ACCUMULATION OF INORGANIC POLYPHOSPHATE IN MUTANTS OF *NEUROSPORA CRASSA*

F. M. HAROLD*

Division of Biology, California Institute of Technology, Pasadena, Calif. (U.S.A.)

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SUMMARY

1. 53 strains of *Neurospora crassa*, representing 4 wild types and 40 mutant loci, were examined for accumulation of acid-soluble inorganic polyphosphate. When grown on a limited supply of growth factor, most of the mutants were found to accumulate polyphosphate, though the amounts varied widely.

2. The time sequence of polyphosphate accumulation was studied in shaken cultures of the histidine-requiring strain, C-84. When growth ceased due to exhaustion of the amino acid, the RNA content of the mycelium dropped sharply. Concomitantly, acid-soluble polyphosphate accumulated; the levels of other phosphorus compounds, including the acid-insoluble polyphosphate, were not affected.

3. By the use of ^{32}P it was demonstrated that, in C-84, extensive degradation of RNA occurred upon exhaustion of the histidine. The phosphorus released from RNA was converted to polyphosphate and constituted a major source of phosphorus for polyphosphate accumulation. Similar observations were made with other mutant strains, though the details of the pattern of growth, RNA degradation and polyphosphate accumulation varied from strain to strain.

4. Inhibition of the growth of *Neurospora* strains by withholding a macronutrient or by addition of a metabolic inhibitor did not, in general, induce either RNA degradation or polyphosphate accumulation. However, when mycelium was transferred to medium lacking the carbon source, sucrose, extensive degradation of RNA took place. Acid-soluble polyphosphate and inorganic orthophosphate accumulated under these conditions.

5. Polyphosphate accumulation was inhibited by certain amino acid analogs. In C-84, the histidine analog 2-thiazolealanine was found to inhibit RNA degradation but had no effect on the accumulation of polyphosphate.

6. The relationship of polyphosphate accumulation to the primary genetic block is discussed. The possible role of RNA degradation as the immediate cause of polyphosphate accumulation is considered.

INTRODUCTION

Cultures of genetically blocked strains of micro-organisms have commonly been found to accumulate substances that occur in the wild type in trace amounts only.

Abbreviations: PCA, perchloric acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Pi, inorganic orthophosphate; TZA, 2-thiazole alanine.

* Present address: Department of Experimental Chemistry, Division of Research and Laboratories, National Jewish Hospital at Denver, Colo. (U.S.A.).

Generally, the substances which accumulate are closely related to the metabolic step affected by the mutation. Examples are known, however, of mutant strains which accumulate compounds bearing no obvious relationship to the primary metabolic deficiency[1].

A striking case of this kind was described by HOULAHAN AND MITCHELL[2]. Several nutritional mutants of *Neurospora crassa* were found to accumulate large amounts of inorganic polyphosphate when grown on a limiting supply of their required growth factor. Genetic analysis indicated that this phenomenon was associated with the mutation giving rise to the nutritional requirement rather than with an independent mutant gene. Polyphosphate accumulation was therefore regarded as a secondary, pleiotropic, effect of the nutritional deficiency.

The multiplicity of physiological consequences brought about by a specific gene mutation is an important aspect of gene action but has received relatively little attention. The present paper presents experiments designed to analyze the metabolic pattern through which a block in the synthesis of various amino acids, vitamins, purines or pyrimidines may bring about the accumulation of inorganic polyphosphate.

A preliminary report describing some of this work has been presented[3].

EXPERIMENTAL

Biological methods

Strains of *Neurospora crassa* were obtained from the culture collection of the California Institute of Technology. They were kept on Fries agar minimal medium[4] supplemented with the appropriate growth factors.

Growth experiments were conducted in both stationary and shaken cultures.

Stationary cultures were initiated by inoculating conidia into 20-ml aliquots of Fries medium[4]. Pads were harvested after 4 days at 25°, washed with acetone, dried, weighed and analyzed as described below.

Shaken cultures were grown in a modified Fries medium designed to permit omission of single nutrients without affecting the other constituents. Stock solutions of the following compositions, per liter, were prepared: (A) $MgSO_4 \cdot 7H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$, 2 g; NaCl, 2 g; trace elements[4], 20 ml; Biotin, 60 μg. (B) Sodium potassium tartrate$\cdot 4H_2O$, 230 g. (C) KH_2PO_4, 40 g. (D) NH_4NO_3, 80 g.

Equal volumes of each stock solution were combined and diluted to 10 volumes. 1.5 % sucrose was added as carbon source; other supplements were added as described for the individual experiments. Conidia were harvested from Fries agar medium, filtered through glass wool and introduced into the above medium at a density of 10^5–10^6 conidia per ml. Flat bottom culture flasks of 2.5 l capacity, containing up to 1 l of the suspension, were then placed on a reciprocating shaker and agitated at 100 cycles/min at 25°.

To transfer growing mycelium from one type of medium to another, the mycelium was harvested by gentle filtration, washed several times with water and resuspended in fresh medium. At intervals, aliquots of the culture were removed and the mycelium was filtered off by suction, washed with acetone and dried. The weighed pads were then analyzed.

Growth of *Neurospora* under these conditions was almost logarithmic for the first four doubling times and then slowed down, presumably because of the increasing size

of the clumps (Fig. 1). The weights of mycelium in volumetric samples of growing cultures were nevertheless generally reproducible to ± 5 %.

