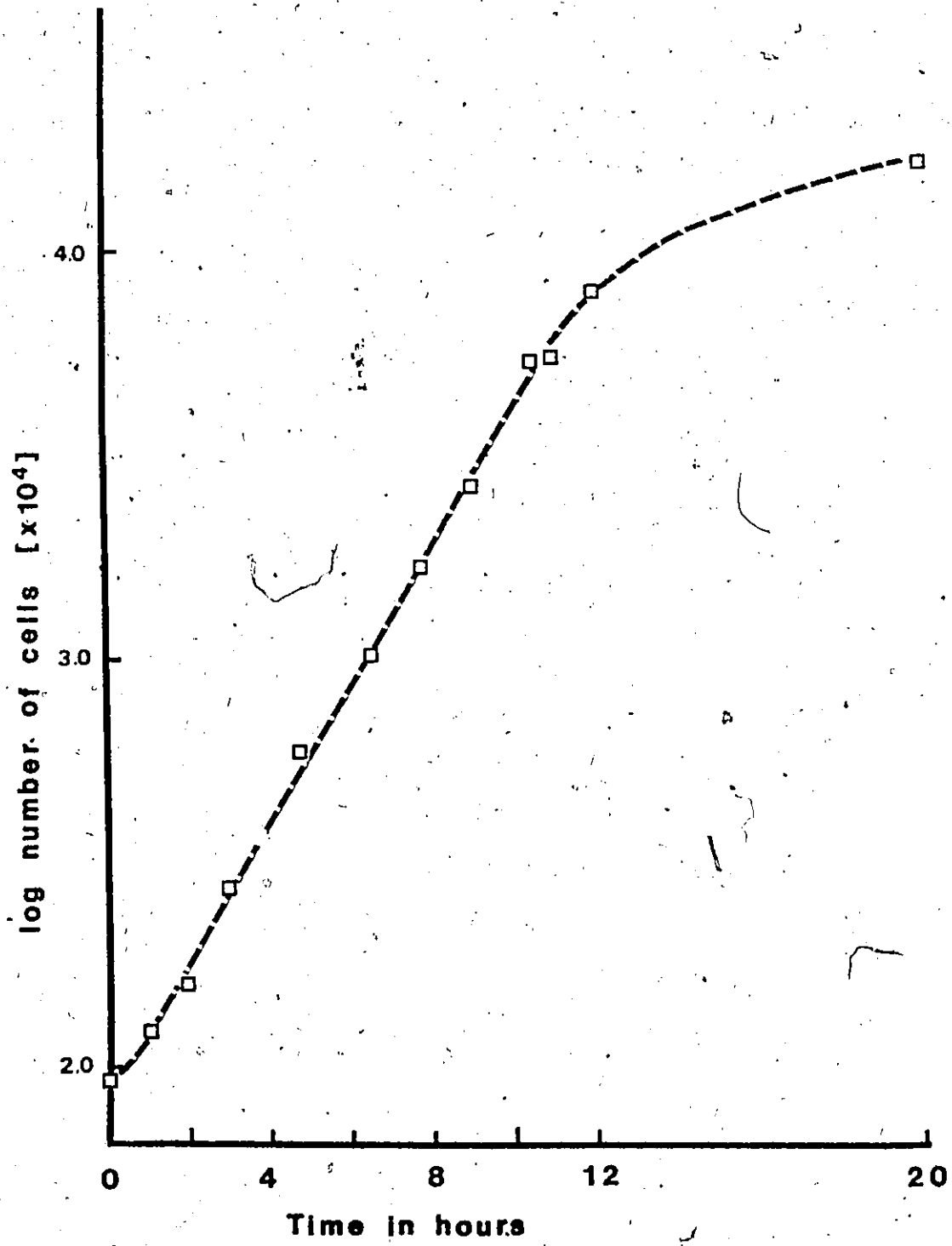
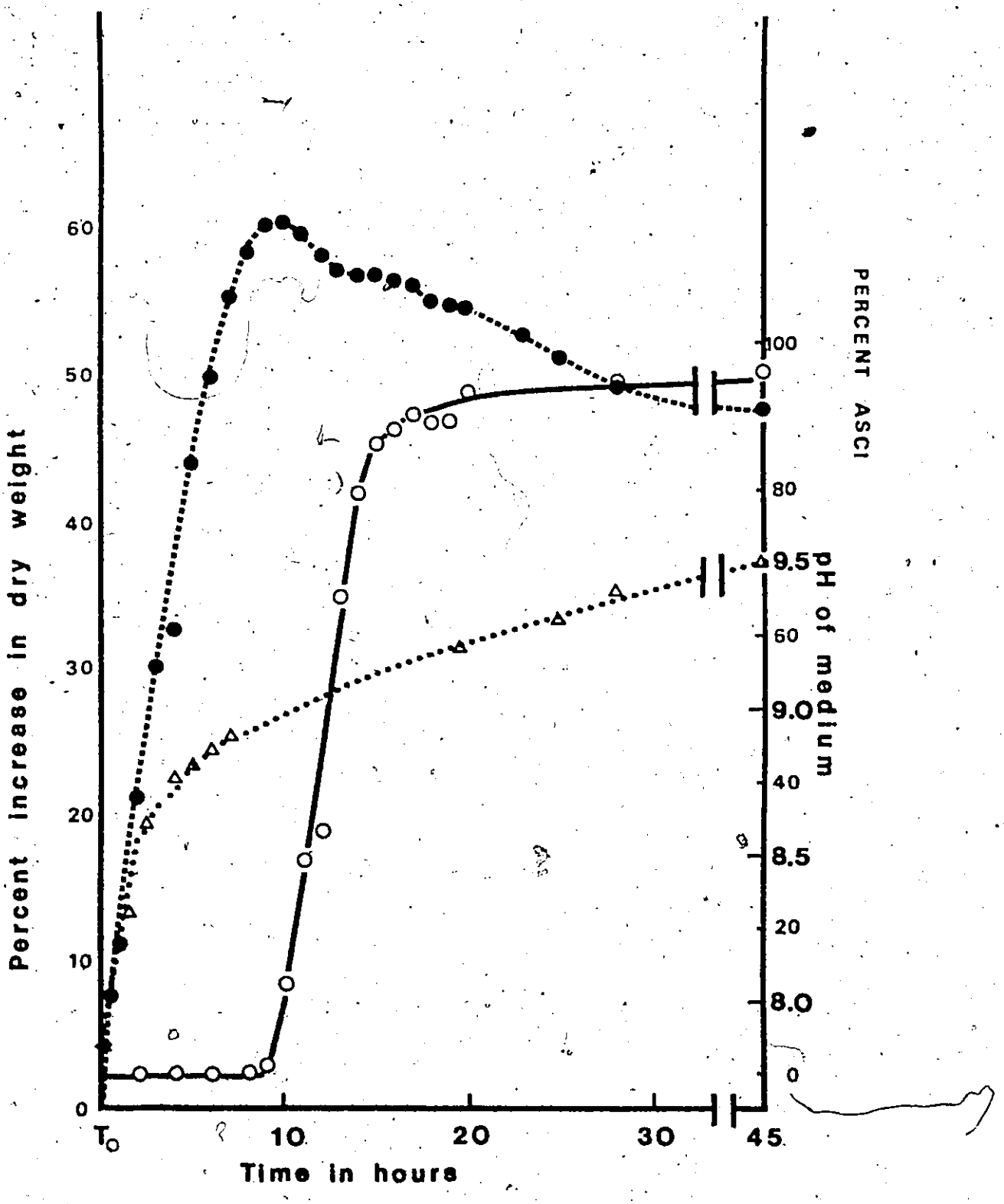

4.0
3.0
2.0
log number of cells [x10⁴]
0
4
8
12
20
Time in hours

Figure 10: Overall changes during sporulation.

●·····● Change in dry weight. Samples of 150 ml of sporulation culture were dried and weighed as described; T_o weight of sample - 157.5 µg.

○—○ Percent asci in the culture; each point represents a count of 400 cells.

△····△ Change of pH of medium during sporulation.


