OBSERVATIONS ON NUCLEIC ACID AND POLYPHOSPHATE IN *TORULOPSIS UTILIS*

by

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INTRODUCTION

In the course of investigations into the role of ribose nucleic acid (RNA) in the biosynthesis of protein, we have been interested in measuring the quantity of RNA and of protein nitrogen present in yeast cultures at various times during the growth cycle, as a preliminary to isotopic work. Phosphorus determinations have been complicated, however, by the association with the RNA of another phosphorus compound (labile phosphate) which was readily hydrolysed by hot acid, and was thought to be the metaphosphate described by WIAME[1]. This necessitated the development of a method for estimating nucleic acid phosphate (NAP) in the presence of the labile phosphate. By application of this method, certain aspects of the metabolism of the labile phosphate and of RNA have been observed, and the findings have stimulated interest in the general structure of these compounds.

METHODS AND MATERIALS

Growth experiments were carried out using the yeast *Torulopsis utilis* (now classified as *Candida utilis*[2]). This was grown in shake culture in 250 ml conical flasks at 25° C in a Warburg tank, each flask containing 100 ml of culture. The culture medium used was as follows:

D-Glucose ($C_6H_{12}O_6$)	3 %
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 %
Ammonium sulphate $(NH_4)_2SO_4$	0.3 %
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.05 %
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.07 %
Sodium chloride (NaCl)	0.05 %

For the inoculum in growth experiments, yeast of the required age was centrifuged, and resuspended in fresh medium in concentration sufficient to give a turbidity reading (extinction), at tenfold dilution, of about 0.15 at 420 mμ in a Unicam absorptiometer S.P. 400. (This corresponds to about 1 mg dry weight of yeast in 1 ml of culture.) 20 ml samples of the suspension were then taken at intervals during the growth cycle, and each sample was fractionated using SCHNEIDER'S[3] technique. The nucleic acid fraction was made up to 25 ml, and the absorption of an aliquot measured at 260 mμ and 310 mμ in a Beckman spectrophotometer. Other aliquots were used to determine orthophosphate and total phosphate by ALLEN'S[4] method. Protein nitrogen determinations were performed by the micro-Kjeldahl procedure of WILLITS AND OGG[5].

The condensed phosphates used in hydrolysis experiments, *viz.* sodium pyrophosphate, tripolyphosphate, trimetaphosphate and Glass 696 (a polyphosphate of chain length about 30) were kindly supplied by Albright and Wilson, Ltd. A highly condensed sodium polyphosphate was provided by Dr. P. ALEXANDER. This had been prepared as follows: potassium dihydrogen phosphate was heated

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to 640° C for 1 hour, and then cooled. An aqueous suspension of the condensed potassium phosphate thus formed was shaken with the sodium form of an anionic resin, and the resulting solution was freeze-dried to give a soluble white powder. The product, which was considered to be a linear sodium polyphosphate, was stated to have a molecular weight of the order of 10^5 as determined by light-scattering.

The commercial yeast nucleic acid used was a sample of BDH "Nucleic Acid from Yeast. Sodium Salt". A portion was reprecipitated twice with 10 volumes of glacial acetic acid.

EXPERIMENTAL AND RESULTS

Heterogeneity of nucleic acid fraction

A culture of *T. utilis* was grown for 36 hours in the synthetic medium described, starved of nitrogen by transference for 36 hours to similar medium devoid of nitrogen, and then inoculated into fresh medium. The nucleic acid and protein in samples taken during the following 24 hours of growth were measured, and the results are shown in Fig. 1.

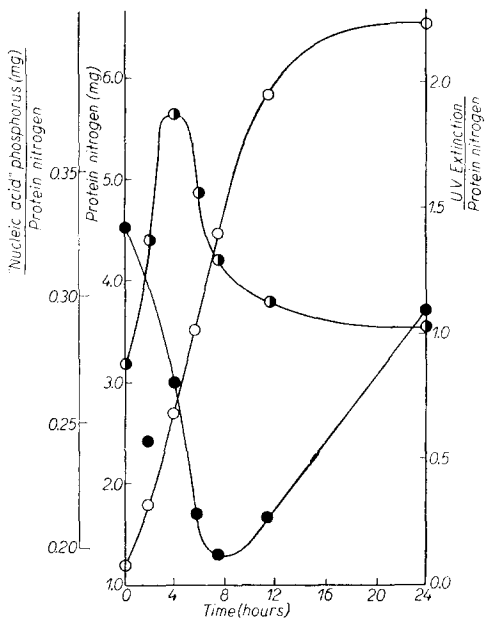

Fig. 1. ○ Protein Nitrogen (mg); ◑ UV Extinction (of nucleic acid fraction) per mg of protein nitrogen; ● mg Phosphorus (of nucleic acid fraction) per mg of protein nitrogen.

