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Authors

Cramer, CL
Vaughn, LE
Davis, RH

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Basic Amino Acids and Inorganic Polyphosphates in *Neurospora crassa*: Independent Regulation of Vacuolar Pools

CAROLE L. CRAMER, LYNN E. VAUGHN, AND ROWLAND H. DAVIS*

Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92717

At least 78%, and perhaps all, of inorganic polyphosphate is shown to be contained within the vesicles (vacuoles) of *Neurospora crassa*, where over 97% of the soluble arginine, lysine, and ornithine pools are known to accumulate. Furthermore, synthetic polyphosphate can concentrate arginine up to 400-fold from dilute (0.01 mM) solutions in equilibrium dialysis. For these reasons and because the molar ratio of basic amino acids and polyphosphate phosphorus is approximately 1, we tested the hypothesis that there was an obligate physiological relationship between them. Experiments in which nitrogen starvation and arginine excess were imposed upon cells showed that polyphosphate content was insensitive to changes in the basic amino acid content. Experiments involving phosphate starvation and restoration showed that basic amino acid content was almost wholly independent of polyphosphate pools. Moreover, the normal high degree of compartmentation of arginine in vesicles was maintained despite polyphosphate depletion, and arginine was still exchanged across the vesicular membrane. We conclude that *N. crassa*, like yeasts, can regulate polyphosphates and basic amino acids independently, and that the accumulation of basic amino acids in vesicles may depend upon an energy-requiring mechanism in addition to the demonstrated charge interaction with polyphosphate.

Neurospora, when grown in minimal medium, maintains large pools of the basic amino acids arginine and ornithine. The intracellular concentrations would be 8 to 10 mM each if they were distributed evenly throughout the cell water (4). However, approximately 97% of arginine, ornithine, lysine, and perhaps histidine is sequestered from the cytosol in osmotically sensitive, membrane-bound vesicles (9, 12, 16, 20). The mechanism allowing this degree of concentration of these basic molecules is unknown.

Yeast vacuoles have also been shown to contain large amounts of arginine (23). The concomitant localization of inorganic polyphosphate, a polyanion, to the yeast vacuole (17) suggested that polyphosphate could function as a fixed negative charge which could concentrate basic compounds through an ion-exchange mechanism. Specificity of the vacuolar contents could be imposed by selective permeability of the vacuolar membrane. This model explains the retention of arginine in isolated vacuoles and predicts that changes in the vacuolar arginine pools would be reflected in changes either in the polyphosphate levels or in the cations bound to polyphosphate (6, 10). This has been confirmed, in part, in studies of yeasts (6).

In this paper we demonstrate the location of polyphosphate within the arginine-containing vesicles of *N. crassa* and the ability of polyphosphate to concentrate arginine in an artificial system. The correlation of polyphosphate and basic amino acid pools in vivo is then studied to determine whether the pools of polyphosphate and basic amino acids are obligately coupled to one another. In a later paper (L. E. Vaughn and R. H. Davis, manuscript in preparation), we will show that the *N. crassa* "vesicles" are in fact vacuoles, as defined by the protease and α-mannosidase activities of purified preparations (10).

MATERIALS AND METHODS

Strains, media, and growth. The wild-type strain, 74A, was used for all experiments involving vesicle isolation and whole-cell pool determination. The ureaseless strain *ure-1* (allele *ur-9*) was used for [^{14}C]arginine tracer experiments. In general, cultures were grown at 25°C with aeration (3) in Vogel medium N (19) supplemented with 1.5% sucrose. Nitrogen-free and phosphate-free media were the same, with either NH_4NO_3 or KH_2PO_4 omitted. Where noted, L-arginine hydrochloride was added at 200 mg/liter or, when used as a nitrogen source, 20 mM.

Isolation of vesicles. The isolation of vesicles followed the procedures, with some modification, by which Matile et al. (11) and Weiss (20) and Weiss and Davis (22) isolated vesicles (vacuoles, "protease particles") from *N. crassa*. Exponentially growing cultures (0.5 to 0.8 mg [dry weight] per ml) were har-