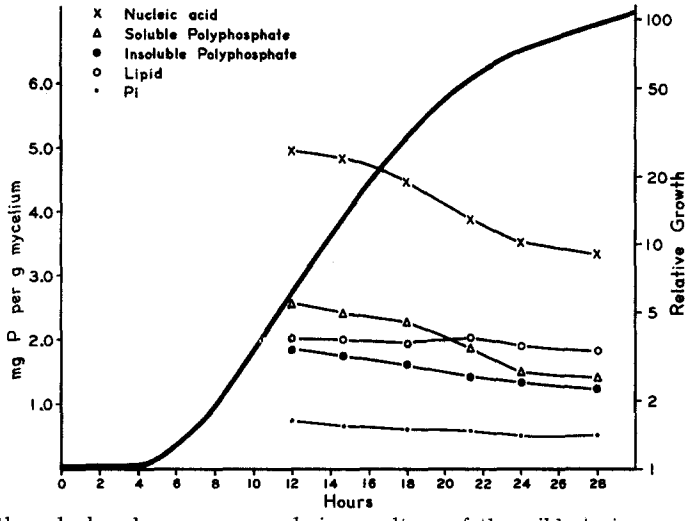

Fig. 1. Growth and phosphorus compounds in a culture of the wild strain, 25a. Conidia were suspended in growth medium at 0 h and the relative mass of mycelium per unit volume of culture is designated as relative growth.

Analytical methods

The analytical methods used in the present study were based upon those developed by others for the fractionation and estimation of phosphorus compounds[5–7], but were simplified to permit the rapid processing of numerous samples.

Fractionation of the mycelium: Samples of acetone-dried mycelium, weighing about 100 mg, were ground with sand and 5 ml cold 0.5 *M* PCA. After standing in the refrigerator for 20 min the mycelium was centrifuged and extracted once more in the same manner. The extracts were pooled and brought to 10 ml.

The residue was then extracted with 5 ml of ethanol at room temperature for 30 min. After centrifugation, 5 ml of ethanol–ethyl ether (3:1) were added to the residue, the mixture was boiled for 1 min, allowed to stand at room temperature for 20 min, and centrifuged again. The two extracts were pooled and brought to 10 ml.

The lipid-free residue was dried by heating in a water bath and then extracted twice with 5-ml portions of hot 0.5 *N* PCA (70°, 15 min). The extracts were pooled and brought to 10 ml.

Estimation of phosphorus compounds: Some or all of the following analytical determinations were performed upon the four fractions.

Cold PCA extract

Inorganic orthophosphate and total phosphorus were determined by ALLEN's method[8]. The ^{32}P contents were measured after extraction into isobutanol[9] and by direct mounting of an aliquot of the extract, respectively.

Acid-labile phosphorus was determined as above after hydrolysis with 1 *N* HCl for 15 min at 100°.

In the early experiments, inorganic polyphosphate extractable with cold PCA ("soluble polyphosphate") was determined by precipitation with Ba^{++} at pH 4.5

(see ref. 6). However, reproducibility of the results was poor and a more reliable method was sought. The procedure finally adopted was based upon the finding of CRANE[10] that Norit A adsorbs most organic phosphorus compounds from acid solution, leaving inorganic orthophosphate and polyphosphate behind. In practice, 50–100 mg washed Norit A (the exact amount was not critical) were added to 6 ml of the PCA extract. After 10 min the suspension was centrifuged and the acid-labile phosphorus in the supernate was taken to represent the soluble polyphosphate. To justify this procedure it was shown that:

Virtually all (80–100 %) the phosphorus remaining after charcoal treatment was acid labile. Nucleotides, which are the major non-polyphosphate, acid-labile phosphorus compounds, were completely removed.

In experiments using ^{32}P, the acid-labile phosphate remaining after charcoal treatment was completely precipitable with Ba^{++} at pH 4.5.

It should nevertheless be pointed out that this method may give high values in cases where large amounts of non-nucleotide, acid-labile phosphorus compounds are present.

The ^{32}P content of the soluble polyphosphate was determined by precipitation with Ba^{++}. To 4 ml of the supernate from the charcoal treatment, 3.5 mg of carrier sodium polyphosphate (average chain length 50) were added, and the polyphosphate was precipitated as described above.

Lipid extract

Total phosphorus and total ^{32}P were determined as described above.

Hot PCA extract

Total phosphorus and total ^{32}P were determined as described above. Inorganic polyphosphate ("insoluble polyphosphate") was determined by adsorbing the nucleic acid on Norit A (about 200 mg Norit A/5 ml extract). The residual phosphorus, all of which was acid-labile, was taken to be inorganic polyphosphate. Total nucleic acids were estimated by difference, all the phosphorus adsorbed on charcoal being considered to be nucleic acid phosphorus. Frequently the extinction at 260 mμ was also determined and in a few cases the nucleic acid was eluted from the charcoal with alcoholic ammonia.

Residue

The residue from the hot PCA extract was analyzed for total phosphorus and total ^{32}P.

Chemicals

Reagent-grade chemicals were used whenever possible. Growth factors and antagonists were purchased from Nutritional Biochemicals Company or from the Sigma Chemical Company. ^{32}P was obtained from Oak Ridge National Laboratories. Norit A, purchased from Braun Chemical Company, was washed with dilute acid and with water before use.

Samples of inorganic polyphosphate were a gift from Dr. C. F. CALLIS of the Monsanto Chemical Company, and 2-thiazolealanine was a gift from Dr. R. G. JONES of the Lilly Research Laboratories. It is a pleasure to acknowledge their generosity.

RESULTS

Accumulation of acid-labile phosphate by Neurospora *strains*

In order to determine whether the accumulation of inorganic polyphosphate is a general property of *Neurospora* mutants or is correlated with specific metabolic blocks, a survey of mutants was undertaken. In all, 4 wild type and 49 mutant strains representing 40 different loci[11] were analyzed. The molds were grown in stationary cultures supplemented with various amounts of the required growth factor; the pads obtained after 4 days were analyzed for acid-labile phosphate extractable with cold PCA. Most *Neurospora* mutants were found to accumulate acid-labile phosphate when their growth was limited by the supply of available growth factor, but the amounts accumulated varied widely. Some of the experimental results are shown in Table I. No obvious correlation with specific metabolic blocks could be discerned.