Percent increase in dry weight
0
10
20
30
40
50
60
PERCENT ASCI
100
80
60
40
20
0
pH of medium
9.5
9.0
8.5
8.0
T0
10
20
30
45
Time in hours

increase in dry weight represents an incorporation of 10% of the total amount of acetate present in the medium into cell components. The effect of acetate utilization is an extensive and rapid change in pH from an initial value of 7.7 to a final of 9.5 (see Fig. 10). It is assumed that the rise in pH is primarily due to the appearance of carbonate and bicarbonate in the medium as a consequence of respiration of the acetate (Esposito et al., 1969).

Observation using a phase contrast microscope showed that a noticeable increase in refractility of a large number of cells occurred within one half hour after transfer to sporulation medium and was concomitant with the disappearance of the initially very prominent vacuole. Spores were first observed after 9 hrs in sporulation medium which corresponds in time with the attainment of greatest dry weight. By T_{15} virtually all cells contained spores. Routinely between 90-95% of all cells produced ascospores.

Examination of thin sections of sporulating yeast cells with the electron microscope indicated that the spores within an individual ascus did not always develop in a completely synchronous manner but rather showed differences, particularly with respect to spore wall development. To see whether asynchrony was reflected in an increase in the average number of spores per ascus as sporulation proceeds, spore counts were routinely made. A typical example is shown in Table 3. Clearly the average spore number per ascus remained constant. It must be mentioned at this time that

Table 3

Number of Spores per Ascus During Sporulation

Time after T_o hours	Number of spores per ascus					Average number of spores per ascus**
	0	1	2	3	4	
12	124	4	40	27	5	2.43
14	105	6	63	20	6	2.27
17	55	7	100	33	5	2.25
20	32	15	100	48	5	2.26
28	15	6	118	53	8	2.34
45	9	11	125	48	7	2.27

* Each count involved 200 cells.

** Only cells containing spores were used to calculate the spores/ascus value.

the scoring system used tended to minimize the number of spores per cell. As was pointed out earlier, all buds regardless of size were counted as separate cells. At the time of transfer of vegetative cells to sporulation medium, a substantial number of them have buds of approximately one half the size of the mother cell, or less. It appears that in most instances these cell pairs only develop four spores between them; in other words, they behave as one physiological unit. If the bud is very small, only one spore develops in it, and it is almost exclusively this circumstance which gives rise to the one-spored asci in Table 3. The same can be said about two spored asci; if the bud is about half the size of the mother cell, four spores will develop - two in the mother cell and two in the daughter cell.

Figure 10 shows that there is a gradual decline in dry weight after T_{10}. This decrease in weight was accompanied by the appearance of phosphorus and UV absorbing material in the medium (Table 4). Ramirez and Miller (1964) and Kane and Roth (1974) reported the appearance of amino acids and carbohydrate in the medium during sporulation. Since the rapid increase in the amount of UV absorbing material and phosphorus in the medium coincides with the attainment of maximum sporulation percentage (see Fig. 10), it is possible that the appearance of metabolites in the medium is an indication of the deterioration of the ascus and the attainment of functional independence by the spores.

Table 4

Appearance of Cell Products in the Sporulation Medium

Time in sporulation medium (hrs)	OD_{260}	OD_{280}	OD_{720}	
			orthophosphate	ash phosphorus
.5	0	0	< 0.01	< 0.01
2.5	< 0.01	< 0.01	< 0.01	< 0.01
5	< 0.02	< 0.02	0.01	0.01
7	0.04	0.04	0.01	0.01
10	0.06	0.05	0.02	0.02
15	0.16	0.11	0.04	0.05
19	0.30	0.21	0.04	0.06
23	0.49	0.36	0.05	0.06
30	0.62	0.46	0.06	0.08
41	0.86	0.66	0.06	0.10
45	0.81	0.60	0.08	0.11
48	0.84	0.64	0.07	0.10

Sporulation culture samples were withdrawn at intervals. The medium was analysed after removal of cells by filtration using 0.45 µm Millipore filters. UV readings were taken using T_o hour culture medium as a blank. The maximum ash phosphorus values observed correspond to 2.5 mg of phosphorus per 10^{10} cells.

Changes in Polyphosphates During Sporulation

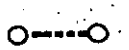
The time required to centrifuge cells from growth medium, wash and resuspend in sporulation medium was between 1 3/4 to 2 1/2 hrs. To monitor the changes which occur during this time, samples of 10^{10} cells were removed just prior to transfer and immediately following resuspension in sporulation medium and analysed in the appropriate manner. The corresponding points on the graphs are designated T_v and T_o respectively and are separated by an arbitrary time interval of 2 hrs.

Total polyphosphate levels were found to be constant for the first two hrs after transfer, showed a decline between 2 and 4 hrs into sporulation and were once again constant until T_{10} (Fig. 11). The initial drop occurs about the time DNA synthesis as a prelude to meiosis takes place.

Polyphosphate fraction 1 (Fig. 12), following an early peak at $T_{\frac{1}{2}}$ becomes stable at slightly reduced levels from T_2 to T_8. The gradual increase occurring between T_8 and T_{12} is followed by a sharp decline which starts at T_{14} and continues until T_{45}.

The changes in fraction 2 levels up to T_4 show a pattern which is somewhat similar to that observed during vegetative growth except that the fluctuations are smaller and compressed in time (Fig. 12). The initial increase in fraction 2 occurring between T_v and T_o may be ascribed to the partial hydrolysis of fractions 3 and 4. The other

Figure 11: Changes in total cell phosphorus, total polyphosphate and orthophosphate during sporulation.


Total cell phosphorus


Total polyphosphate


Orthophosphate


Appearance of spores (as % asci; see Fig. 10).