In view of the demonstration by WIAME[1] of the occurrence of a labile phosphate in the nucleic acid fraction of baker's yeast, it is not surprising that the phosphorus determinations of the nucleic acid fraction gave results differing from the UV values. As a rough guide to the nature and quantity of the phosphorus compounds in this fraction, the trichloracetic acid (TCA) SCHNEIDER extracts were allowed to stand for a period of about six weeks, and the resulting orthophosphate measured. By subtracting orthophosphate from total phosphate, values were obtained for the strongly bound "Fixed Phosphate", and the figures are given in Table I.

The UV values are roughly proportional to fixed phosphate, giving a ratio of about 11, and it appears probable that the fixed phosphate is, in fact, nucleic acid phosphate.

TABLE I

Time (h)	*Total P (mg)*	*Labile P (mg)*	*Fixed P (mg)*	*UV (extinction)*	*UV Fixed P*
0	0.39	0.28	0.11	1.04	9.5
4	0.72	0.27	0.45	5.06	11.2
5¾	0.81	0.28	0.53	5.90	11.1
7½	0.89	0.39	0.50	5.80	11.6
11½	1.25	0.62	0.63	6.60	10.4
24	1.94	1.28	0.66	6.80	10.3

The above method, however, is inelegant and long; a speedier and more refined analysis was therefore sought. Since the SCHNEIDER extraction of the nucleic acid involves a

15 minute hydrolysis with 5% TCA at 90° C, whereby much of the labile phosphate is hydrolysed, it seemed that continued hydrolysis under the same conditions, making use of the differing rates of hydrolysis of the labile phosphate and nucleic acid phosphate might be employed. WIAME's[6] work had indicated that the labile phosphate was probably a condensed phosphate; in particular the kinetics of its hydrolysis by *N* HCl were typical of this class of compound. The hydrolysis of four different condensed phosphates was therefore examined, using 5% TCA at 90° C; they all gave similar rate curves, and were 94% hydrolysed to orthophosphate after 75 minutes.

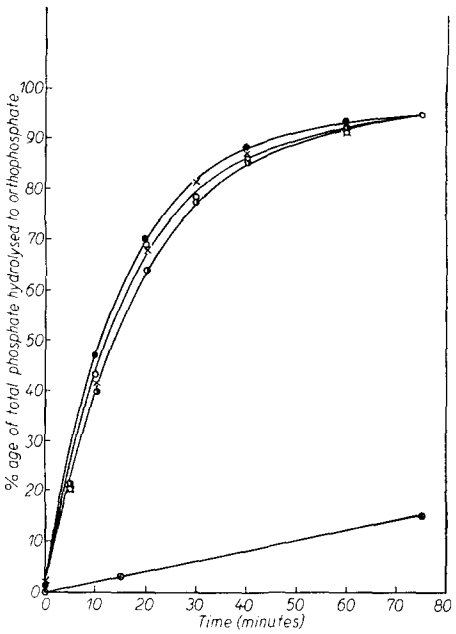

Fig. 2. Rate of hydrolysis of condensed phosphates and of bakers yeast nucleic acid. × Pyrophosphate; ◑ Tripolyphosphate; ○ Trimetaphoshate; ● Glass 696; ⊗ Baker's yeast nucleic acid.

Since four different condensed phosphates were hydrolysed to the same degree by the 75 min hydrolysis, it seems reasonable to suppose that the hydrolysis under these conditions is typical of condensed phosphates in general, and may be due merely to the fission of the P–O–P bond. Under similar conditions, a SCHNEIDER nucleic acid extract of baker's yeast, free from labile phosphate, and a sample of herring sperm deoxyribose-nucleic acid (DNA) were hydrolysed only to the extent of 15%. It may be considered that this low degree of hydrolysis is typical of fission of the nucleotide C–O–P bond, and that hydrolysis of such bonds in a similar molecular environment would always liberate the same percentage of phosphorus.