From the work of HOULAHAN AND MITCHELL[2] it was already known that inorganic polyphosphate was the major constituent of the acid-soluble, acid-labile

TABLE I

ACID-LABILE PHOSPHATE IN PCA EXTRACT OF VARIOUS *Neurospora* STRAINS[11]

Locus	*Strain number*	*Supplement, mg/20 ml*		*Dry wt. of mycelium mg/20 ml*	*Acid-labile phosphate, mg/g mycelium*
Tryp-2	75001	Tryptophan,	2	100	1.9
			0.02	38	3.8
Tryp-4	C-151	Tryptophan,	2	25	1.4
			0.25	9	8.9
Nt	C-86	Tryptophan,	2	55	6.0
			0.15	13	14.2
Nt	39401a i	Tryptophan,	2	60	2.7
			0.25	30	12.7
Nic-1	3416	Niacin,	0.1	86	2.2
			0.005	52	7.9
Nic-1	44801	Niacin,	0.1	95	2.3
			0.005	60	4.1
His-1	C-84	Histidine,	2	77	5.6
			0.25	20	12.5
His-2	C-94	Histidine,	2	80	3.5
			0.25	15	10.5
His-3	C-140	Histidine,	2	73	5.4
			0.5	10	12.0
His-4	C-141	Histidine,	2	83	4.4
			0.5	13	8.5
Leu-1	33757	Leucine,	2	45	4.0
			0.25	11	5.9
Met-1	38706	Methionine,	2	92	4.8
			0.05	15	10.8
Met-5	9666	Methionine,	2	80	2.7
			0.1	12	12.5
pdx-p	39106	Pyridoxamine,	$12 \cdot 10^{-3}$	96	3.2
			$0.25 \cdot 10^{-3}$	35	5.3
pdx-q	35405	Pyridoxamine,	$12 \cdot 10^{-3}$	91	2.1
			$0.25 \cdot 10^{-3}$	41	4.3
Wild	25a	Biotin	$8 \cdot 10^{-5}$	80	2.0
			$1.6 \cdot 10^{-7}$	46	1.7
Wild	Em5297a	Biotin	$8 \cdot 10^{-5}$	94	2.5
			$1.6 \cdot 10^{-7}$	17	1.4

phosphorus fraction. For more specific identification of the material, cultures of the wild strain, 25a, and of 39401a (on limiting tryptophan) were grown on a large scale. Inorganic polyphosphate was isolated by precipitation with Ba^{++} at pH 4.5 as described in the experimental section. The washed barium salt was converted to the free acid by stirring with Amberlite resin IR-120 (see ref. 12); the latter was then analyzed by titration[13] and (after neutralization) by paper chromatography[14]. Both samples had an average chain length of about 20 by titration. Polyphosphates of chain length less than 7 were practically absent, in agreement with the results of MULLER AND EBEL[15] in other fungi.

Phosphorus compounds in shaken culture

To study the accumulation of polyphosphate as a function of time, shaken cultures of *Neurospora* were employed.

Wild type, 25a: As is the case in other micro-organisms, two types of inorganic polyphosphate occurred in *Neurospora*. One was extractable with cold PCA ("soluble polyphosphate") while the other was insoluble in cold PCA but was extracted by hot PCA ("insoluble polyphosphate"). The levels* of these and other phosphorus compounds at various times in a growing culture of the wild strain, 25a, are shown in Fig. 1. This pattern, remarkable only for the absence of dramatic changes, was quite typical of growing cultures of *Neurospora*, whether wild or mutant growing in the presence of excess of growth factor.

Histidineless, C-84: The histidine-requiring strain C-84 accumulates inorganic polyphosphate when grown on a limiting supply of histidine. To study the effect of histidine deprivation upon the levels of phosphorus compounds in C-84, a culture of C-84 was grown for 16 h in medium supplemented with 100 μg/ml histidine·HCl. The mycelium was then harvested and divided into two portions. One was resuspended in medium supplemented with histidine (flask A) while the other was placed in medium devoid of histidine (flask B). The subsequent behavior of these two cultures with respect to growth and phosphorus metabolism is shown in Fig. 2.

The culture in flask A continued to grow at a decreasing rate and exhibited no striking changes in the levels of the various phosphorus fractions. The starving culture in flask B grew normally for a time but growth ceased abruptly after 6 h. Coincident with the cessation of growth, there was a sharp rise in the level of soluble polyphosphate and a marked drop in the RNA content. The other phosphorus fractions, including the insoluble polyphosphate, were unaffected except for a slow rise in the level of inorganic orthophosphate.

The process of polyphosphate accumulation could be stopped at any time by addition of histidine to the starving culture. Thus, when histidine was added to a culture of C-84 which had been starved for 24 h, growth and RNA synthesis resumed while the accumulation of polyphosphate ceased. The polyphosphate content of the mycelium decreased, primarily due to admixture with newly synthesized mycelium of low polyphosphate content (Fig. 3). In experiments using ^{32}P, no extensive degradation of the accumulated polyphosphate was detected under these conditions. However, the polyphosphate was subject to degradation when no exogenous phosphate was supplied.

* Throughout this paper, "level" refers to the amount/g dry weight.