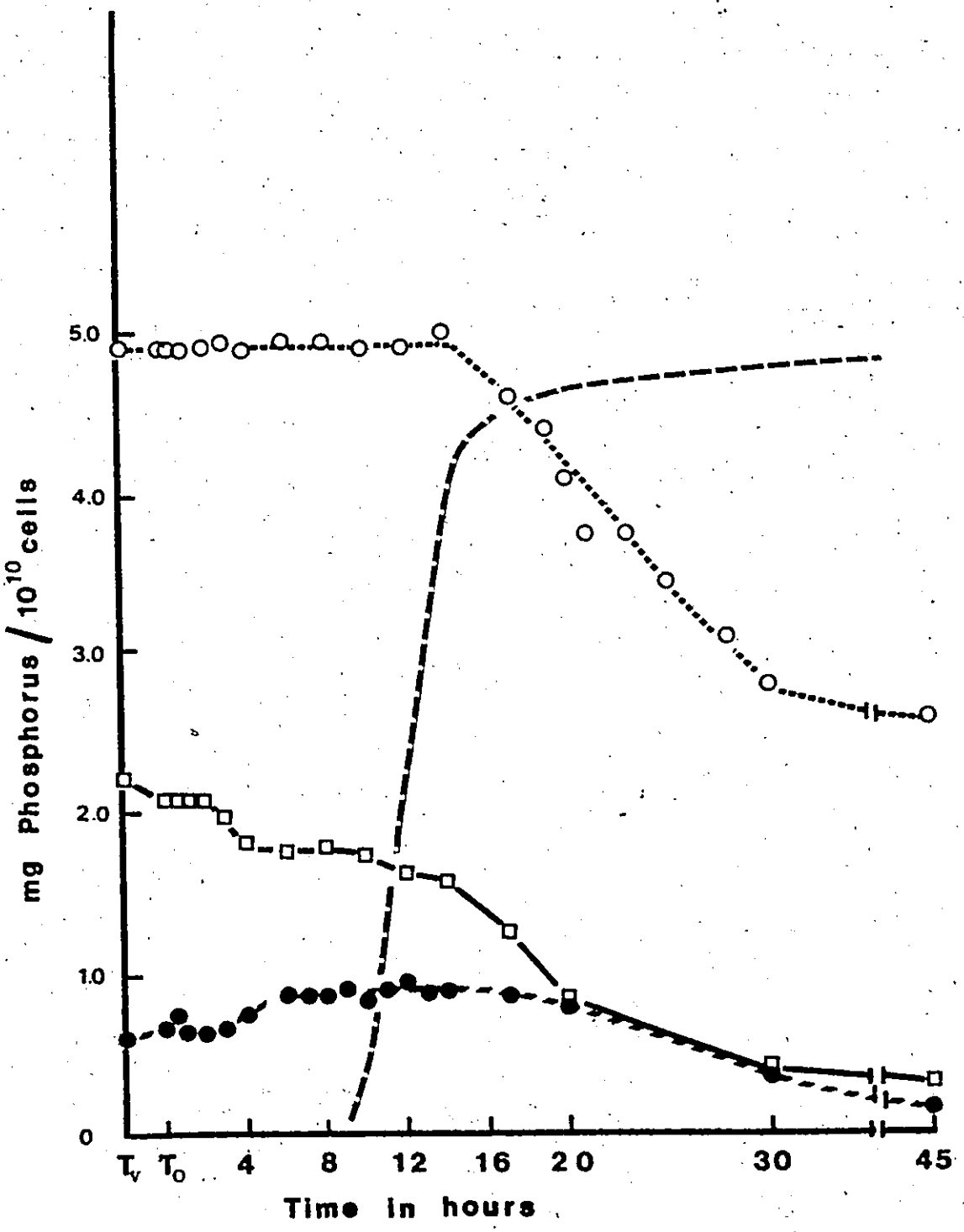
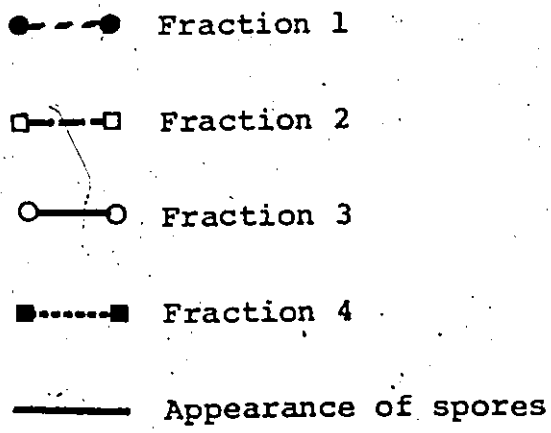
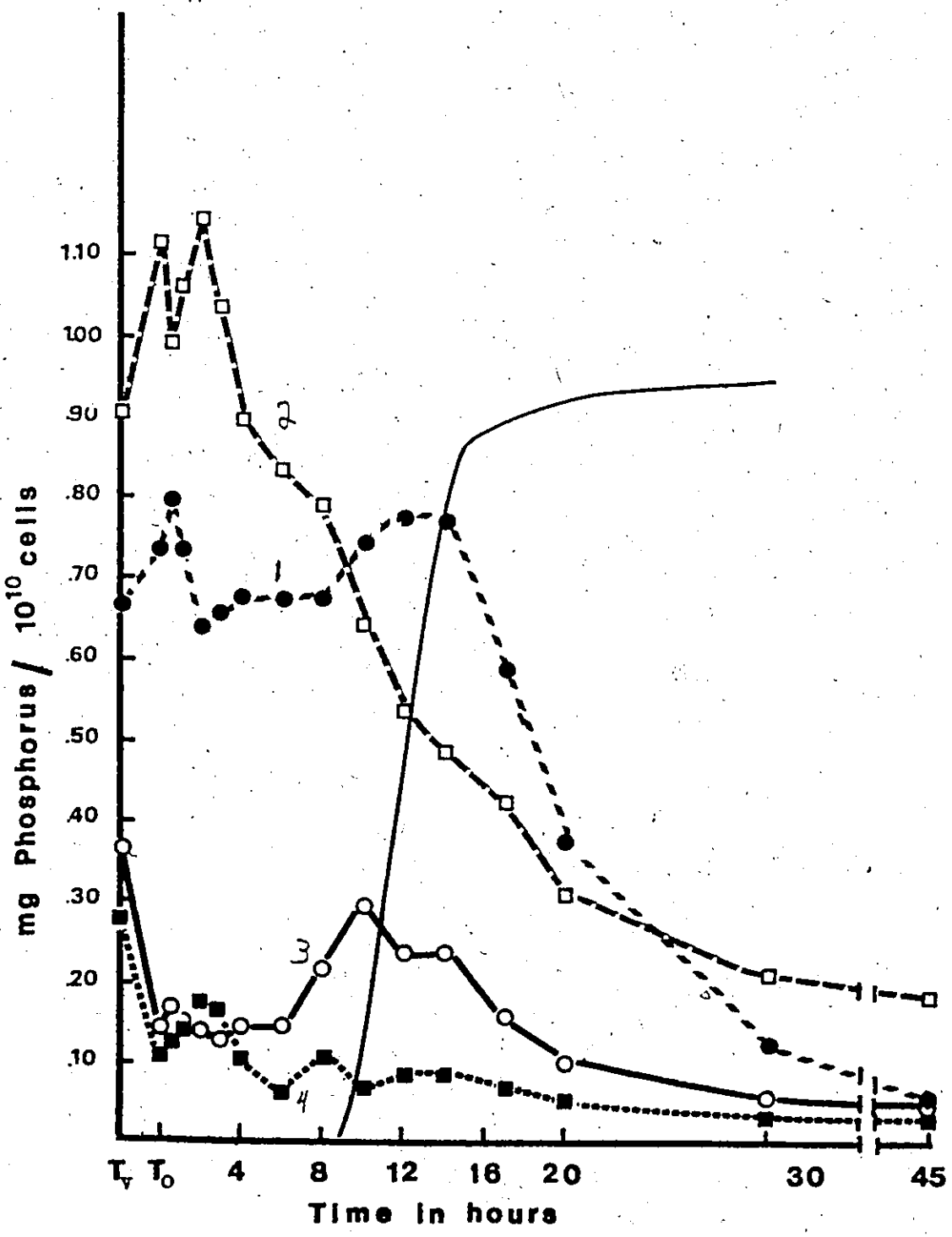

5.0
4.0
3.0
2.0
1.0
0
mg Phosphorus / 10^{10} cells
T_v T_o 4 8 12 16 20 30 45
Time in hours

Figure 12: Changes in the various polyphosphate fractions during sporulation.




1.10
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
mg Phosphorus / 10^{10} cells
1
2
3
4
5
T_v T_o 4 8 12 16 20 30 45
Time in hours

changes occurring thereafter, including the continued rapid decline after T_2 cannot be related to changes occurring in other polyphosphate fractions by a simple cause-effect relationship.

Fraction 3 shows a sharp decline during the time the cells are changed from growth- to sporulation-medium, remains constant between T_o and T_6 and shows a transient increase thereafter with a peak at T_{10}. The increase begins about the time DNA synthesis is completed.

Fraction 4 decreases between T_v and T_o analogous to the change shown by fraction 3 but shows a modest recovery following exposure to sporulation medium. From T_4 onward the fraction declines gradually.

Changes Occurring in Other Phosphorus Containing Fractions During Sporulation

Orthophosphate

After a transient increase in orthophosphate level during the first hour after transfer of cells to sporulation medium a larger increase occurs between T_3 and T_6. Orthophosphate then remains constant until virtually all cells have sporulated (see Fig. 11). The amount of orthophosphate present during this time is approximately 50% greater than the maximum level observed during vegetative growth.

RNA

Extraction of RNA from sporulating cells by the method of Herbert et al. (1971) brings considerable quantities of carbohydrates into solution. They react with the orcinol reagent to varying degrees and thus render an accurate ribose analysis impossible.

