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Polyphosphate-Cation Interaction in the Amino Acid-containing Vacuole of *Neurospora crassa**

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The vacuoles of *Neurospora crassa*, grown in minimal medium, contain a 1:1 ratio of basic amino acids and phosphate, the latter in the form of long-chain, inorganic polyphosphate-P. Vacuoles isolated from cells depleted of polyphosphate retain basic amino acids despite the absence of over 90% of their polyphosphate. Thus, vacuolar retention of basic amino acids is not dependent upon binding to or charge neutralization by polyphosphate. Polyphosphate was found to be the only macromolecular polyanion in vacuoles of normal or phosphate-depleted cells. Gel filtration experiments revealed that about half the polyphosphate of normal vacuoles is bound strongly by vacuolar spermidine, Mg^{2+}, and Ca^{2+}. The polyphosphate thus occupied was not available for basic amino acid binding. We have identified about 90% of the cations of isolated vacuoles; in addition to spermidine, Mg^{2+}, and Ca^{2+}, the cation pool consists mainly of arginine, ornithine, histidine, lysine, and Na^+, with a small amount of K^+. Isolated vacuoles appear to be almost wholly impermeable to all these ions, and *in vivo*, vacuoles appear to be highly selective in ion uptake by an active process. The interaction of basic amino acid with the available polyphosphate was found to reduce the chemical activity of the former. In keeping with this effect, cells with abnormally high basic amino acid-polyphosphate ratios displayed greatly swollen vacuoles, indicating considerable osmotic activity of the basic amino acids and their counterions under these conditions.

Fungal vacuoles have been compared to plant vacuoles and animal lysosomes owing to their content of hydrolytic enzymes (1, 2). Not previously stressed is their resemblance to amine-storage organelles of mammals such as chromaffin granules of the adrenal medulla, histamine-containing granules of blood platelets, and synaptic vesicles of neurons. These organelles characteristically contain (*a*) large amounts of low molecular weight, cationic amines; (*b*) phosphate-containing polyanions; and (*c*) a proton-translocating ATPase required for amine accumulation. The polyanions may play a role in neutralizing both the charge and the osmotic activity of the amines (3, 4). In fungi, vacuoles sequester large pools of basic amino acids, which serve as a nitrogen reserve. Fungal vacuoles also contain inorganic polyphosphate and have a membrane ATPase very similar to that of chromaffin granules.

More than 95% of the cellular basic amino acids are within the vacuoles of *Neurospora* cells growing in minimal medium (5–9). An equivalent amount of polyphosphate-P is also present within the vacuole, suggesting its possible role in basic amino acid concentration and storage. An obligate relationship between polyphosphate and arginine in isolated yeast vacuoles was suggested by Durr *et al.* (10). They found that arginine leakage from yeast vacuoles *in vitro* was concomitant with polyphosphate degradation and its release as inorganic phosphate. On the other hand, *Neurospora* cultures, starved for phosphate, lose 90% of their vacuolar polyphosphate but continue to sequester large pools of basic amino acids (8). This result suggests that ionic interactions with polyphosphates cannot be the sole mechanism of basic amino acid concentration and retention in *Neurospora* vacuoles. To test the importance of polyanions in concentration and retention of amino acids, we here identify the bulk of the cations and polyanions in purified vacuoles and study some of their interactions *in vitro*.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

Arginine Retention in Isolated Vacuoles—Purified vacuoles isolated from cultures grown in minimal medium retain the 1:1 ratio of basic amino acids and polyphosphate-P found in whole cells (8). (Arginine represents 30% of this basic amino acid pool.) We wished to alter the polyphosphate-P:basic amino acid ratio by nutritional means and to determine whether vacuoles isolated from the resulting cultures retained this ratio. A culture starved for phosphate had an arginine:polyphosphate ratio of 2.4, as opposed to the normal ratio of 0.3. Vacuoles isolated from the P-depleted culture retained the high ratio, suggesting no leakage of arginine. The arginine was retained during incubation of the organellar preparation at 25 °C for 1–3 h and during further purification of the vacuoles by gradient centrifugation. Cultures were also grown in 20 mM arginine as the sole nitrogen source, which greatly elevates the cellular arginine pool. Arginine (85% of