vested in cheesecloth and treated with the cell wall-degrading enzyme β-glucuronidase (1 ml/g of cells [dry weight]). Because vesicle yield was poor when we used the previously described buffer for the cell wall digestion step, we used a pH 5.8 buffer containing 10 mM citrate ion (citric acid, adjusted to pH 5.8 with solid K_2HPO_4), 1 mM EDTA, 0.14 M β-mercaptoethanol, and 0.6 M sorbitol. After incubation with shaking at 30°C for 30 min, the suspension was centrifuged for 5 min at 500 × *g*. The cells were washed twice by suspension in 1 M sorbitol followed by centrifugation. The cells were then suspended in 15 ml of a fractionation buffer containing 1.0 M sorbitol, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminomethane sulfonate–NaOH (TES-NaOH) (pH 7.5), and 1 mM EDTA. The cells were lysed at 4°C with six strokes in a Teflon-glass homogenizer at 1,600 rpm. Unbroken cells and debris, removed by centrifugation at 600 × *g* for 5 min, were suspended in 15 ml of fractionation buffer, rehomogenized (six strokes), and centrifuged again at 600 × *g* for 10 min (referred to as S600). The crude lysate, consisting of the combined supernatants of homogenized cells, was filtered through two layers of Miracloth (Chicopee Mills, Inc., Miltown, N.J.). The contents of this filtrate were a reasonable measure of cell breakage, since arginine, polyphosphate, and a cytosolic enzyme, ornithine transaminase, were found in the same proportions as they occur in whole cells (Vaughn and Davis, in preparation). The filtrate was centrifuged at 15,000 × *g* for 20 min to obtain a crude organelle pellet (P15000).

Density gradient centrifugation was used to purify vesicles. All gradient materials contained 10 mM TES-NaOH (pH 7.5) and 1 mM EDTA. First, the P15000 was suspended in 0.2 to 1.0 ml of this buffer, containing 1.0 M sorbitol, and 0.2 ml of the suspension was layered on a 12-ml, continuous 30 to 60% (wt/wt; 1.0 to 2.25 M) sucrose gradient. The gradient was centrifuged at 100,000 × *g* for 3 h in a Beckman SW41-Ti rotor to obtain a vesicle pellet, free from mitochondria. This will be called the sucrose gradient pellet. In a second type of gradient, the sucrose gradient pellet was suspended in 2.25 M sorbitol buffer and layered on an iso-osmotic sorbitol-Metrizamide gradient (2.25 M sorbitol at the top to 1.59 M sorbitol plus 0.66 M Metrizamide at the bottom). The vesicles were sedimented to their equilibrium density for 6 h at 100,000 × *g* in a Beckman SW41-Ti rotor. Fractions were collected from a puncture in the bottom of the tube.

To study organelle buoyant density as a function of molarity, continuous, iso-osmotic sorbitol-Metrizamide gradients were used. All contained Metrizamide ranging in concentration from 0 to 0.66 M, and sorbitol was used to make the gradient iso-osomotic throughout at 1.0 or 2.0 M. Equilibrium was reached after 4 h of centrifugation at 100,000 × *g*.

Dialysis experiments. Boiled dialysis bags containing 1 ml of Na^+ polyphosphate (10 μmol of phosphorus; chain length of 200 ± 5, M_r = 20,000) were exposed to L-arginine hydrochloride solutions (10 μmol in varying volumes). The flasks were shaken at 4°C. After 24 and 46 h, samples of the medium expected to contain 0.1 to 0.2 μmol of arginine were concentrated on short AG-50 ion-exchange columns (0.7 by 3 cm), eluted with 0.2 M NaOH, and measured. The dialysis bags were cut and thoroughly mixed in water, which was brought to 10 ml. The arginine and polyphosphate of these solutions were determined (see below). Recovery of arginine was 87 to 95%, and no systematic loss or degradation of polyphosphate was detected. Controls established that arginine was not appreciably adsorbed to glass; one-third of the loss indicated above could be attributed to the dialysis bag. Equilibrium was reached in 24 h.

Estimation of cellular polyphosphate and amino acid content. Whole cells were extracted by the procedure of Harold (7). Samples containing 30 to 40 mg (dry weight) of mycelia were extracted twice with 0.5 N $HClO_4$ at 4°C for 20 min. After centrifugation, the residue was extracted once with ethanol (30 min, 25°C) and then with ethanol-ethyl ether (3:1, vol/vol; 70°C for 3 min and then 25°C for 20 min). The insoluble residue was finally extracted twice with 0.5 N $HClO_4$ at 70°C for 15 min. The cold $HClO_4$-soluble fraction contained all amino acids and the "soluble" polyphosphate. The hot $HClO_4$-soluble fraction contained the partially hydrolyzed, remaining polyphosphate.