Extracts prepared by the method of Ogur and Rosen (1950) contained less carbohydrate but one extraction was not sufficient for the recovery of all RNA present. It was found that up to the time of the first appearance of spores two extractions were sufficient but thereafter (i.e. after T_8) a third extraction was needed to ensure total recovery of all RNA present. Exposure of purified commercial yeast RNA (Schwarz Bioresearch) to the same conditions did not result in an increase in OD_{260} for the first 72 hrs. This rules out the likelihood of RNA estimates which are too high as a consequence of nucleic acid hydrolysis. The OD_{260} of extracts prepared by the method of Herbert et al (1971) was virtually identical with that of extracts prepared according to Ogur and Rosen (1950) at least up to T_8. On the other hand, ribose analysis of the same extracts by the orcinol method gave consistently higher RNA estimates. The comparison shown in Table 5 shows a small systematic difference of the OD_{260} values between the two extraction methods which is within the limits of variation normally found among equivalent

Table 5

Comparison of RNA Estimates

RNA estimates of sporulating yeast cells

Time in sporulation medium	Extracts prepared according to Ogur and Rosen (1950)		Extracts prepared according to Herbert et al. (1971)	
	OD_{260} analysis (mg RNA)	Ribose analysis (mg RNA)	OD_{260} analysis (mg RNA)	Ribose analysis (mg RNA)
0	13.5	27.2	12.3	43.8
1	14.0	23.5	12.8	46.9
2	14.0	22.8	12.3	46.9
3	13.2	22.4	12.0	46.2

Extracts were prepared as stated in Methods on samples of 1×10^{10} cells. In each case Yeast RNA was used as a standard.

samples. But it is clear that RNA estimates based on the orcinol method are much too high. As shown in Fig. 13a, RNA levels were found to increase slightly during the first two hrs of sporulation, decline to approximately 65% of the initial value by the time 80% of the cells contain spores (i.e. at $T_{1\,4}$) and then remain almost constant up to $T_{4\,5}$. A small but consistently noticed transient increase after $T_{1\,4}$ does not appear to be correlated with any overt change in the culture.

Phospholipids

Phospholipid content (Fig. 13b) was found to be unchanged for the first 4 hrs, increased nearly 50% between T_4 and T_9 and remained at this new level for approximately 10 hrs. By $T_{4\,5}$ a reduction to 60% of the initial level has occurred. The increase occurring between T_4 and T_9 corresponds developmentally with the onset of meiosis and prospore formation. The decrease indicated after $T_{1\,7}$ may be the consequence of breakdown of phospholipids in the epiplasm and subsequent leakage into the medium (see Fig. 11 and Table 4) or it may be a reflection of altered extraction behaviour as the spores mature.

Residue phosphorus

The effect of two different extraction procedures on the amount of phosphorus remaining in the cell residue is shown in Fig. 14. Extraction by the method of Ogur and Rosen (1950) up to and including the extraction of RNA

Figure 13a: Changes in RNA levels during sporulation.

Extraction of RNA was carried out according to the scheme of Ogur and Rosen (1950) as described under Methods. Ribonucleic acid was estimated by measuring the OD_{260}. Yeast RNA was used as a standard.

Figure 13b: Changes in the level of phospholipid during sporulation.

Extraction of phospholipids was performed according to Herbert et al. (1971) as described under Methods. Lipid phosphorus was determined by measuring the orthophosphate content of aliquots of extracts after ashing.

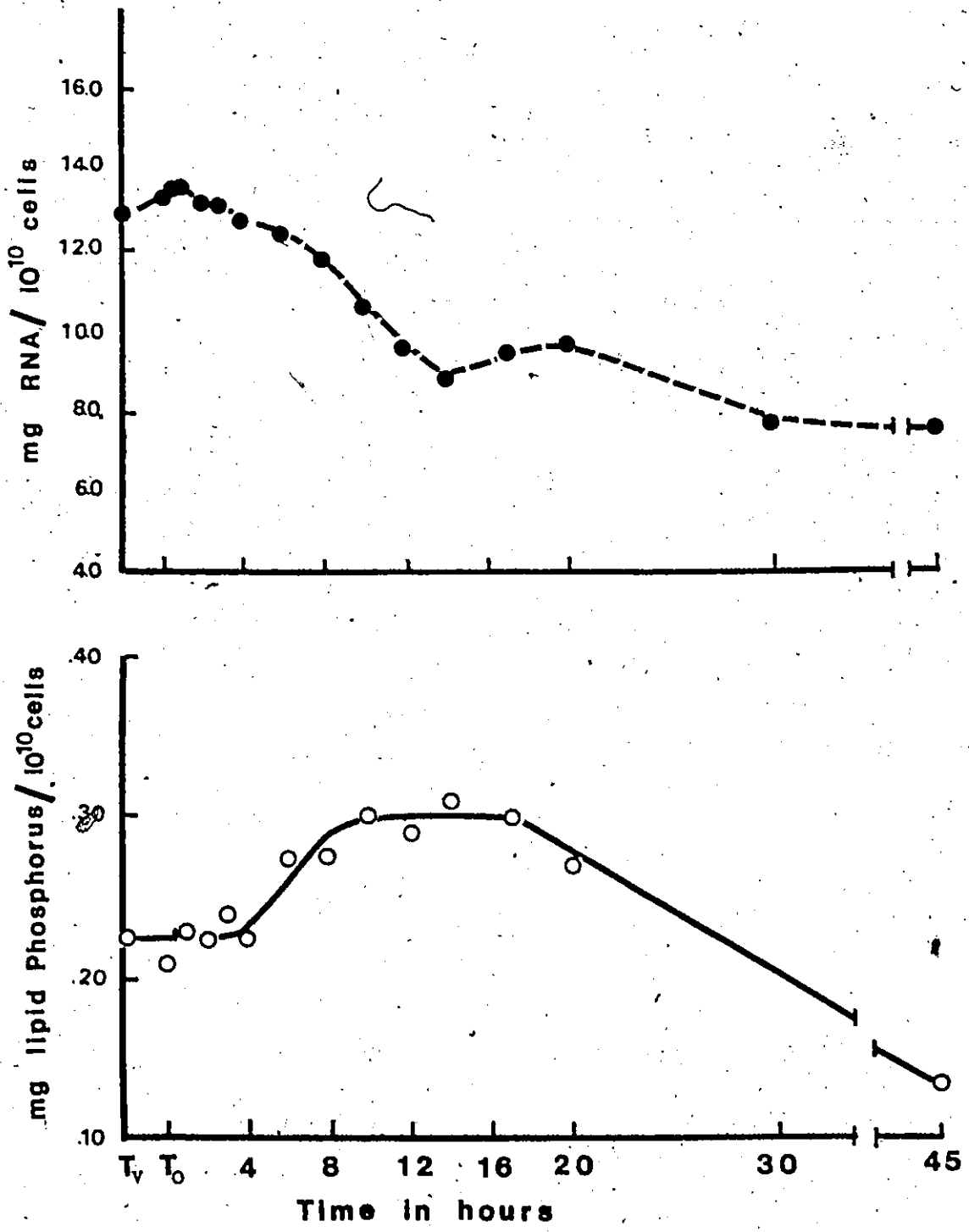

16.0
14.0
12.0
10.0
8.0
6.0
4.0
mg RNA / 10^{10} cells
.40
.30
.20
.10
mg lipid Phosphorus / 10^{10} cells
T_V T_0 4 8 12 16 20 30 45
Time in hours