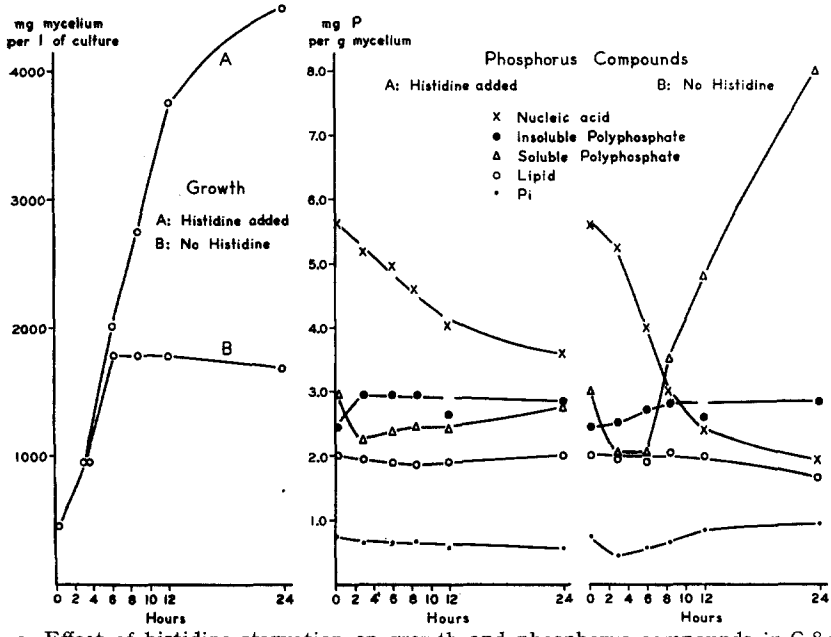

Fig. 2. Effect of histidine starvation on growth and phosphorus compounds in C-84. At 0 h, mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A was supplemented with 100 μg/ml histidine while flask B received no histidine.

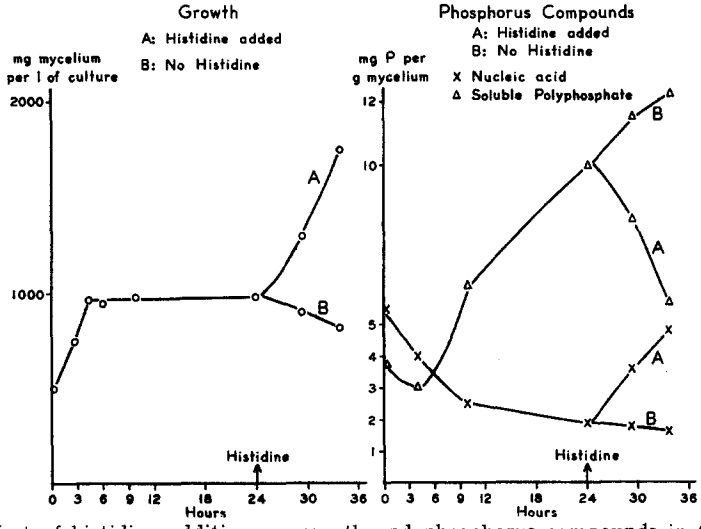

Fig. 3. Effect of histidine addition on growth and phosphorus compounds in C-84 starved of histidine. At 0 h, mycelium of C-84 was suspended in medium devoid of histidine. At 24 h, this culture was divided into two portions; histidine, 100 μg/ml, was added to flask A, while flask B received no histidine.

Results similar to those presented above were also obtained with other nutritional mutants. In general, polyphosphate accumulation in shaken cultures was less pronounced than in stationary ones. However, strains 38706 (methionineless) and 39401-RIA (nt-type, requiring either tryptophan or niacin), accumulated significant

amounts of polyphosphate and showed the same reciprocal relationship of RNA and polyphosphate levels.

Growth inhibition and polyphosphate accumulation

The finding that polyphosphate accumulation was associated with a condition that limited growth, prompted numerous experiments on the effect of growth inhibition on polyphosphate metabolism. Most of these were performed with the wild strain 25a and with the tryptophan-requiring 39401-RIA, and were designed as described in the previous section. Inhibition of growth by depriving the cultures of nitrogen, sulfur, biotin or divalent metal ions or by the metabolic inhibitors *p*-fluorophenylalanine, azaserine, 5-methyltryptophan or ethionine failed to induce polyphosphate accumulation. These findings support the conclusion reached by HOULAHAN AND MITCHELL[2] that while the inhibition of growth may be a factor in polyphosphate accumulation it is not, in itself, sufficient to induce it.

A relationship between RNA and soluble polyphosphate

The results discussed in a previous section had hinted at a possible reciprocal relationship between RNA and polyphosphate. This matter was consequently explored by the use of ^{32}P.

Incorporation of ^{32}P into C-84 in presence and absence of histidine: A culture of C-84 which had been grown for 17 h in medium supplemented with 100 μg/ml histidine was harvested and divided into two equal portions. One was resuspended in medium supplemented with histidine (flask A) while the other (flask B) was placed in medium lacking histidine. When growth in flask B had ceased, ^{32}P was added to both cultures and its incorporation into various compounds was followed.

Some of the results are shown in Fig. 4. On a volumetric basis, the two cultures synthesized approximately equal amounts of soluble polyphosphate—culture A because it continued to grow and culture B because polyphosphate accumulated after growth had ceased. However, the incorporation of ^{32}P into the soluble polyphosphate was far greater in flask A than in flask B. It is clear that the bulk of the soluble polyphosphate synthesized by the starved mycelium was derived, not from exogenous phosphate, but from unlabeled, endogenous precursors.

^{32}P conservation in histidine-starved C-84: The nature of the endogenous source of phosphorus for polyphosphate synthesis became clear when the reverse experiment was performed. Mycelium of C-84 was grown in ^{32}P-labeled medium supplemented with 100 μg/ml histidine for 17 h, harvested, resuspended in fresh, unlabeled medium and divided into two portions. Flask A was supplemented with histidine while flask B received none. The redistribution of the ^{32}P was followed and is shown in Fig. 5.