The method of analysis therefore adopted was to continue the SCHNEIDER hydrolysis (5% TCA at 90° C) on an aliquot of the nucleic acid fraction for a further hour, and the orthophosphate produced (1 hour P) was determined. Another aliquot was analysed for total phosphate. From these data, the nucleic acid phosphate (N) and labile phosphate (M) could be calculated from the simultaneous equations:

$$1 \text{ Hour P} = 0.94\,M + 0.15\,N.$$
$$\text{Total P} = M + N.$$

Application of hydrolysis method and composition of yeast nucleic acid

The differential hydrolysis technique was applied to a growth experiment in order to substantiate the constant ratio of UV absorption to nucleic acid phosphorus (NAP) found previously in the nucleic acid fraction. For this experiment a two-week-old culture was used as inoculum, which was found to have a low content of labile phosphate.

The growth curve (Fig. 3) shows a long lag in protein synthesis, during which time there was a drop in nucleic acid content measured by UV absorption. Table II demonstrates an unexpected change in ratio of UV absorption to NAP, and it seems that the magnitude of the ratio may be correlated with synthetic activity.

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If the differential hydrolysis technique is valid, the change in ratio of the nucleic acid fractions at different times during the growth cycle must reflect either a change in composition of nucleic acid or a variation in lability of some of the phosphorus. To determine the nature and degree of variation in the composition of the nucleic acid, it is necessary to know the composition of an authentic sample of yeast nucleic acid, about which there appears to be some contradiction in the literature. Thus WIAME[7] obtained an extinction of 0.61 for a solution of reprecipitated RNA containing 0.57 $mM \cdot 10^{-4}$ of phosphorus per ml. Converting the latter into mg P per 25 ml solution (the units used in this communication), the ratio of extinction to phosphorus is 13.9.

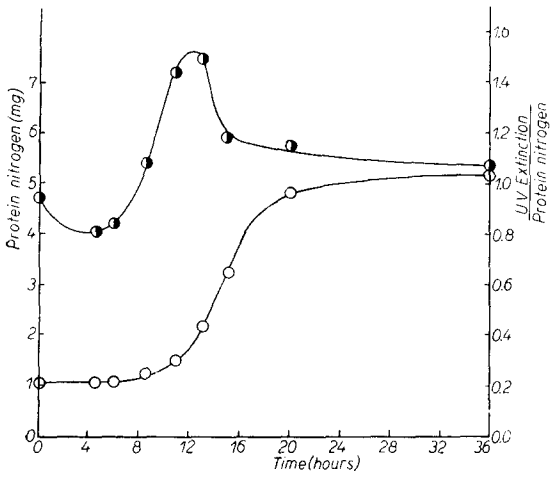

Fig. 3. ○ Protein Nitrogen (mg); ◑ UV Extinction (of nucleic acid fraction) per mg of protein nitrogen.

On the other hand, DI CARLO AND SCHULTZ[8] have given a calibration graph for the UV absorption of yeast nucleic acid; by extrapolation, an extinction value of about 1.05 may be derived for a solution containing 1 mg nucleic acid in 25 ml of solution. Since the theoretical value for the phosphorus content of RNA is considered to be about 9.5%[3], the UV to phosphorus ratio is about 11.0.

TABLE II

Time (hours)	*UV Extinction (A)*	*NAP (B)*	*Ratio A/B*
0	1.00	0.070	14.3
4½	0.865	0.084	10.3
6	0.910	0.092	9.9
8½	1.37	0.123	10.9
11	2.17	0.218	10.0
15	3.78	0.31	12.0
20	5.55	0.386	14.4
36	5.50	0.42	13.1

In view of the discrepancy in composition between the values given by the two different sources, it seemed advisable to analyse a sample of yeast nucleic acid which had not been subjected to the usual harsh extraction treatments of the commercial material. A piece of commercial baker's yeast was therefore fractionated by SCHNEIDER's technique, and labile phosphate was shown to be absent by the fact that the nucleic acid fraction contained only 3% of the total phosphate in the form of orthophosphate after the 15 minute TCA hydrolysis. The UV absorption and the total phosphate were then measured, and the ratio found to be 10.3. This was therefore taken to be the true value for yeast nucleic acid. The ratio was also determined for a sample of BDH "Nucleic Acid from Yeast. Sodium Salt", and for the reprecipitated material. The figures obtained were 13.7 and 13.8 respectively. The percentage of phosphorus in the sodium salt was 6.9, which is equivalent to 7.4% of the free acid.