Polyphosphate was estimated as acid-labile inorganic phosphate (13) after the $HClO_4$ extracts were brought to 0.8 N H^+ with HCl and boiled for 15 min. Nucleotide and nucleic acid phosphate (which can contribute to the acid-labile phosphate fraction) were eliminated by adsorption to acid-washed Norit A before the 0.8 N acid step. Controls using ATP, DNA, RNA, and synthetic polyphosphate (chain lengths of 200 and 45) assured us of the accuracy of separation and estimation procedures. (Polyphosphates, even of fairly great chain length, are soluble in cold acid. As noted by Harold [8], however, whole cells extracted with cold $HClO_4$ leave over half of the polyphosphates complexed with polygalactosamine of the cell wall. Thus, where polyphosphates of whole cells are largely "insoluble," polyphosphates of isolated organelles are largely soluble in cold $HClO_4$ and will not sediment at 12,000 × *g*.)

Amino acids were estimated in the cold $HClO_4$ extracts (1). The extracts were neutralized with NaOH, brought to 5 ml with 0.116 M Na^+ citrate buffer (pH 5.3; 0.35 M in sodium ion), and loaded onto AG-50-X8 ion-exchange columns (Na^+ form, 200/400 mesh, 0.7 × 7.5 cm). This was followed by 5 ml of citrate buffer and 5 ml of 0.2 N NaOH. All neutral and acidic amino acids emerged in the citrate buffer fractions; all basic amino acids and polyamines emerge in the NaOH wash. Arginine was estimated by the method of van Pilsum et al. (18). Total amino acids of fractions and of the unfractionated acid-soluble pool were measured with the ninhydrin reaction (15).

Enzyme assays. Succinate dehydrogenase was estimated by the method of Pennington (14). Isocitrate lyase was estimated by the method of G. H. Dixon and H. L. Kornberg (Biochem. J. **72**:3P, 1959).

[^{14}C]arginine tracer experiments. To estimate the degree of compartmentation of arginine, the experiment of Subramanian et al. (16) was performed on phosphate-sufficient and phosphate-starved cells. In essence, the method is to give a very small, chemically negligible (0.1 μM final concentration) pulse of [*guanidino*-^{14}C]arginine and to estimate the specific radio-

activity of new protein arginine and of acid-soluble arginine at short intervals after addition of label. At each time, two 3-ml samples of culture were taken. One was added to 3 ml of 20 mM arginine hydrochloride (4°C), filtered immediately, dried, and counted as a measure of uptake. The other sample was added to 3 ml of 10% trichloroacetic acid (4°C), filtered after 1 h, dried, and counted as a measure of [^{14}C]arginine incorporation into protein. The acid-soluble [^{14}C]arginine was calculated as the difference between samples. The specific radioactivity of the acid-soluble pool was determined by dividing the acid-soluble radioactivity (counts per minute) by the amount of arginine (in micromoles) known to be in the same sample at that particular time. The specific radioactivity of the "new" protein arginine was determined by dividing the increment of radioactivity in a given interval (e.g., 20 s, 1 min, etc.) by the increment, in micromoles, of arginine in protein during that interval. The last term was calculated from the growth equation $\Delta A = A_0 (e^{kt} - 1)$, where ΔA is the increment of arginine in protein; A_0 is the protein arginine (micromoles per sample) at the beginning of the interval; t is the duration of the interval (minutes); and k is the growth constant (ln2/doubling time in minutes). If there is no compartmentation of arginine, the acid-soluble pool and the new protein arginine will have the same specific radioactivity. If, as is found, there is compartmentation, the earliest points in this experiment will show the new protein arginine to have much higher specific radioactivity than the pool at that time. This is because the labeled arginine passes through the cell membrane into the cytosol, where the protein synthetic machinery has preferential access to it; the bulk of the unlabeled arginine of the cell is in the vesicles. Thus, if the specific radioactivity of new protein is 100-fold that of the soluble pool, it is clear that the cytosol has at most only 1% of the cell's arginine, the rest being sequestered and thus unable to dilute the radioactivity which enters protein.

In the later part of the experiment, the radioactivity which had not entered protein will have been withdrawn into the vesicles, where it emerges at a very low specific radioactivity. The radioactivity which enters protein at that time will be further diluted by unlabeled arginine from biosynthesis. Because the biosynthetic rate is known, the degree to which vesicular arginine (equivalent to acid-soluble radioactivity) is diluted by biosynthesized arginine can be used in a simple fashion to determine the efflux rate of arginine from the vesicles (2). Experimental methods and calculations are described in more detail elsewhere (2, 16).

Chemicals. Most chemicals and β-glucuronidase were purchased from Sigma Chemical Co., St. Louis, Mo. Metrizamide (analytical grade) was obtained from Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y. Isotopes were obtained from New England Nuclear Corp., Boston, Mass. (^{3}H and ^{14}C), and International Chemical and Nuclear Co. Irvine, Calif. (^{32}P).