In the growing culture (flask A), the nucleic acids retained virtually all of the ^{32}P incorporated into them. This behavior is typical of most growing micro-organisms[16,17]. In flask B, growth ceased after 6 h and rapid degradation of nucleic acid set in. The bases were apparently excreted into the medium, but the phosphorus was retained within the mycelium and converted to soluble polyphosphate. The ^{32}P content of the insoluble polyphosphate fraction did not increase, but a slow rise in the inorganic orthophosphate fraction was generally seen. The behavior of other fractions, not shown in Fig. 5, was the same in both cultures. Nucleic acids thus constituted a major source of phosphorus for polyphosphate accumulation, though it should be noted that

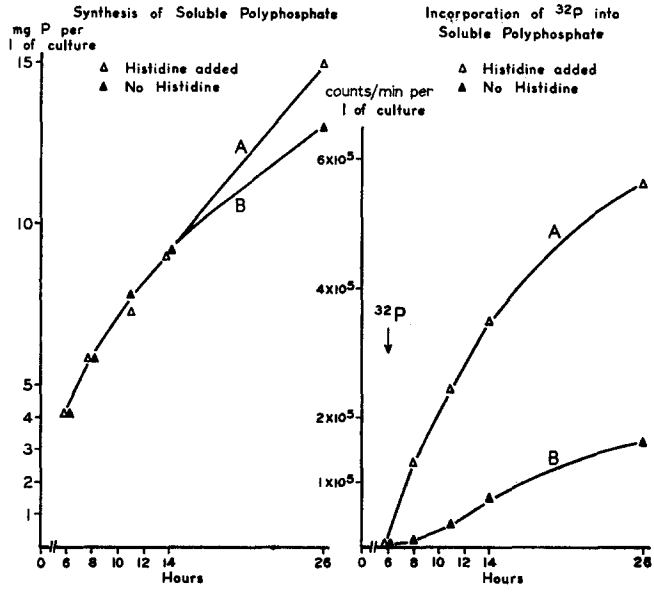

Fig. 4. Incorporation of ^{32}P into the soluble polyphosphate of C-84 in the presence and absence of histidine. At o h, mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A was supplemented with histidine while flask B was not. ^{32}P was added to both at 6 h. Ordinates refer to amounts of phosphorus or ^{32}P in polyphosphate per unit volume of culture.

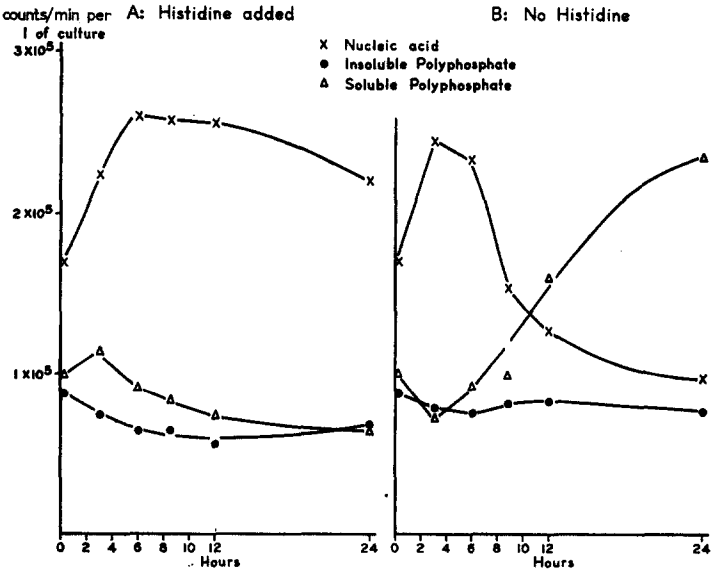

Fig. 5. Retention of ^{32}P in phosphorus compounds of C-84 in presence and absence of histidine. At o h, ^{32}P-labeled mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A received histidine while B did not. To facilitate quantitative comparisons, the data on growth and phosphorus fractions pertaining to this experiment were plotted in Fig. 2. Ordinates as in Fig. 4.

the amount of polyphosphate synthesized was greater than that of phosphorus released from the nucleic acids.

Since DNA is a relatively minor component of the nucleic acids of *Neurospora*, amounting to less than 10 % of the total[18], it is clear that RNA must be undergoing degradation. In fact, no net degradation of DNA has yet been observed as measured by BURTON's method[19].

Polyphosphate accumulation as a consequence of RNA degradation

The finding that RNA degradation constitutes a major source of phosphorus for polyphosphate accumulation raises the question as to whether these two phenomena are related as cause and effect. To test this hypothesis a more detailed investigation of the correlation between RNA degradation and polyphosphate accumulation was undertaken.

Reversal of RNA degradation by readdition of histidine: When histidine was added to a culture of C-84 which had been starved of histidine, the degradation of RNA ceased. In fact, growth resumed and the accumulation of polyphosphate also ceased (see a previous section).

Failure of various starvation conditions and growth inhibitors to induce RNA degradation: In a previous section, a number of treatments were listed which inhibited growth but did not induce polyphosphate accumulation. In general, RNA degradation also failed to occur under those conditions*. The effect of nitrogen deprivation on C-84 illustrates this conclusion. When mycelium of C-84 was transferred to medium devoid of nitrogen, the synthesis of protein and of RNA ceased abruptly, and that of other phosphorus compounds soon followed suit. However, no significant loss of ^{32}P from RNA was observed. It is of interest that the mass of the mycelium continued to increase for some time, often doubling or tripling before "growth" ceased. This is presumably due to accumulation of "reserve materials" such as lipids or polysaccharides[20].

RNA degradation and polyphosphate accumulation in other Neurospora *mutants*: Studies on ^{32}P conservation, analogous to those described for C-84, were also carried out with a number of other mutants. In every case, RNA degradation was observed when mycelium of an auxotrophic strain was grown on fully supplemented medium and then transferred to minimal medium. However, the pattern of growth, RNA degradation and polyphosphate accumulation varied from strain to strain.