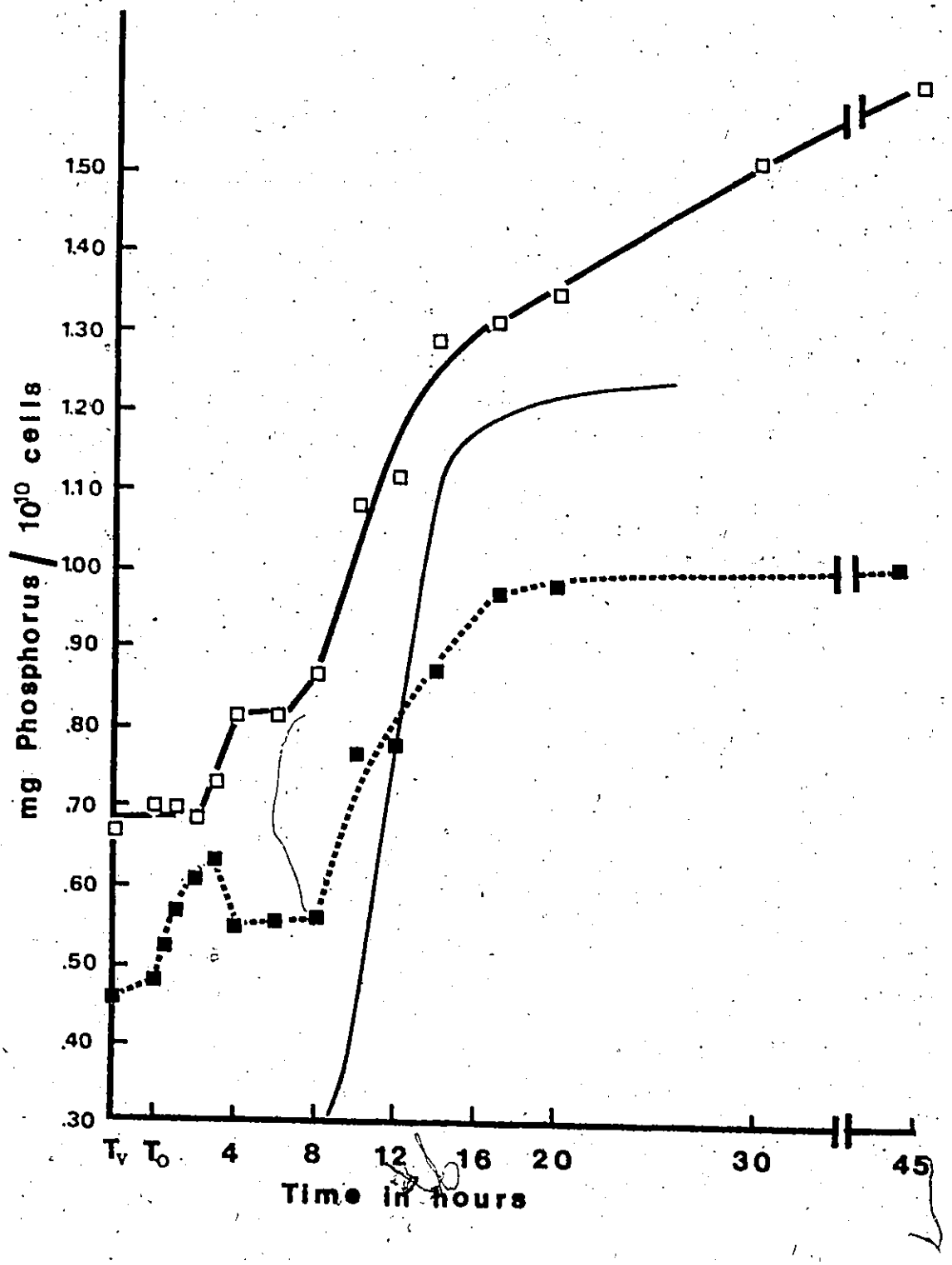
Figure 14: Changes in residue phosphorus during sporulation.

Changes in residue phosphorus were determined after extraction of cells by two different extraction methods as described in Methods.

■·····■ Residue phosphorus determined after extraction by the method of Ogur and Rosen (1950) (curve #1).

□——□ Residue phosphorus determined after extraction of polyphosphates according to Liss and Langen (1960) followed by extraction of RNA according to Trevelyan and Harrison (1956) (Curve #2).

——— Appearance of spores.


mg Phosphorus / 10^{10} cells
1.50
1.40
1.30
1.20
1.10
1.00
.90
.80
.70
.60
.50
.40
.30
T_v T_o
4
8
12
16
20
30
45
Time in hours

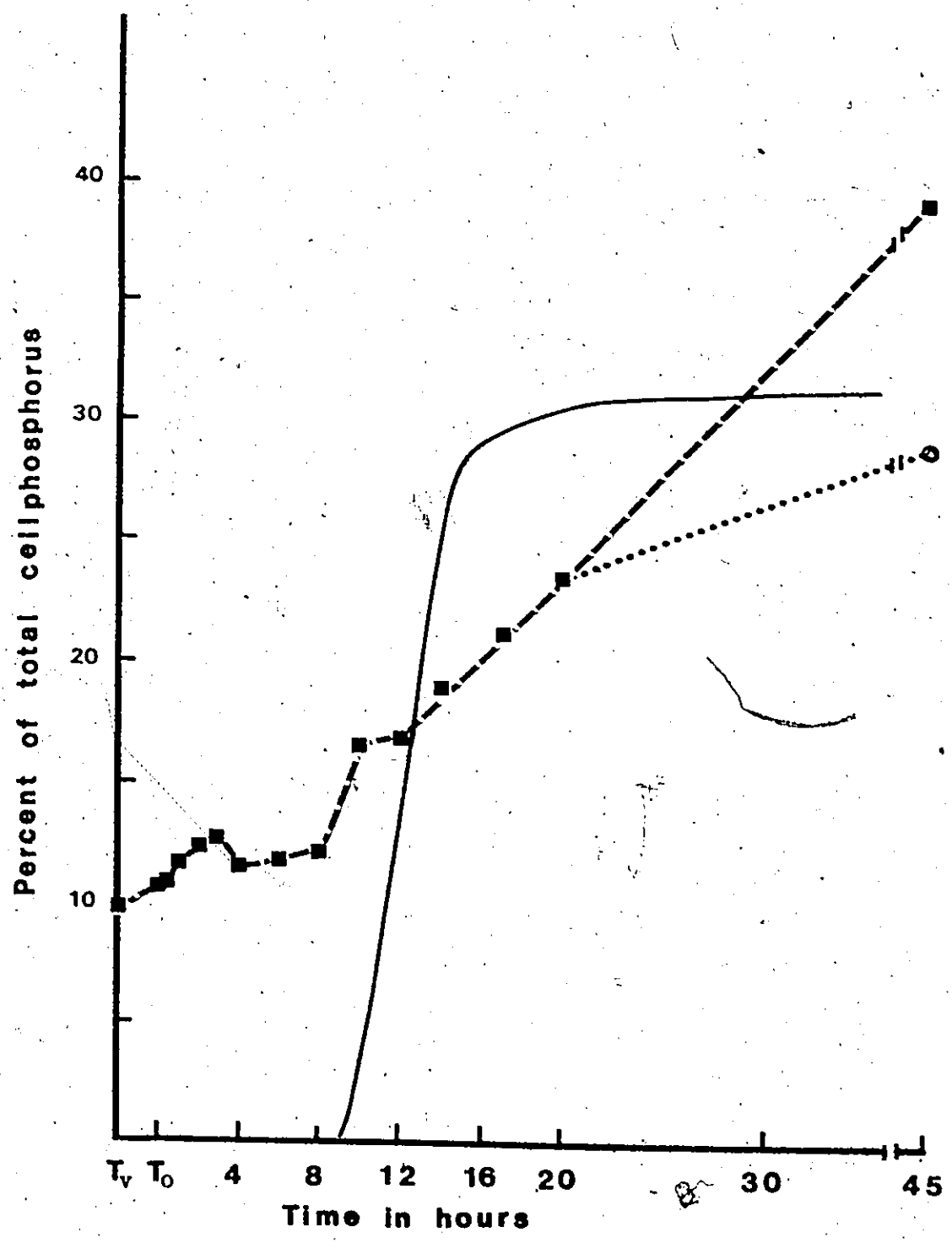
would be expected to result in the removal of all phosphorus with the exception of that contained in DNA and phosphoproteins. Determination of residue phosphorus by ashing shows that the amount of phosphorus in this fraction increases as sporulation proceeds (Fig. 14; curve 1). The increase becomes more pronounced about the time spores first become visible. The implication is that either a new phosphorus containing spore component is synthesized which is not extracted with the method employed or that the spores are much more resistant to extraction. The magnitude of the increase in residue phosphorus becomes clearer when the latter is expressed as percent of total cell phosphorus (Fig. 15). It was found that three extractions to remove DNA (according to the same method) resulted in the removal of an additional 25% of the residue phosphorus, at least one half of which was not DNA associated and appeared as orthophosphate in the extract. The presence of an acid labile phosphorus containing component may indicate that long chain polyphosphates which are difficult to extract may still be present. Evidence that polyphosphate is not always removed by the extraction scheme used comes from a comparison of the above results with those obtained by a different method of extraction. Samples extracted by the method of Liss and Langen (1960), which removes polyphosphates, were subsequently extracted with 0.5 N $HClO_4$ for 2 hrs at 37°C to remove ribonucleic acids

Figure 15: Residue phosphorus expressed as percent of total cell phosphorus.
The values of curve #1 (Fig. 14) are expressed in terms of the percentage of the total cell phosphorus.