RESULTS

Vesicular location of polyphosphate. In the best S600 preparations of β-glucuronidase-treated cells, 78% of the polyphosphate liberated from cells was sedimentable at 20,000 × *g* (Table 1). A similar proportion of the arginine was also sedimentable in these preparations. We conclude that at least 78% of these components is organellar. The ratio of polyphosphate phosphorus to arginine was the same in whole cells and the pellet (Table 1), indicating exact copurification. Previous work has established that over 97% of acid-soluble arginine is vesicular (16, 20), and therefore the copurification suggests that virtually all polyphosphate is also in an organelle. This conclusion is compromised by a demonstrable ionic interaction between arginine and polyphosphate (see equilibrium dialysis experiment below): arginine may leak from vesicles during isolation, and only that which remains bound to polyphosphate will sediment at 20,000 × *g*. The ratio of the components in this fraction could thereby coincidentally match that of whole cells, even if some of the polyphosphate in vivo were not organellar. Thus we cannot rigorously prove that more than 78% of the polyphosphate is organellar, but it is entirely possible that all of it is.

To identify the polyphosphate with the vesicle, a double-label experiment was performed. A culture labeled continuously with $^{32}PO_4^{3-}$ was given a 10-min pulse of [^{3}H]arginine, conditions in which the vesicle will take up substantial amounts of radioactive arginine (16). After harvesting, a P15000 fraction was prepared and run on a 30 to 60% sucrose gradient (see Materials and Methods). The majority (>99.9%) of the succinate dehydrogenase (mitochondria) and the majority (>99.7%) of isocitrate lyase (glyoxysomes) banded isopycnically in the gradient or

TABLE 1. *Distribution of polyphosphate and arginine during cell fractionation*

Fraction	Polyphosphate		Arginine		Ratio: polyphosphate/arginine
	nmol of P	%	nmol	%	
Whole cells	2,400		604		4.11
S600[a]	1,430	100	339	100	4.22
S20000[b]					
I	335	23	70	21	4.79
II	330	22	70	20	4.71
P20000[b]					
I	1,095	77	269	79	4.07
II	1,140	78	283	80	4.03

[a] Sum of S20000 and P20000.

[b] Sedimentation was accomplished by loading duplicate 9-ml samples (I and II) of the S600 onto 3 ml of 1.1 M sorbitol in ultracentrifuge tubes and centrifuging in a Beckman SW41-Ti rotor for 20 min at 20,000 × *g*.

remained in the sample zone. The very small pellet, containing the bulk of vesicular arginine, was resuspended in iso-osmotic sorbitol and centrifuged to isopycnic equilibrium in a Metrizamide-sorbitol gradient. The [^{32}P]polyphosphate and [^{3}H]arginine, plotted as percentages of radioactivity recovered in the gradient, are shown in Fig. 1. The curves are superimposable, suggesting that polyphosphate and arginine are in the same organelle. Colorimetric determinations (not shown) established that 98% of the phosphate in the acid-soluble fraction of vesicles was acid-labile polyphosphate. The remainder was organic, acid-stable phosphate.

As a further test of the vesicular location of polyphosphate, we asked whether organellar polyphosphate and arginine responded similarly in gradients of different osmotic strength. A P15000 fraction from cells grown in minimal medium was applied directly to Metrizamide-sorbitol gradients which were either 1.0 or 2.0 M throughout. After centrifugation to equilibrium (4 h at 100,000 × *g*), the distributions of arginine, polyphosphate, and succinate dehydrogenase were determined (Fig. 2). Both arginine and polyphosphate responded similarly to the difference in molarity, and both responded differently than the mitochondrial marker. We conclude that arginine and polyphosphate are in the same organelle, the vesicle.

Accumulation of arginine by polyphosphate. To determine whether polyphosphate could lead to concentration gradients of arginine across a membrane, an equilibrium dialysis experiment was performed. One milliliter of sodium polyphosphate solution (chain length, 200; 10 μmol of phosphorus) was added to dialysis bags. The bags were placed in cold, unbuffered solutions containing 10 μmol of arginine hydrochloride. The volume of these solutions varied from 10 to 1,000 ml, yielding initial arginine concentrations from 1 mM down to 0.01 mM. (The cytosolic concentration of arginine is estimated to be in the neighborhood of 0.05 mM [5].) If sodium and arginine have equal affinities for polyphosphate, a maximum of 5 μmol of arginine would move into the bag in exchange for sodium. This would lead to an arginine/phosphorus ratio of 0.5. The extent to which the Donnan or ion-binding ratio was maintained in dilute solution would be a measure of the efficiency of polyphosphate as a concentrating agent.