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We therefore have two conflicting ratios for yeast nucleic acid, 10.3 for the native material and 13.9 for the commercially extracted sample. This difference may possibly be explained on the basis of the electrometric titration evidence of FLETCHER[9] *et al.*, who showed that yeast RNA contains three primary and one secondary phosphoryl –OH dissociations for every four atoms of phosphorus. They indicated that the secondary phosphorus atoms, being only singly bound in nucleotide linkage, would be readily labile, and suggested that this lability was the cause of the low content of phosphorus in many commercial samples of RNA. Now if all the labile phosphorus atoms are removed entirely during the alkaline extraction of commercial nucleic acid, such material will contain only 75% of the requisite amount of phosphorus, and the ratio of UV absorption to phosphorus will correspondingly be $33^1/_3$% greater than the normal value. Both of these effects are observed in the BDH nucleic acid examined.

It is thus seen that two ratios of UV absorption to phosphorus, of about 10 and 14 respectively, may be obtained, depending apparently on whether or not the secondary phosphoryl group of the nucleic acid has been removed. It will be noted that the ratios obtained during the different stages of the growth cycle fluctuate between these values, and it might not be unreasonable to suggest that the changing ratios do, in fact, demonstrate an *in vivo* variation in the binding of this same phosphate group.

It must be emphasised that both in the case of the literature values quoted, and in all the experimental work described, the nucleic acid fraction was always subjected to the SCHNEIDER hydrolysis prior to analysis. In consequence, the state of the nucleic acid in all the cases cited should be precisely similar.

The behaviour of labile phosphate during the growth cycle

Early experiments had been carried out in which only UV measurements and total phosphorus determinations had been performed on the nucleic acid fraction. In view of the demonstration of the proportionality of UV absorption to nucleic acid phosphorus being 10.3, the labile phosphate could be determined by calculating NAP from UV values and subtracting the resulting figures from the total phosphate. Data obtained in this manner showed a decrease in the total amount of labile phosphate during the early part of growth, followed by an increase during the decline of protein synthesis, with continued increase after nitrogen assimilation had ceased. As a rough approximation, it may be said that the labile phosphate reached a minimum at the time of maximum rate of protein synthesis, at which time the nucleic acid extinction per unit protein was at its maximum value. An example of this effect is shown in Fig. 4, in which labile phosphate has been calculated from the results of the experiment represented by Fig. 1.

The behaviour of labile phosphate during the growth cycle has also been followed by the differential hydrolysis technique, applying the hour's TCA hydrolysis to the SCHNEIDER nucleic acid fraction. The results are shown in Fig. 5, and it will be seen that they are in accordance with the expected effect.

Metachromasy

WIAME[10,11] has suggested, on the basis of the metachromatic reaction with toluidine blue given by the acid-insoluble labile phosphate and the fact that sodium "hexametaphosphate" prepared by the method of JONES[12] also gives the reaction, that the labile phosphate might be a hexametaphosphate, possibly in polymerised form.

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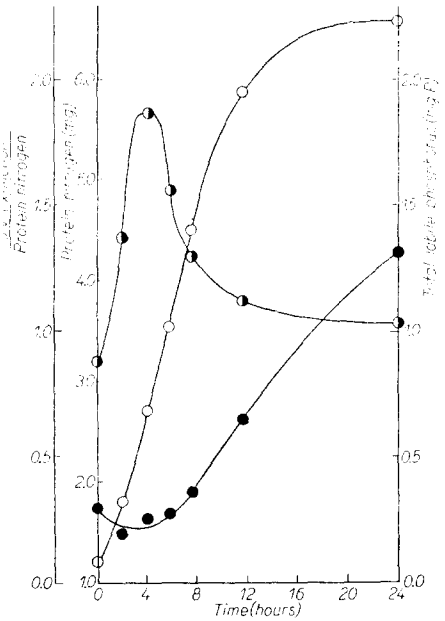

Fig. 4. O Protein Nitrogen (mag); ◑ UV Extinction (of nucleic acid fraction) per mg of protein nitrogen; ● Total labile phosphorus (mg).