The methionineless strain, 38706, continued to grow very slowly in the absence of methionine. Polyphosphate accumulation was comparable to that in C-84 and transfer of ^{32}P from RNA to polyphosphate was demonstrated, but the stoichiometry was obscured by the continued synthesis of RNA.

A different pattern was found in the leucineless strain, 33757. When mycelium of this strain was transferred to minimal medium, degradation of nucleic acid set in immediately. The phosphorus was largely converted to soluble polyphosphate, resulting in limited accumulation of this substance (Fig. 6). Increase of the mycelial mass partly obscured this accumulation when the polyphosphate content was expressed as mg P/g mycelium (maximum value observed: 3.0 mg P/g).

* Sulfur starvation did induce some RNA degradation in C-84, and there was a concomitant increase in the ^{32}P content of the soluble polyphosphate fraction. Net accumulation of polyphosphate was not observed, perhaps because the culture continued to grow slowly.

The behavior of a double mutant requiring both leucine and histidine was also studied. This strain was prepared by crossing C-84a and 33757A, using the latter as the conidial parent. The pattern of growth, RNA degradation and polyphosphate accumulation depended upon which of the two amino acids was withheld: when histidine alone was absent, it behaved like C-84 while in leucine deficiency it behaved like 33757. When neither amino acid was supplied, the pattern seen in leucine starvation was obtained.

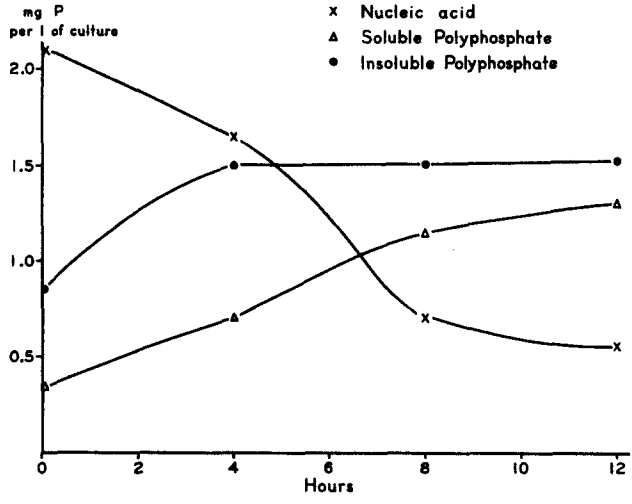

Fig. 6. Effect of leucine starvation on intracellular phosphorus compounds in 33757. Mycelium of 33757 was transferred to medium lacking leucine at o h. Ordinate as in Fig. 4.

Finally, the tryptophanless strain C-83, degraded RNA but accumulated practically no polyphosphate.

Induction of RNA degradation by sucrose starvation: As pointed out above, starvation of *Neurospora* with respect to most of the major nutrients did not induce RNA degradation. However, in all the strains tested, RNA degradation did ensue when the mycelium was suspended in medium lacking the carbon source, sucrose. It thus became possible to study the fate of RNA-phosphorus in non-growing cultures.

A culture of C-84 was grown in ^{32}P-labeled, supplemented medium for 17 h. The mycelium was then harvested and resuspended in unlabeled medium, containing histidine but lacking sucrose. No increase in mass occurred under these conditions—in fact, a small loss of weight was observed. As shown in Fig. 7, considerable degradation of nucleic acid took place. The insoluble polyphosphate and the phospholipid also showed some degradation while orthophosphate and soluble polyphosphate accumulated.

These changes were due almost entirely to a redistribution of the intracellular phosphorus, and resulted in its retention within the mycelium. Generally, 80–90 % of the ^{32}P originally present in the mycelium was conserved. Also, parallel experiments, in which unlabeled mycelium was resuspended in labeled medium lacking sucrose showed that virtually no incorporation of phosphorus into the mycelium took place under these conditions. Moreover, the results of Fig. 7 could be duplicated by suspending the mycelium in buffer lacking both nitrogen and phosphorus as well as sucrose.

Parallel experiments were carried out with several other strains of *Neurospora*, both wild and mutant. RNA degradation could be demonstrated in all strains, but the amount of polyphosphate accumulated varied. Some strains behaved in a manner analogous to C-84: 38706 and 39401 RIA, and the wild types Em5297a and 1A fell into this category. Other strains, including C-83 and the wild type 25a, degraded RNA extensively yet synthesized little or no polyphosphate. Intermediate responses were also found.

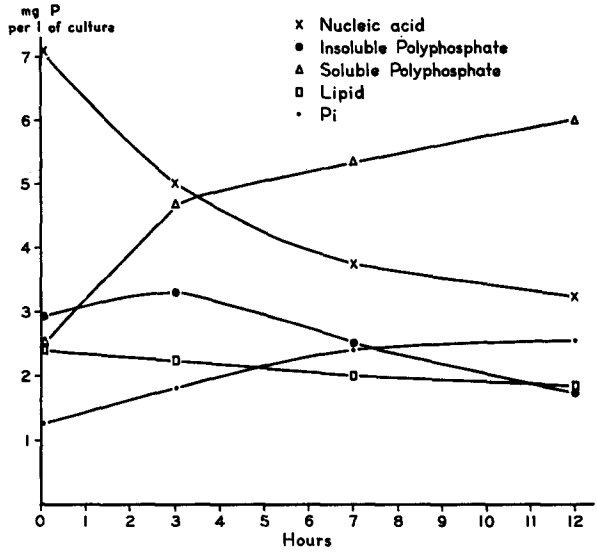

Fig. 7. Effect of sucrose starvation on intracellular phosphorus compounds in C-84. Mycelium of C-84 was resuspended in medium lacking sucrose at o h. Ordinate as in Fig. 4.