The data (Fig. 3) show that at the highest initial concentrations of arginine, the expected equilibrium ratio of arginine to phosphorus was seen. This ratio is the most accurate representation of the data, because it is internally controlled for losses of both components during

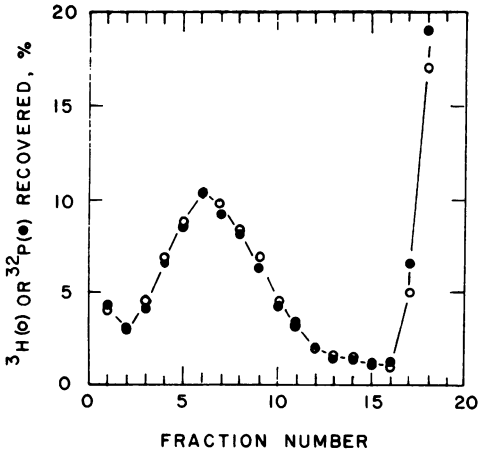

FIG. 1. *Distribution of [^{3}H]arginine and [^{32}P]-polyphosphate in a sorbitol-Metrizamide gradient. A sucrose-gradient pellet (see text) was collected in 2.25 M sorbitol and applied to the gradient, which was 2.25 osM throughout.*

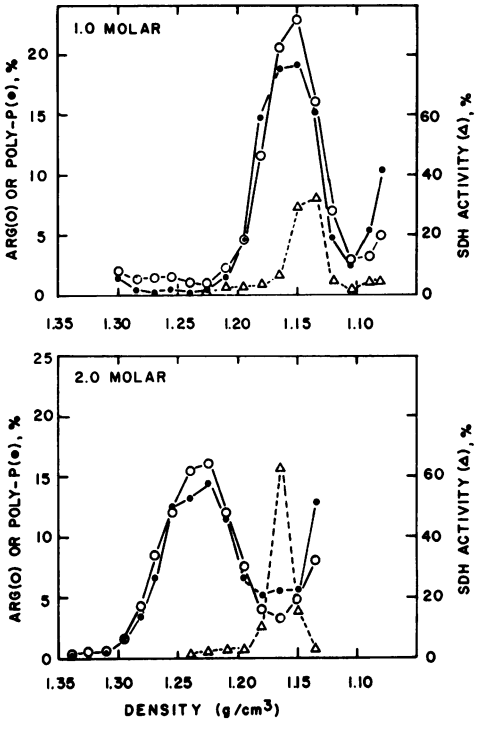

FIG. 2. *Response of equilibrium density of vesicles to change of osmotic strength of the gradient. P15000 pellets were applied to sorbitol-Metrizamide gradients which were either 1.0 M (upper panel) or 2.0 M (lower panel) throughout. Polyphosphate, arginine and succinate dehydrogenase (SDH, a mitochondrial marker) were determined on each fraction.*

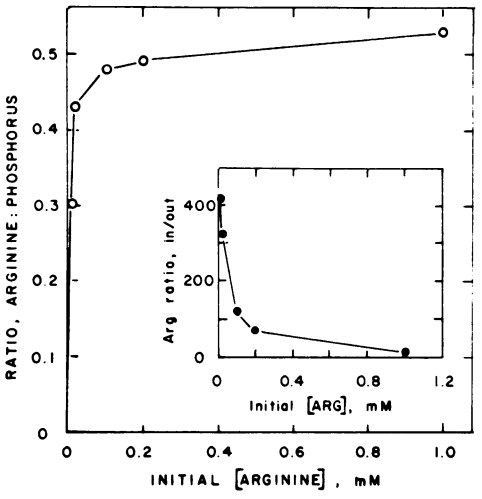

FIG. 3. *Dialysis of 1 ml of Na^+ polyphosphate (10 μmol of phosphorus) against solutions containing 10 μmol of arginine HCl. (Main axes) Relation of the arginine/phosphorus ratio in the dialysis bag to the initial arginine concentration. (Inset) Final ratio of arginine concentrations in and out of the bag as a function of the initial arginine concentration.*

manipulations. At the lower concentrations of 0.01 and 0.02 mM, the arginine/polyphosphate phosphorus ratio fell to 0.44 and 0.30, respectively. It is noteworthy that at these concentrations, the ratio of arginine concentrations inside and outside the bag was 300 to 400 (Fig. 3, inset). A control experiment, using sodium phosphate in the dialysis bag in place of sodium polyphosphate, showed no accumulation of arginine and the expected loss of the phosphate to the dialysis medium.