■---■ Residue phosphorus

——— Appearance of spores

····· Residue phosphorus after 3 successive extractions for DNA (.5 N $HClO_4$ 70°C for 20 mins.) at T_{45}.


40
30
20
10
Percent of total cell phosphorus
T_v T_0 4 8 12 16 20 30 45
Time in hours

(Trevelyan and Harrison, 1956). The residue remaining still contains DNA, phosphoproteins and phospholipids. In other words, it ought to be very similar in terms of phosphorus content to the fraction obtained by the method of Ogur and Rosen (1950) except for the additional phospholipid phosphorus. Examination of the result obtained (Fig. 14; curve 2) shows an initial difference which is equal to the initial phospholipid phosphorus content determined earlier (Fig. 13b). The transient increase between T_o and T_4 seen in curve 1 is absent. From this evidence, and the similarity in both size and duration of the increase noted in Figure 14, curve 1; and that found for polyphosphate fraction 4 (Fig. 12), it was concluded the difference in the nature of curve 1 and 2 was due to incomplete polyphosphate extraction. The increase in the amount of residue phosphorus occurring between T_3 and T_4 follows the pattern of increase in phospholipids (Fig. 13b) in magnitude and time. The increasing discrepancy between the two curves in later sporulation stages is due to the incomplete extraction of RNA by the method of Trevelyan and Harrison (1956) (Fig. 14; curve 2).

DISCUSSION

The growth characteristics of the vegetative culture (Fig. 1) are very similar to those reported by Croes (1967) who used undefined medium of similar composition to that employed in this study. Deceleration begins when cell density reaches approximately 5×10^7 cells per ml and continues for 4 - 5 hrs, following which a new and much slower growth rate is maintained for at least 10 hrs. Katchman and Fetty (1955), using a defined medium showed essentially the same pattern but found the deceleration phase to be considerably shorter. Since deceleration is believed to be the consequence of the sequential disappearance of "non essential, but growth accelerating nutrients" (Herbert, 1961), the longer deceleration period reported here may reflect the complex medium used. The average dry weight per cell during stationary phase was approximately 75% that of the mid log phase value which is in good agreement with the results of Keup (1967) and appears to be a general phenomenon in microbial organisms (Herbert, 1961). In contrast, Katchman and Fetty (1955) found that the dry weight per cell remained constant throughout. The explanation for the discrepancy probably involves the difference in growth medium composition.

The latter authors used defined medium with a higher than usual glucose content (40 g/l). This makes it probable that the limiting factor for growth was nitrogen and not glucose. Under these conditions considerable quantities of glycogen are deposited (Herbert, 1961) which would lead to an increase in the dry weight as compared to the condition in which glucose is the (growth) limiting factor. Croes (1967) and Keup (1967) found that the beginning of deceleration coincided with the disappearance of glucose from the growth medium. The new and much slower growth rate which follows deceleration is attributed to aerobic utilization of the accumulated ethanol.

Polyphosphate Changes Occurring During Growth

As has been shown in Figure 2 the amount of polyphosphate increases throughout the early monitored stages, i.e. during the logarithmic growth phase as well as the period of deceleration. Only with the beginning of stationary phase do polyphosphate levels begin to decline. The rate of accumulation is fastest during the fermentative stage of growth and declines as the growth rate declines. These findings are similar to those of Katchman and Fetty (1955) except that in their case the accumulation and the subsequent decline of polyphosphate appears to be compressed into a smaller time interval and maximal values are reached at

the beginning of deceleration. From the results obtained here it is evident that polyphosphate accumulation is supported by fermentation as well as respiration. During stationary phase the polyphosphate level remains unchanged but the molecular composition undergoes a marked shift toward a lower average chain length. The striking changes in the levels of the four constituent fractions (Figs. 3-6) suggest that their interrelationship may not be as clear cut as Langen et al. (1962) report. Their results indicate that polyphosphate synthesis in phosphorus starved cells after transfer to fresh medium containing phosphorus leads to an immediate build-up of fraction 4 followed by a sequential increase in the levels of fractions 3,2 and 1 over a three hr period (this is supported by their results with short term ^{32}P labelling mentioned in the Introduction). Unfortunately the experimental conditions these authors employed were not clearly stated. It is presumed that the reported increase in polyphosphates occurred under conditions differing from those reported in an earlier paper (Liss and Langen, 1961) because although the basic description of the experiments is the same, the results are not. In the above mentioned paper (Liss and Langen, 1961) it is reported that fractions 1 - 3 increase linearly with time, differing only in the rate of accumulation whereas fraction 4 increases at a constantly diminishing rate and begins to decline approximately 2 hrs

after transfer to phosphorus containing medium in which nitrogen was limiting. The discrepancy in results between seemingly very similar experiments emphasizes the difficulty involved in establishing a cause and effect relationship based on different approaches to the same problem. Evidence for this was also provided by Ehrenberg (1961) who showed that the pattern of polyphosphate accumulation depends to a significant extent on the concentration of phosphorus in the medium.

Before an attempt of interpretation of the data shown in Figures 3 - 6 is made, the following stipulations have to be introduced: (1) Polyphosphate synthesis involves the addition of orthophosphate to existing polymer molecules of intermediate chain length; (2) Breakdown of polyphosphates occurs to a significant extent by random scission of the polymer; (3) The temporal sequence of events is a reflection of their functional relationship.

The first assumption is based on the evidence of a number of workers (Kuhl, 1960; Harold, 1966) involving a variety of different organisms; they have shown that long chain polyphosphates are the first to accumulate or to become most heavily labelled, under the appropriate conditions. *In vitro* experiments by Kornberg et al. (1956) using polyphosphate kinase from *Escherichia coli* showed that only long chain polyphosphates accumulated and the necessity of a primer was postulated (Harold, 1966).

The second assumption is based on the results of labelling experiments, prihcipally those of Liss, Langen and Lohmann (1958; 1960; 1962; 1962) which showed a gradual shift of label from (long chain) fraction 4 to the other fractions during net polyphosphate synthesis. It is also supported by the findings of Mattenheimer (1956a; 1956b; 1956c) who showed that breakdown of polyphosphates by polyphosphatases brought about a gradual reduction in molecular size with little or no liberation of orthophosphate. Lastly, it is probable that at least part of the breakdown of long chain polyphosphates is non-enzymatic and is mediated by divalent cations in the cell.

The third stipulation needs the additional qualification that changes in the size of the four fractions are primarily the result of internal shifts. These shifts may involve the addition or outflow of orthophosphate to or from the polyphosphate pool during periods of net synthesis or degradation.

As can be seen from Figure 5, fraction 3 increases throughout the early monitored growth phase. It is proposed that this fraction acts as the primer for polyphosphate synthesis. Elongation of fraction 3 molecules leads to a net increase in fraction 4. Simultaneous random polyphosphatase action leads to a reduction of the average polymer size of all fractions. In the case of fraction 4, the majority of polymer scissions will result in fragments

of a size which can act as a primer for continued synthesis. Fraction 3 and 4 thus may represent at least in part a cyclic system during net polyphosphate synthesis. This is reflected in the rapid increase in fraction 4 (Fig. 6). Scission products of fraction 4 which are too small to act as a primer constitute additions to fractions 1 and 2 as well as the orthophosphate pool. The partial recycling of fraction 3 leads to reduced addition to fraction 2 and therefore to a reduction in the pool size (Fig. 4). This in turn leads to a (much less pronounced) lowering of fraction 1 levels (Fig. 3). As net polyphosphate synthesis slows, the outflow from fractions 3 and 4 exceeds that from the proportionately much smaller pools of fractions 1 and 2, and consequently an increase in the low molecular weight fractions occurs. This trend continues until steady state conditions are established with the onset of stationary phase.