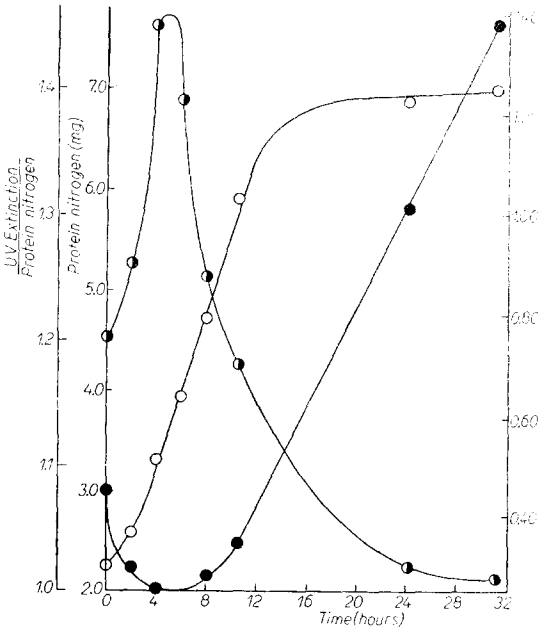

Fig. 5. O Protein Nitrogen (mg); ◑ UV extinction (of nucleic acid fraction) per mg of protein nitrogen; ● Total labile phosphorus (mg).

The metachromatic reaction with toluidine blue was performed on the following sodium phosphates: pyrophosphate, tripolyphosphate, trimetaphosphate, Glass 696, and a polyphosphate of molecular weight about 100,000. Both Glass 696 and the high molecular weight polyphosphate gave the reaction. It would therefore appear that the metachromatic reaction is given by highly condensed phosphates in general. In addition, metachromasy was obtained using alginic acid, sodium polymethacrylate, and the sodium salt of deoxyribosenucleic acid, which indicates that the reaction is a property of polyanions. Furthermore, "sodium hexametaphosphate" is a term applied generally to Graham's salt, or "Glassy metaphosphate", the material obtained by rapid cooling of a phosphate melt from above 628° C—the term does not imply a view about the degree of polymerisation[13,14,15], and, in fact, end-group determinations indicate that "metaphosphate" glasses have a molecular weight of the order of 10,000–20,000[16]. Both Glass 696 and the sodium "hexametaphosphate" prepared by the method of JONES are therefore similar compounds, being polyphosphates of high molecular weight.

It seems reasonable, therefore, to suggest that the labile phosphate occurring in the SCHNEIDER nucleic acid fraction of yeast is a highly condensed polyphosphate. In this respect it is of interest to note that high molecular weight "polymetaphosphate" has been isolated from the mould *Aspergillus niger*[17]. In addition, EBEL[18] has investigated a number of micro-organisms containing metachromatic granules, and has shown in all cases that the material responsible for the metachromatic reaction is, in fact, a polyphosphate.

The term "metaphosphate", although often used as synonymous with polyphos-

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phate, may be considered misleading when applied to such a compound. Thus metaphosphates have the formula $(MPO_3)_n$, where M is a univalent cation. There are only two proven metaphosphates, the sodium tri- and tetra-metaphosphates, which have cyclic structures and therefore correspond exactly to $(NaPO_3)_3$ and $(NaPO_3)_4$ respectively. The sodium polyphosphates have the formula $(Na,H)_{n+2}P_nO_{3n+1}$ and form a series of linear molecules. Admittedly, as the chain length of a polyphosphate increases and n tends to infinity, the formula tends to $[(Na,H)PO_3]_n$, *i.e.* to the composition required for metaphosphates. Such a compound, however, cannot be classed as a true metaphosphate since the chain must always start and terminate with a secondary phosphoryl group.

DISCUSSION

SPIEGELMAN AND KAMEN[19] have shown that when yeast is grown in a medium containing only glucose and phosphate labelled with P^{32}, the "nucleoprotein" fraction builds up a store of labelled phosphate. This flows out of the fraction when ammonium salts are added, but the outflow is stopped when agents such as sodium azide and dinitrophenol are added in concentrations which prevent enzyme formation and protein synthesis without inhibiting fermentation. In later experiments[20, 21], it was shown that most of the activity of the "nucleoprotein" fraction was due to the acid-insoluble "metaphosphate".