The data show, in this restricted experiment, that polyphosphate within a semipermeable membrane is a cation exchanger and can generate large gradients of arginine "concentration." It is not possible with these data to determine the extent to which arginine was bound to polyphosphate (as an osmotically inactive species) and to what extent it simply established a Donnan equilibrium with sodium ion, chloride, and polyphosphate.

Polyphosphate pools: effect of arginine. The experiments described so far suggest that polyphosphate may play a role in the ability of vesicles to concentrate basic amino acids. During growth on minimal medium, wild-type cultures contain about 100 nmol of basic amino acids per mg (dry weight) and about 110 nmol of phosphorus, as polyphosphate, per mg (dry weight). The approximate 1:1 stoichiometric ratio is consistent with the notion that polyphosphate might determine the capacity of the vesicle for basic amino acids. If this is an obligatory relationship, we would predict that expansion of the vesicular basic amino acid pool would be correlated with an increase in polyphosphate content.

A test of this hypothesis was made by growing wild-type *N. crassa* in Vogel minimal medium supplemented with 1 mM arginine. The basic amino acid pool, the bulk of which is vesicular (21), was 200 nmol/mg (170 nmol of which was arginine). Under these conditions, polyphosphate phosphorus remained at the level of 100 nmol of phosphorus per mg. A more severe test was made by growing the organism in nitrogen-free Vogel medium to which 20 mM arginine was added as the nitrogen source. Here, basic amino acids reached 306 nmol/mg; polyphosphate phosphorus remained at 115 nmol/mg. To more fully demonstrate the independence of polyphosphate and basic amino acids, a nitrogen starvation experiment was performed. Wild-type cultures transferred from Vogel minimal medium to nitrogen-free medium retained 93% of their polyphosphate after 3 h, even though the basic amino acid pool had declined to less than 6 μmol/mg (dry weight). The ratio of basic amino acids to polyphosphate phosphorus, 0.9 initially, declined at 0.07 at 3 h.

Basic amino acids: effect of phosphate limitation. The model which requires polyphosphate to trap basic (cationic) amino acids within the vesicle predicts that decreases in polyphosphate will result in a loss of basic amino acids from the vesicles to the cytosol. Because catabolic enzymes for ornithine and arginine are present in the cytosol (22), loss of these amino acids from the vesicle should be reflected in a decrease of the total intracellular pool of these compounds. The effect of limiting cellular polyphosphate was determined.

Wild-type cells grown in Vogel medium were collected, washed, and suspended in phosphate-free medium. At this time, there were 95 nmol of basic amino acids and 120 nmol of polyphosphate phosphorus per mg (dry weight). The polyphosphate pool decreased greatly upon phosphate starvation, to 12% of normal after 3 h. Total basic amino acids maintained 86% of their initial level after 3 h of starvation. Thus at 3 h, the ratio of basic amino acids to polyphosphate phosphorus was 5.6. (The inorganic phosphate pool remained constant for 1.5 h of phosphate starvation and then decreased to 55 percent by 3 h.) The polyphosphate pool returned to normal within 1 h of restoration of phosphate. The basic amino acid levels showed no concomitant increase at that time.

Another strain, *ure-1*, demonstrated more dramatically the indifference of basic amino acid pools to polyphosphate depletion. Whereas both

wild-type and *ure-1* strains lost 90% of their polyphosphate pools after 3 h, phosphate starvation caused a twofold decrease in the growth rate of *ure-1* and only a 25% decrease in that of wild type. This was correlated with a substantial increase of the amino acid pools, including the basic amino acids, in *ure-1*. Thus during starvation of *ure-1*, the ratio of basic amino acids to polyphosphate phosphorus changed from 1:1 to approximately 13:1.

Because the level of basic amino acids was not correlated with extreme changes in polyphosphate pools, it was important to determine whether depletion of vesicular polyphosphate impaired the sequestration of arginine from the cytosolic compartment or the movement of arginine across the vesicular membrane. Conceivably, polyphosphate depletion could leave high pools of basic amino acids trapped as a nonexchangeable pool within the vesicle during starvation, or could in some way lead to an altered distribution of amino acids without reducing their level in the cell as a whole.