Role of Polyphosphates in Cellular Metabolism

The rapid accumulation of polyphosphates by previously phosphorus starved cells has been described as "overcompensation" (Liss and Langen, 1962). This phenomenon is even more accentuated when the phosphorus starved cells are incubated in a "no growth" medium containing orthophosphate and glucose (Harold, 1966). This was interpreted by Schmidt (1966) to be a consequence of the abundant supply of ATP,

more specifically, a high ATP/ADP ratio. The question in the present instance is, what factors are involved during "normal" growth which bring about the increase in polyphosphates observed?

The rapid changes in fraction 2 and 4 begin just prior to deceleration of the growth rate (Fig. 1) and consequently coincide with the disappearance of glucose from the medium and the attainment of respiratory capacity by the cells (Croes, 1967; Keup, 1967). More importantly it coincides with the beginning of a sharp decline in RNA content (Fig. 8). This antagonistic behaviour between polyphosphates and nucleic acids has been observed by a number of workers (Kuhl, 1960; Harold, 1966), but the relationship has remained obscure. Bailey and Parks (1972) measured ATP levels in yeast during "normal" growth and found a sharp increase at the end of log phase, peak levels at the beginning of deceleration and a decline thereafter. The rise in ATP level thus coincides with the decline in RNA and the increase in polyphosphate synthesis.

The functional relationship of these changes is interpreted to be as follows: The sharp decline in total RNA is indicative of reduced ATP demand while a high orthophosphate level (Fig. 2), possibly elevated by nucleotide hydrolysis, and undiminished phosphorylation capacity would tend toward high ATP levels as found by Bailey and Parks (1972). Under these conditions the polyphosphate pool

becomes a sink for excess phosphorylating capacity. In other words, it acts as a buffer to absorb excess ATP produced. Unfortunately no information is available concerning changes in the level of ADP during growth. As Kornberg (1957) demonstrated, the reaction catalysed by polyphosphate kinase *in vitro* is readily reversible even at low ADP levels. In order to determine whether this is the case with yeast *in vivo*, ADP levels during growth - analogous to the experiments by Bailey and Park for ATP - will have to be determined.

Changes in Polyphosphates During Sporulation

As can be seen from Figure 11, the phosphorus content per cell remains unchanged for at least the first 14 hrs of sporulation. Any changes in the magnitude of the various phosphorus fractions must, therefore, be the result of shifts in the internal pools. During the period between T_v and T_o the net change in polyphosphate level is negligible (Fig. 11) but there is a significant reduction in the pool size of fractions 3 and 4 and a concomitant increase in fractions 1 and 2 (Fig. 12). This shift occurs during a period when the energy balance of the cells would be expected to deteriorate in the absence of an external energy source. The reduction in fractions 3 and 4 may thus be a consequence of increased utilization and/or a lowered rate of synthesis. Surprisingly, during the first 2 hrs after transfer of the

cells to sporulation medium, total polyphosphates (Fig. 11) remain unchanged. This is also true for orthophosphate with the exception of a small and transient initial increase (Fig. 11). This may indicate that (1) the energy (ATP) generating system of the cells is sufficient to satisfy the increased demand and the energy potential of the polyphosphates is therefore not utilized, or (2) the polyphosphates are not an energy storage system and the constant level reflects a lack of demand for stored phosphorus. The small increase seen in fraction 4 between T_o and T_2 (Fig. 12) may indicate that the shift in the energy balance is toward an increased polyphosphate synthesis and lack of net accumulation of polyphosphate is the result of increased turnover. Determination of ATP/ADP ratios, as mentioned above, may provide a partial answer.

The functional interrelationship of the four polyphosphate fractions (Fig. 12) suggested earlier for the growth phase is not readily apparent from the fluctuations in the pool sizes. The changes in the level of orthophosphate (Fig. 11) and polyphosphate fractions 1 and 2 (Fig. 12) during the first 2 hrs of sporulation are likely the consequence of rapidly changing pool demands caused by an increased turnover and (although small) increase in RNA content; in the process the normal pattern of the precursor-product relationship of the four fractions may be obscured.

There are changes in the various fractions beyond this time however which suggest that there may be differences in the metabolic function of the different pools. The main indication is found in the rapid decline of fraction 2 between T_2 and T_{14} which occurs at a time when other polyphosphate fractions remain constant or increase in amount suggesting a donor function for this fraction which is not shared by the other pools during this period.

The increase in fraction 3 between T_6 and T_8 begins at a time when orthophosphate reaches maximal levels, but is not followed by an increase in fraction 4 as might be expected. An explanation for this discrepancy will be offered later.

Changes in RNA Levels During Sporulation

Reports in the literature present contradictory results concerning changes in RNA content during sporulation. Esposito et al. (1969) found a large increase in RNA during the first 10 hrs of sporulation followed by a gradual decline such that after 50 hrs the RNA level exceeded the T_o value by 20%. For RNA analysis these authors used the orcinol method which, as has been stated earlier, is susceptible to carbohydrate interference. Their RNA determinations are therefore probably not reliable. Support for this criticism is the coincidence that the time of maximal RNA level they reported corresponds with the period of maximum soluble glycogen accumulation (Kane and Roth, 1974; Fig. 3b).

Chaffin et al. (1974) report an increase in RNA beginning about T_4. Sando and Miyake (1971) report a small initial increase followed by a gradual decline and Croes (1967) failed to detect an increase and found a gradual decrease to approximately 50% of the T_o value after 24 hrs. The latter results are similar to the pattern shown in the present work. The more rapid decline in RNA level (Fig. 13A) may be ascribed to the higher sporulation percentage (90 - 95% as compared to their 70 - 75%) and the shorter time course of sporulation obtained with the yeast strain used in this study. The transient increase in RNA level following $T_{1\frac{1}{2}}$ has not been reported elsewhere, possibly because of the much longer time intervals between successive readings taken by other investigators. It is not believed to be an artifact since it was a consistent feature of all analyses done. It is of interest in this connection that Esposito et al. (1969) found two peaks of protein synthesis during sporulation. The second peak occurred at a time when sporulation approached the maximum percentage of asci observed and coincided with a period of extensive lipid synthesis. In terms of the present work this may correspond to the period during which the increase in RNA level occurred. It is possible that these events are associated with the deposition of a fatty layer on the spore surface as reported by Miller and Hoffman-Ostenhof (1964).

Changes in Residue Phosphorus

It is unlikely, in the writer's opinion, that the increasing amount of residue-associated phosphorus is indicative of the accumulation of a new sporulation-specific compound. Evidence has been presented earlier which suggests that polyphosphates are not always completely removed by standard extraction procedures. A marked increase in the amount of residue phosphorus parallels the appearance of spores in the culture (Fig. 14); no further increase during spore maturation is observed. This is interpreted as evidence that the spore envelope is considerably more resistant to extraction than the vegetative cell envelope and that of developing ascus. The greater resistance may be imparted by the lipids associated with the outer spore coat (Illingworth et al.1973).

During three successive extractions for DNA according to the method of Ogur and Rosen (1950), it was found that while the amount of UV absorbing material present in the third extract was negligible, a considerable quantity of phosphorus appeared in this extract, approximately half of it as orthophosphate. Since phosphoproteins are resistant to hydrolysis under the conditions employed the inference is that the phosphorus extracted was part of an unextracted polyphosphate fraction, presumably of high molecular weight since they are the most difficult to solubilize. In other words, we are probably dealing with fraction 4 polyphosphates

which were not extracted with the normal method because of the additional resistance posed by the spore envelope. The true level of fraction 4 would then be not as represented in Figure 12 but to it would have to be added part or all of the increase in residue phosphorus which occurs from T_8 onward (Fig. 14, curve 1). If this is so, then the fluctuations in the four polyphosphate fractions during sporulation, with the exception of the initial period from T_o to T_2, conform with the precursor-product relationship proposed earlier although the magnitude of the changes is much smaller than that occurring during the growth phase.