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 1, and Table III) are presented in miniprint at the end of this paper. The abbreviation used is: TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2784, cite authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

the basic amino acid pool under these conditions) was in 3-fold excess over polyphosphate-P (8). Purified vacuoles from these cultures retain the 3:1 excess of arginine to polyphosphate-P. Hence, in both cases in which the vacuolar basic amino acid pool exceeded polyphosphate-P, the amino acid is retained within the vacuoles during cell breakage, and vacuole purification. Ionic trapping by polyphosphates therefore, cannot be the sole mechanism of amino acid retention *in vitro*. Either basic amino acids cannot permeate the membrane under these conditions or they are trapped by an unrecognized impermeant anion in the vacuole.

Gel Filtration Binding Assays of Vacuolar Components—We used the gel filtration binding assay of Hummel and Dreyer (11) to study the interaction of arginine and polyphosphate and to detect other polyanions. Polyphosphate or vacuolar lysates were applied to Sephadex G-25 columns equilibrated with the eluant, 3 mM L-arginine HCl and 3 mM NaCl. An arginine elution profile of synthetic polyphosphate is shown in Fig 2*A*. Bound arginine is the amount of arginine above the 3 mM base-line in the macromolecular portion of the profile (Fig. 2, fractions 4 and 5). This is hereafter expressed as the arginine:polyphosphate-P ratio. The ratio of 0.5 is expected because equimolar amounts of Na^+ and arginine are present (see "Experimental Procedures," Miniprint). In using the method with biological samples, a ratio exceeding 0.5 would indicate the existence of another polyanion; a ratio less than 0.5 would indicate the existence of a bound competitor of arginine for polyphosphate sites. A lysate of whole vacuoles isolated from a culture grown in minimal medium was applied to a column. Over 75% of vacuolar polyphosphates eluted in the void volume (Fig. 2*B*); smaller polyphosphates (chain length < 5) were delayed. The arginine:polyphosphate-P ratio in the macromolecular peak was 0.28 (Fig. 2*B*). This is considerably lower than the ratio characteristic of synthetic polyphosphate. We assume that an amount of Na^+ equal to arginine is associated with the polyphosphates because of its presence in the eluting buffer. The result suggests that 44% (100 − (2 × 28)) of the polyphosphate charge is associated with components which do not dissociate during gel filtration. Because such competitors may mask alternative polyanions, further investigation required (*a*) removal of "competing" vacuolar cations and (*b*) identification of vacuolar components strongly bound to polyphosphate.

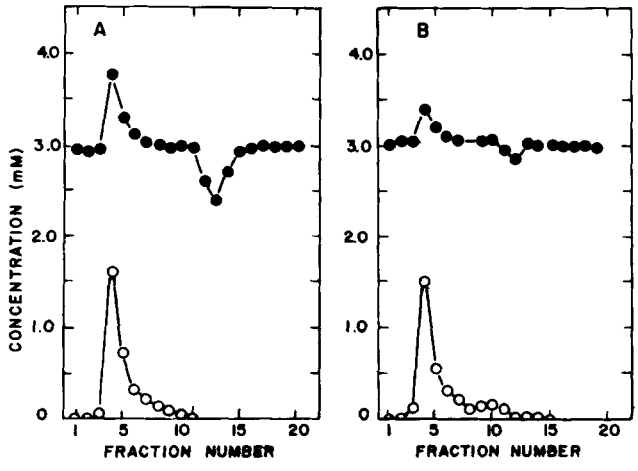

FIG. 2. **Gel filtration assay for arginine binding.** Sephadex G-25 (fine) columns were equilibrated with a solution of 3 mM arginine HCl and 3 mM NaCl. Samples were brought to 3 mM arginine, applied to columns, and eluted with 3 mM arginine HCl and 3 mM NaCl at 0–4 °C. The concentrations of arginine (●) and polyphosphate-P (○) were determined for each fraction. *A*, synthetic polyphosphate (chain length 200); *B*, vacuolar lysate of culture grown in minimal medium.