To test these possibilities, a [^{14}C]arginine pulse experiment was performed on phosphate-sufficient and phosphate-starved cultures of the *ure-1* strain. From such experiments (see Materials and Methods), the degree of compartmentation, the flux across the vesicular membrane, and the rate of arginine catabolism can be inferred. The phosphate-sufficient *ure-1* culture (Fig. 4A) yielded data almost identical to those of Subramanian et al. (16). Uptake of the [^{14}C]arginine pulse was complete by 5 min, moving immediately into both protein and the soluble arginine pool (Fig. 4, inset). Because protein synthesis occurs in the cytosol, the specific radioactivity of new protein arginine represents the specific radioactivity of the cytosolic pool from which protein-synthetic reactions draw. The specific radioactivities of new protein arginine and the soluble arginine pool are shown in Fig. 4A (main axes). The great disparity between these specific radioactivities in the earliest interval (0 to 20 s) after the pulse suggests that most of the arginine pool is sequestered from the cytosol. In fact, these earliest points allow us to calculate that the cytosolic pool is no more than 3% of the total cellular pool of soluble arginine. (If protein synthesis were drawing randomly from the entire soluble arginine pool, the two curves in Fig. 4A [main axes] would be identical.) Once uptake is complete, the specific radioactivity of the soluble arginine pool (97% of which is vesicular) remains high and radioactivity enters protein more slowly. The specific radioactivity of new protein arginine diminishes and by 7 min equilibrates at about one-third of the soluble pool. This implies that ultimately one-third of the arginine entering protein is from the vesicles and two-thirds is contributed directly by arginine biosynthesis.

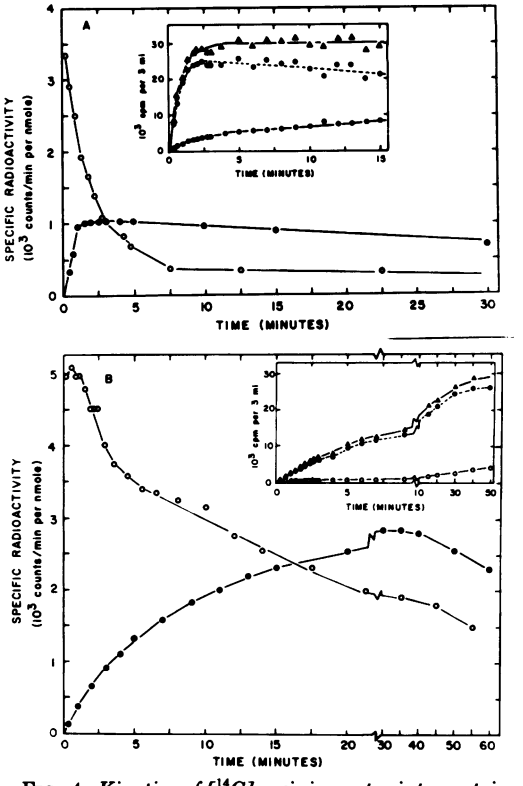

FIG. 4. *Kinetics of [^{14}C]arginine entry into protein and the soluble arginine pool in logarithmically growing ure-1 cultures in phosphate-sufficient medium (A) and after 3 h of phosphate starvation (B). Polyphosphate levels at time zero were 100 and 9.5 nmol of phosphorus per mg (dry weight) in phosphate-sufficient and phosphate-starved cultures, respectively. (Main axes) Specific radioactivity of soluble arginine (●) and the arginine which is being used for protein synthesis (○) after the [^{14}C]arginine pulse (time zero). The latter is calculated for each interval sampled as the ratio of the increment in counts divided by the increment in micromoles of arginine entering protein, and the points are placed in the midpoint of the interval (see reference 16). (Inset) Raw data for entry of [^{14}C]arginine into whole cell (△) and protein (○). The counts entering the soluble arginine pool (●) are determined by subtracting radioactivity in protein from that in whole cells. Note the break in time scales in (B). The phosphate-sufficient culture (A) had a doubling time of 150 min and steady-state arginine values of 187 nmol/mg (dry weight) (protein) and 28 nmol/mg (dry weight) (soluble). The phosphate-starved culture (B) had a 320-min doubling time, 111 nmol of protein arginine per mg (dry weight), and a soluble arginine pool which increased from 52.5 to 66 nmol/mg (dry weight) in the 60 min of the experiment.*