LINDEGREN[22] suggests that volutin, which WIAME[23] has identified with "metaphosphate", is essential for cell division. He considers that the loss of volutin in yeasts after division ceases is responsible for the lag period that often occurs when "dissimilated" cells are introduced into fresh medium.

All these observations are consistent with the view that the nucleic acid-associated polyphosphate of yeast is involved in protein synthesis, and it has often been suggested that the function of this material is to act as an energy supply by virtue of the high standard free energy associated with the hydrolytic fission of the P–O–P bonds. Our observations may be interpreted in accordance with such a hypothesis. Thus it has been shown that the polyphosphate decreases in quantity during the early growth phase. When the rate of protein synthesis decreases, polyphosphate is stored, and apparently is not utilised again until the yeast is suspended in fresh culture medium. Furthermore, it has been observed in experiments where there is a low content of labile phosphate initially that there is a correspondingly long lag phase as regards protein synthesis, and it may well be that the length of the lag phase in yeast cultures can be correlated with the initial amount of labile phosphate present.

A further significant feature in the phosphorus metabolism of the nucleic acid fraction is the change in ratio of UV extinction to NAP during the growth cycle. High ratios are obtained at the end of the cycle, after the cessation of protein synthesis, the low values being reached soon after inoculation. This effect also may be connected with energy relationships, since it has been found[24] that nucleic acid from an actively growing culture of *T. utilis* is capable of supporting luminescence in firefly extracts, whereas that from a dormant culture will not. Since luminescence depends on the expenditure of energy, the low-ratio nucleic acid may be considerd an "energy-rich" form. If analogy with adenosine triphosphate (ATP) is permissible, such a compound might be expected to act by transference of phosphate; *i.e.* donation of phosphate, followed by replacement of the deficiency from a phosphorus store. In this respect, it has been shown that the

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acid-insoluble polyphosphate becomes depleted during the early growth phase, namely during the same period as the low-ratio nucleic acid form is attained. In addition, the nucleic acid and polyphosphate occur in the same fraction (acid-insoluble, non-lipid, non-protein fraction) possibly as a complex[25,6], which BRACHET[26] has termed "phosphorylated nucleic acid". It would therefore not be surprising if there existed a nucleic acid phosphate which served as a phosphorylating agent in the same way as ATP, yet provided specificity in protein synthesis by aligning the amino acids along the polynucleotide chain. This is basically the hypothesis proposed by CHANTRENNE[27] (see also DOUNCE[28], and KONINGSBERGER AND OVERBEEK[29]) in which he suggests that the carboxyl groups of the amino acids could be activated by acylphosphate formation prior to peptide synthesis. The importance of such phosphorylations in peptide bond formation has been stressed by LIPMANN[30,31], and referred to by many authors recently[32].

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SUMMARY

1. The nucleic acid fraction of yeast as obtained by the SCHNEIDER technique contains an additional phosphorus compound; it is suggested that it is a linear polyphosphate of high molecular weight.

2. This has necessitated the development of a modified phosphorus determination for the Schneider fraction whereby the nucleic acid phosphate and the polyphosphate are determined in the presence of each other. The method evolved consists of a differential hydrolysis in 5% trichloracetic acid at 90° C for 1 hour. The rate of hydrolysis of the two phosphates has been determined.

3. By the application of the hydrolysis technique, the behaviour of the two phosphorus components of the nucleic acid fraction has been followed throughout the growth cycle. It has been shown that the total amount of polyphosphate decreases during the early active growth phase, then increases rapidly, increasing even after protein synthesis has finished. Fluctuation in the ratio of UV absorption to NAP, as determined by this method, has suggested that the composition of the nucleic acid itself may vary during the growth cycle; the highest values for this ratio were obtained at the termination of protein synthesis, and the lowest values during early growth. The significance of these observations is discussed.

4. Studies have been made on samples of yeast nucleic acid which were free from polyphosphate. The evidence regarding the ratio of UV to P in these investigations and in those on *T. utilis* is discussed with respect to the structure of the nucleic acid.