It is clear from these results that RNA degradation can be a sufficient cause of polyphosphate accumulation by both mutant and wild strains. The efficiency with which phosphorus released from RNA is converted to polyphosphate appears to vary with the strain and may be the resultant of a number of competing reactions.

Inhibition of polyphosphate accumulation and of RNA degradation

Amino acid analogs: In the course of the experiments discussed in a previous section it was discovered that certain amino acid analogs inhibited the accumulation to polyphosphate. In general, an analog of the amino acid required by the mutant was most effective. Thus polyphosphate accumulation in strain 39401-RIA was inhibited by 5-methyl tryptophan (400 μg/ml) which had little effect on C-84 or 38706. Conversely, polyphosphate accumulation in 38706 was strongly inhibited by ethionine (100 μg/ml) while C-84 and 39401-RIA were scarcely affected. No inhibition of RNA degradation was observed.

The degree of inhibition of polyphosphate accumulation by a given concentration of analog was strongly dependent upon the time of addition of the inhibitor. This is illustrated in Fig. 8, which describes the effect of ethionine, added at various times to a culture of 38706 (methionineless) starved for methionine. It is perhaps significant that early addition of ethionine also caused a marked loss of mycelial mass.

The mechanism of this effect remains to be clarified. However, the above results seem to suggest that protein synthesis must be allowed to proceed during the early stages of polyphosphate accumulation. This suggestion receives support from the finding that when mycelium of C-84, deprived of histidine, was transferred at various times to fresh medium devoid of nitrogen, a similar effect was observed. Nitrogen

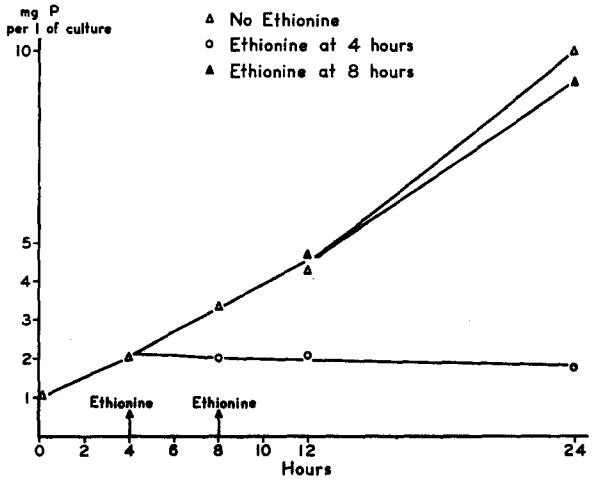

Fig. 8. Effect of ethionine on accumulation of acid-labile phosphate in 38706. Mycelium of 38706 was transferred to medium lacking methionine at o h. Ethionine (100 μg/ml) was added at 4 and 8 h to aliquots of the culture. Ordinate as in Fig. 4.

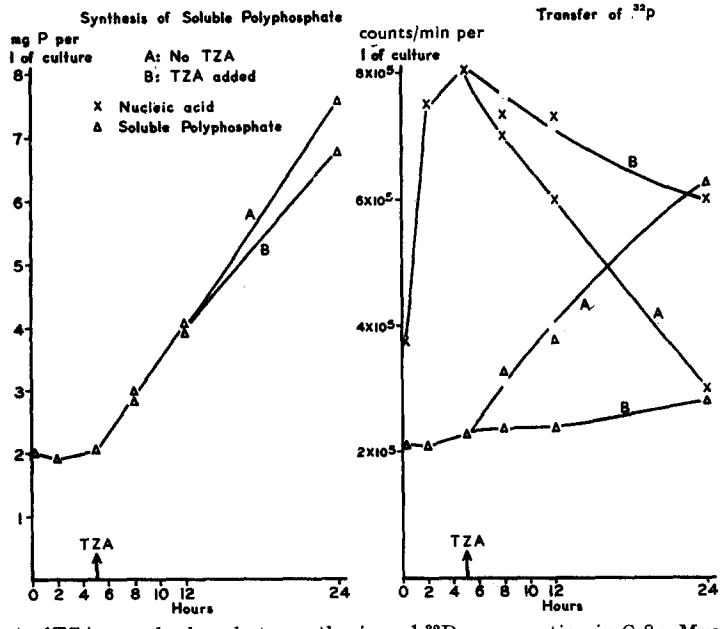

Fig. 9. Effect of TZA on polyphosphate synthesis and ^{32}P conservation in C-84. Mycelium of C-84 was transferred to medium lacking histidine at o h (A). At 5 h, 200 μg/ml of TZA were added to an aliquot (B) of the culture. Ordinates as in Fig. 4.

deprivation inhibited the onset of polyphosphate accumulation but had little effect at later times. It seems likely that the role of protein synthesis is an indirect one, perhaps involving leakiness of the cell as loss of intracellular constituents was often observed.

2-thiazolealanine: This analog of histidine[21] proved to have the disturbing property of inhibiting RNA degradation in C-84 with little effect on polyphosphate accumulation. The results of an experiment in which 200 μg/ml 2-thiazolealanine were added to a culture of C-84 deprived of histidine are shown in Fig. 9. As was the case with the analogs discussed above, the efficacy of 2-thiazolealanine as an inhibitor of RNA degradation depended upon the time of its addition to the culture. In the experiment illustrated, the loss of ^{32}P from the nucleic acid fraction, and its transfer to polyphosphate, were strongly inhibited. However, net synthesis of polyphosphate was the same in both cultures.

In a single experiment in which the effect of 2-thiazolealanine on the incorporation of ^{32}P into C-84 was studied, no stimulation of RNA synthesis was observed; incorporation of ^{32}P into soluble polyphosphate was, of course, enhanced. The amount of available thiazolealanine was unfortunately too limited to permit more extensive investigation.