Data for the same experiment, performed on *ure-1* cultures which had been phosphate starved for 3 h, are shown in Fig. 4B. The polyphosphate levels were less than 10% of normal throughout the experiment. Phosphate starvation had a profound effect on both growth rate (320-min versus 150-min doubling time) and [^{14}C]arginine uptake (Fig. 4B, inset). The resulting expansion of the time scale did not alter the general relationship of protein synthesis to the soluble pool. The disparity of the specific radioactivities of new protein arginine and soluble arginine (Fig. 4B, main axes) displayed an even greater degree of arginine compartmentation; no more than 1.5% of the arginine pool was cytosolic. The kinetics of [^{14}C]arginine efflux from the vesicle, once uptake was complete (40 min), suggest that vesicular arginine was making a greater contribution to newly synthesized protein than in the case of the phosphate-sufficient culture. This may have been due to the slower growth rate, which could have allowed more extensive equilibration of cytosolic and vesicular pools. We conclude that in conditions where the basic amino acid pool exceeds polyphosphate phosphorus by 13-fold, arginine is effectively sequestered and is exchanged across the vesicular membrane.

Degradation of arginine was monitored in these experiments by the accumulation of label in urea. (The strain used lacks urease.) The radioactivity found in urea in phosphate-sufficient cultures (at 15 min) and phosphate-starved cultures (at 30 min) was 0.4 and 0.2% of the total radioactivity, respectively. Thus, depletion of polyphosphate levels led to no increase in arginine degradation.

DISCUSSION

Early observations in this laboratory showed that basic amino acids and polyphosphate phosphorus were approximately equimolar in minimal medium-grown *N. crassa*. The present work was designed to test the hypothesis, first advanced for yeast by Urech et al. (17), that basic amino acid accumulation is coupled to the availability of polyphosphate in an organellar compartment.

Our results show that the majority, and perhaps all, polyphosphate in *N. crassa* resides in the vesicles, which also contain the bulk of the basic amino acids. In addition, we demonstrated that a dialysis bag containing polyphosphate can generate high concentration ratios of arginine in external concentrations even less than the cytosolic levels of arginine of minimal medium-grown *N. crassa*. The artificial dialysis system, which works by an ion-exchange mechanism, is a very crude approximation of the vesicle. This is because sodium ions, which accompany the polyphosphate, impose a stoichiometric limit on arginine accumulation, whereas selective permeability of the vesicular membrane in vivo could easily lead to great specificity and even larger amino acid gradients.

Our physiological experiments were initially surprising in showing almost complete independence of polyphosphate and basic amino acid content. Polyphosphate levels remained relatively constant in the face of great excess or depletion of basic amino acids; the molar ratio of basic amino acids to polyphosphate phosphorus ranged from 0.07 to 2.7. Similarly, basic amino acid levels were little affected as polyphosphates are depleted or restored; the ratio of basic amino acids to polyphosphate phosphorus ranged from 0.9 to 12.8. The most critical test of the role of polyphosphates in basic amino acid compartmentation came from the [^{14}C]arginine pulse experiments. Cells containing only 10% of the normal polyphosphate levels remained capable of maintaining high levels of arginine, compartmenting this arginine within the vesicles, and transporting [^{14}C]arginine both into and out of the vesicles. The results demonstrate that polyphosphate has no obligatory role in basic amino acid accumulation. This is not to say that polyphosphates and basic amino acids do not interact; they clearly do in a chemical sense. Indeed, impairments of the regulation of one of these components may make its concentration subject to influence by the other.

Experiments with *Saccharomyces cerevisiae* similar to those reported here have been described recently by Wiemken and his co-workers (6, 17). They have shown that all polyphosphate lies within the vacuoles, where the pool of arginine, the predominant basic amino acid, is found; that arginine retention in isolated vacuoles requires polyphosphate; and that polyphosphate can generate an arginine gradient across a dialysis membrane. Unlike the results shown here for *N. crassa*, their physiological experiments (6) demonstrate a strong correlation of polyphosphate and basic amino acid content in conditions which specifically elevate the level of either compound. Nevertheless, in conditions of phosphate or nitrogen depletion, arginine and polyphosphate were shown to be stored independently.

The independence of polyphosphate and basic amino acid mobilization is not surprising in view of their distinct roles as phosphorus and nitrogen sources in limiting environments. Because of this independence, *N. crassa* affords an ideal system to study the physiology of this storage organelle. Pertinent questions that remain are: (i) What energy source drives amino acid transport in the

absence of polyphosphate? (ii) What proportion of the ionic content of the vesicles is represented by polyphosphate and basic amino acids? (iii) What counterions and counterion fluxes prevail in the vesicle at various phosphorus-nitrogen ratios? (iv) What is the osmotic state of the vesicular contents, especially when polyphosphate is depleted and basic amino acids are present in high amounts?

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