Some General Comments on Problems of Data Interpretation

In any field of endeavour specific methods become established with time, often with the precedent of much usage obscuring their inherent limitations. Similarly, it is often found that hand in hand with the creation of an established way of interpreting experimental data, or looking at a specific problem, goes a tendency to try to make new findings fit into the accepted pattern and much less critical thinking is employed in examining alternate possibilities of interpretation.

In the study of polyphosphates several examples which illustrate this attitude exist. The purpose of these closing remarks is to point out instances where a more critical attitude would be desirable.

To this day reports on polyphosphates abound with references to (TCA-) soluble and (TCA-) insoluble polyphosphates, terminology which goes back to Juni et al. (1947) and Wiame (1949), notwithstanding the fact that all polyphosphates are (TCA-) soluble. It has been recognized early that the "soluble" polyphosphates are of low molecular weight (i.e. short chain), and the "insoluble" polyphosphates of much larger average molecular weight. As Katchmann and van Wazer (1954) have shown, polyphosphates and proteins co-precipitate in solution. The degree of co-precipitation depends primarily on the chain length of the polyphosphates but also on the pH of the medium and the type and size of proteins present. In fact, proteins have been used to precipitate the synthetic polyphosphates known as Graham's salt and Kurrol's salt since the 19th century. Similarly, Kulaev (1958) has shown nucleic acids and (long chain) polyphosphates form insoluble complexes during extraction. From this information it seems reasonable to suspect that the results obtained using an artificial separation of the polyphosphates into "soluble" and "insoluble" may be influenced significantly by the physiological state of the organisms which are analyzed. During periods of growth-limiting conditions such as exist at stationary phase, the size of the "soluble" polyphosphate pool may appear relatively larger simply because the amount of complexing or precipitating agent (RNA and proteins) present in the cell

is much reduced. Apparent shifts in pool sizes may therefore be misleading unless a chain length distribution analysis of each fraction is made concurrently each time an assay is performed. To the writer's knowledge all average chain length determinations of the various polyphosphate fractions have been performed using extracts from cells which were grown according to the scheme preferred by each individual investigator and the answers obtained have varied widely. It may well be that part of the variance was the consequence of the growth regime (and thus the complexing capacity of the cells).

Quite apart from the influence of complexing agents it is not at all certain that the resistance of the cell envelope to extraction remains the same during growth (and sporulation). The evidence in the present case is that spores are much more resistant to extraction than vegetative cells. It seems not unreasonable to suspect that this added resistance may affect some components of a heterogeneous population of molecules more than others; that, for instance, transfer RNA may be affected less than ribosomal RNA. With polyphosphates it may mean that some fractions are incompletely extracted (as has been suggested in the present work) or, that perhaps the average molecular weight of the molecules making up a particular extract changes. In either case misleading conclusions are likely drawn.

Lastly, the writer feels far too little attention has been paid to the problem of localization and compartmentation in relation to polyphosphate metabolism. The general attitude of most researchers in the field has been to treat the synthesis and degradation (including utilization) of polyphosphates as a unified or closed process, yet there is evidence that we may be dealing with a very heterogeneous system. The exact location of the polyphosphates within the cell has been the subject of much early speculation. There is now good evidence that some of the polyphosphates are localized in distinct granules (for review, see Widra, 1959) and in the nucleus (Grossman and Lang, 1962; Griffin, Davidson and Penniall, 1965; Griffin and Penniall, 1966; Penniall and Griffin, 1964). Indge (1968) has shown that most long chain polyphosphates are associated with the vacuolar fraction and Weimberg and Orton (1965) have presented evidence that some polyphosphates may be associated with the outer cell membrane. Most recently Kulaev (1973) has reported that different polyphosphate fractions can be found in distinct locations, but the evidence presented is far from conclusive.

Even by the most critical standards it appears that there exists a spatial segregation of the polyphosphates in the cell and it seems reasonable to suspect that the compartmentation reflects a difference in function. The

question of course is: does each compartment represent, at least essentially, one of the fractions we recognize as an extraction entity (e.g. fraction 1) as Kulaev (1973) suggests, or does it represent, esentially, a cross section of all of the extractable polyphosphates? If the former is the case the specific functional involvement of each fraction in the general metabolism ought to be recognizable in terms of pool size changes and labelling patterns. Very little work along these lines has been done. If the second assumption is correct, and in the writer's opinion, it is the more reasonable alternative, then answers to the functional specificity of different compartments will be very difficult to obtain. Some of the evidence indicating the possibility of functional diversity has already been referred to earlier (Matsuhashi, 1963; Ehrenberg,1961). The labelling patterns of the four polyphosphate fractions which were the basis of the precursor-product relationship postulated by Langen and Liss (1958) and Langen, Liss and Lohmann (1962) showed that polyphosphate fraction 1 never approached that of fractions 2,3 and 4 either in specific activity nor in total cpm. This was explained as being due to "pool dilution" by the above authors, but the more reasonable answer would be the partial utilization of fraction 3 and 4 polyphosphates or the existence of more than one pathway and/or locus of polyphosphate synthesis. It was mentioned earlier that Ehrenberg (1961)

observed an accumulation of fraction 2 polyphosphates which could not be ascribed to breakdown of long chain fraction 3 and 4. The "pool dilution" of Langen and Liss (1958) may be due to simultaneous synthesis via two separate pathways which may be spatially separated in the cell, one utilizing labelled, the other unlabelled precursor. An example of partial separation of precursor pools was provided by Langen and Liss (1960) with respect to orthophosphate. After pulse labelling yeast cells they monitored the specific activity of the cellular orthophosphate and the terminal phosphate group of ATP following transfer of the washed cells to phosphate free medium. It was found that the specific activity of the extracted orthophosphate even initially (i.e. at T_o) was substantially lower than that of the orthophosphate in the medium and declined rapidly during the first five minutes in phosphate free medium. The specific activity of the terminal phosphate group of ATP during this time increased initially to the level of the labelled orthophosphate in the medium and subsequently declined gradually but remained significantly higher than the activity of the extracted orthophosphate pool for at least 10 minutes. The authors explained these results by postulating the existence of the functional separation of the orthophosphate into two pools one of which is much more actively involved in the metabolic processes of the cell (and which includes the

labelled phosphorus) while the other, unlabelled pool participates only marginally (i.e. has a negligible turnover). It would appear that the same argument can be used to explain the labelling pattern reported by Langen and Liss (1958); Langen, Liss and Lohmann (1962) and Liss and Langen (1962) for polyphosphate fraction 1. The rapid decline of fraction 2 during sporulation observed in the present work can also be interpreted in terms of the specific involvement of this fraction in other metabolic processes as has been mentioned earlier.

From the foregoing remarks it is clear that clarification of these uncertainties will require a substantial investigative effort. The contribution of the present work has been to show that large scale changes in the proportion of the various polyphosphate fractions occur during growth and sporulation and that these changes appear to be correlated with specific changes in the metabolism of the cells. The exact nature of this interrelationship remains to be determined.

SUMMARY

Polyphosphates accumulate during growth and reach peak levels at the end of the growth phase. At this stage they represent approximately 50% of all cell phosphorus.

The initial increase is the result of the accumulation of long chain polyphosphates and occurs at a time when RNA levels begin to decline sharply. During the continuing increase in total polyphosphate content the intermediate and short chain components increase rapidly while the long chain fractions decline. A scheme is proposed which attempts to explain the functional interrelationship of the different fractions.

During the period of polyphosphate accumulation orthophosphate declines to about 65% of the peak value observed during log phase, while phospholipids remain unchanged throughout the growth phase.

During sporulation yeast cells increase in dry weight by approximately 60%. The attainment of largest dry weight coincides with the first appearance of spores in the culture. All four polyphosphate fractions decline during sporulation to a low level; the apparent decline becomes most pronounced about the time maximal sporulation is attained.

Both orthophosphate and phospholipids increase by 50% within 6 hrs after transfer of cells to sporulation medium and remain unchanged thereafter until sporulation is complete. RNA levels decline to approximately 65% of the T_o value within 15 hrs and remain almost constant thereafter, while the amount of extraction resistant phosphorus remaining in the cell residue increases. The rate of increase of this fraction becomes more pronounced with the appearance of spores and reflects the increasing extraction resistance of the cells.

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