DISCUSSION

When the growth of *Neurospora* mutants was restricted by limiting the supply of their required growth factors, accumulation of inorganic polyphosphate within the mycelium was generally observed. The level of polyphosphate that accumulated varied from strain to strain, ranging from twice to ten times the amount present in the wild type. We must therefore inquire how genetic blocks in the synthesis of a wide range of cellular constituents bring about the accumulation of this substance. The obvious suggestion that limitation of growth per se is the condition that induces polyphosphate accumulation, seems to be ruled out by the experiments presented here. This conclusion reinforces that already reached by HOULAHAN AND MITCHELL[2]. At the same time, the very generality of the phenomenon argues against the view that polyphosphate accumulation is a direct consequence of specific metabolic blocks. It appears most plausible to suppose that the various nutritional deficiencies act by inducing a common disturbance of phosphorus metabolism.

A major disturbance of phosphorus metabolism, which was observed in all the mutants studied when the supply of growth factor was exhausted, was the degradation of intracellular RNA. Acid-soluble polyphosphate accumulated in many mutants at the same time. The phosphorus released from RNA was thereby retained within the mycelium and served as a major source of phosphorus for polyphosphate synthesis. As polyphosphate could also be synthesized from exogenous phosphate, without significant RNA turnover, (*e.g.*, during normal growth) it is reasonable to postulate a pool, or pools, of intermediates common to both pathways. The nature of these intermediates is as yet unknown, but ATP in particular is a likely candidate[22–24]. These interrelations are schematically represented in Fig. 10.

Since degradation of RNA constituted a major source of phosphorus for polyphosphate accumulation, the question arises to what extent RNA degradation may be regarded as the immediate cause of this phenomenon. In terms of the histidine-

requiring mutant, C-84, this formulation of the problem implies the following hypothesis: Histidine deprivation induces the breakdown of RNA (reaction 1). Phosphorus liberated from RNA would then flow into the pool of precursors postulated above and polyphosphate accumulation would follow by virtue of the increased production of precursors.

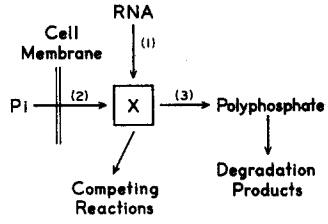

Fig. 10. Metabolic interrelations between RNA and soluble polyphosphate.

This hypothesis offers a ready explanation for the correlation of polyphosphate accumulation with RNA degradation. Strong evidence for the validity of the proposed chain of reactions comes especially from the finding that sucrose starvation induced degradation of RNA and that polyphosphate accumulated under these conditions. This was the case, not only in C-84 and other mutants, but even in many wild strains. Thus, release of phosphorus from RNA appears to be a sufficient cause for polyphosphate accumulation. The observation that the amount of polyphosphate accumulated in histidine starvation exceeded that of RNA degraded can be accounted for, at least in part, by the simultaneous synthesis and breakdown of RNA which were found under these conditions.

While RNA degradation is thus most probably an important factor in polyphosphate accumulation it nevertheless does not seem to be the whole story. This is brought out clearly by the observation that 2-thiazolealanine inhibited RNA degradation in C-84 without affecting polyphosphate accumulation. Whatever the explanation of this finding may be, it implies that other processes, besides RNA degradation, may bring about polyphosphate accumulation. For example, it is conceivable that histidine deprivation in C-84 not only induces RNA degradation but also affects other steps leading to polyphosphate synthesis (reaction 2 or 3). If this were the case, RNA phosphorus entering the precursor pool might be used preferentially for polyphosphate synthesis, but would not be an obligatory precursor: when RNA degradation is artificially inhibited, phosphate would be taken up from the medium. Experiments now in progress on the nature of the precursor, X, may clarify this point.

The complex metabolic control of polyphosphate accumulation suggested by the experiments with strain C-84 provides a reasonable basis for speculation on the wide range of polyphosphate levels attained by various other mutants. Some strains, such as the methionine-requiring 38706, appear to be analogous to C-84 and accumulate large amounts of polyphosphate. Whether this polyphosphate is entirely derived from RNA turnover, or arises in part from other processes, remains to be determined. In other mutants, such as the leucineless strain 33757, polyphosphate accumulation is more limited and can be completely accounted for by the phosphorus liberated from RNA. In this connection, the results with the double mutant that requires both histidine and leucine are of interest. Apparently the nature of the metabolic block,

by influencing the pattern of growth, and RNA degradation, is one of the parameters that determine the amount of polyphosphate accumulated. Finally, in mutants such as the tryptophanless strain C-83, which fail to accumulate polyphosphate, one may expect to find reactions that compete for RNA-phosphorus, resulting in the ultimate loss of phosphorus from the mycelium. Leakiness of the cell membrane and degradation of polyphosphate may also limit, or prevent, polyphosphate accumulation.

It remains to consider the accumulation of polyphosphate by other microbial species in the light of the present findings in *Neurospora*. Polyphosphate accumulation is frequently encountered in cultures of yeast, bacteria and algae under conditions unfavorable to growth (see *e.g.*, ref. 7, 24, 25) and is commonly interpreted as resulting from excessive production of ATP which is not utilized in synthetic reactions[23,26,27]. A reciprocal relationship between the synthesis of RNA and of polyphosphate has also been postulated[28,29]. The results presented here suggest that degradative reactions, particularly the degradation of RNA[30–32], may have to be taken into account in some of these cases.

A unique feature of polyphosphate accumulation in *Neurospora* is that it is confined to the acid-soluble fraction. This observation must be added to the growing evidence supporting the view that the acid-soluble and -insoluble polyphosphates constitute physiologically distinct entities[33,34].

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