5. It has been demonstrated that the metachromatic reaction with toluidine blue is given by a number of polyanions and is not restricted to "hexametaphosphate". The structure and nomenclature of this substance are discussed.

RÉSUMÉ

1. La fraction acides nucléiques obtenue à partir de levure par la méthode de SCHNEIDER contient aussi un autre composé phosphoré; les auteurs pensent que c'est un polyphosphate à poids moléculaire élevé.

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2. La méthode de dosage du phosphore dans cette fraction a dû être modifiée, afin de pouvoir doser le phosphore nucléique et celui du polyphosphate en présence l'un de l'autre. La méthode mise au point dans ce but repose sur une hydrolyse différentielle d'une heure à 90° dans l'acide trichloracétique 5%. La vitesse d'hydrolyse des deux composés phosphorés a été déterminée.

3. Grâce à cette technique, il a été possible de suivre le comportement des deux composés phosphorés pendant toute la croissance de la levure. Les résultats montrent que la quantité totale de polyphosphates décroît au début de la phase de croissance active, puis qu'elle augmente rapidement et continue même à augmenter après l'arrêt de la synthèse des protéines. La variation du rapport de l'absorption dans l'U.V. à la teneur en phosphore nucléique suggère que la composition de l'acide nucléique lui-même changerait pendant le cycle de croissance; les valeurs les plus élevées de ce rapport sont atteintes à la fin de la période de synthèse des protéines, les valeurs les plus basses se placent au début de la croissance. Les auteurs discutent la signification possible de ces observations.

4. Des échantillons d'acide nucléique de levure exempt de polyphosphates ont été examinés également. Les auteurs considèrent la signification, en ce qui regarde la structure de l'acide nucléique, des valeurs du rapport de l'absorption dans l'U.V. à la teneur en phosphore obtenues pour ces acides nucléiques et pour ceux de *T. utilis*.

5. La réaction métachromatique avec le bleu de toluidine est obtenue avec plusieurs substances polyanioniques, elle n'est pas limitée au cas de l'hexamétaphosphate.

ZUSAMMENFASSUNG

1. Die Nukleinsäurefraktion aus Hefe, wie sie mit der Methode nach SCHNEIDER gewonnen wird, enthält ausser den Nukleinsäuren noch eine andere phosphorhaltige Verbindung. Es wird vermutet, dass es sich dabei um ein lineares Polyphosphat höheren Molekulargewichtes handelt.

2. Die Methode zur Bestimmung des Phosphats in dieser Fraktion musste modifiziert werden, um das Phosphat der Nukleinsäuren und des Polyphosphates in beider Gegenwart bestimmen zu können. Die entwickelte Methode besteht in einer einstündigen Differentialhydrolyse in 5% Trichloressigsäure bei 90° C.

3. Durch die Anwendung der Hydrolysentechnik war es möglich, das Verhalten der beiden Phosphatkomponenten während des Wachstums der Hefe zu verfolgen. Es konnte gezeigt werden, das die Gesamtmenge des Polyphosphates zu Beginn der Phase des aktiven Wachstums abnimmt dann schnell zunimmt und selbst nach Abschluss der Proteinsynthese noch weiter anwächst. Die Schwankungen im Verhältnis der UV Absorption zum Gehalt an Nukleinsäurephosphat lassen vermuten, dass sich die Zusammensetzung der Nukleinsäuren während des Wachtumzyklus ändern, die höchsten Werte dieses Verhältnisses wurden am Ende der Periode der Proteinsynthese erhalten, und die niedrigsten Werte zu Beginn des Wachstums. Die Bedeutung dieser Beobachtungen wird diskutiert.

4. In gleicher Weise wurden Proben von Hefenukleinsäuren untersucht, die keine Polyphosphate enthielten. Die Bedeutung des Verhältnisses der UV Absorption zu dem Gehalt an Phosphor bei diesen Nukleinsäuren und bei denen von *T. utilis* wird im Hinblick auf die Struktur der Nukleinsäuren diskutiert.

5. Es wird gezeigt, dass die metachromatische Reaktion mit Toluidin-Blau bei einer Reihe von Polyanionen auftritt und nicht auf "Hexametaphosphat" beschränkt ist. Struktur und Nomenklatur dieser Substanz wird diskutiert.

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