Vacuolar Polyanions—Inorganic polyphosphate accounts for about 99% of the total vacuolar phosphate (8). We wished to know whether another macromolecular polyanion was present in vacuoles of normal or phosphate-starved cultures. A detergent-lysed vacuole preparation from a culture grown in minimal medium was mixed with Dowex 50-H^+ and centrifuged to remove cations, and the supernatant was tested by the gel filtration assay for arginine binding. The arginine:polyphosphate-P ratio in the macromolecular peak was 0.49, suggesting that cations had been removed and that the only macromolecular polyanion was polyphosphate.

In a second experiment, a vacuolar lysate containing 15 μmol of endogenous polyphosphate-P was heated for 10 min at 90 °C (to destroy endogenous enzymes), brought to 5 mM TES Na^+, pH 8.0, and digested for 4 h at 37 °C with 6.25 units of *Escherichia coli* alkaline phosphatase. The lysate lost 95% of its polyphosphate and concomitantly its macromolecular arginine-binding capacity, confirming the conclusion that polyphosphate was the only macromolecular anion. Using this extract, the molecular size class of the competing cations was determined. Synthetic polyphosphate ($n = 200$) was added back to the phosphate-digested extract, and the latter was rerun on the gel filtration column. The arginine:polyphosphate-P ratio of the preparation was 0.34, indicating that competitors had bound again to polyphosphate. Another digested extract was passed through Sephadex G-25 before polyphosphate was added. Polyphosphate was then added to the macromolecular portion of the column eluate. The arginine:polyphosphate-P ratio of this preparation, when tested by gel filtration, was 0.48. This result suggested that the competing cations were not macromolecular. Lysates of vacuoles isolated from polyphosphate-depleted cells, when tested by gel filtration, showed little arginine binding whether or not the lysates were treated with Dowex 50. We conclude that polyphosphate is the only significant macromolecular polyanion normally in the *Neurospora* vacuole. In addition, an alternative polyanion does not replace polyphosphate under conditions of phosphate starvation. Our tests do not exclude smaller, non-phosphate polyanions such as polyglutamic acid.

Multivalent, Vacuolar Cations—The vacuolar cations which compete with Na^+ and arginine in the arginine-binding assay were not displaced from polyphosphate on gel filtration columns run with 100 mM NaCl, but they were removed from polyphosphate by Dowex-50. This suggested that they were multivalent. Indeed, when the polyphosphate-containing fraction of the Sephadex eluate of normal vacuoles was studied, it was found to contain spermidine, Mg^{2+}, and Ca^{2+}. Traces of putrescine and basic peptides were found, but in amounts that could occupy no more than 1% of the vacuolar polyphosphate-P. No polyglucosamine, polygalactosamine, ethanolamine or *S*-adenosylmethionine was detected in the macromolecular (bound) fraction.

The relative amounts of polyphosphate, Mg^{2+}, spermidine, and Ca^{2+} in purified vacuoles are shown in Table I. If we consider the valence of the cations, they account for over 90% of the reduction in arginine-binding capacity of vacuolar polyphosphates (Table I). A reconstruction experiment was done in which Mg^{2+}, spermidine, and Ca^{2+} were added to synthetic polyphosphate in order to mimic their vacuolar ratios. The cations diminished the arginine-binding capacity of the polyphosphate (from 0.5 to 0.29), confirming the expected stoichiometric relationships (Table I). Experiments in which increasing amounts of Mg^{2+} or spermidine were related

TABLE I

Cations competing with arginine for polyphosphate-P

Comparison of arginine-binding capacities of a vacuolar extract and a reconstruction approximating the vacuolar ratios of polyphosphate (poly-P), Mg^{2+}, spermidine, and Ca^{2+}.

Ionic component	Vacuolar extract[a]		Reconstruction[b]	
	Found	Expected poly-P occupied	Found	Expected poly-P occupied
	μmol		*μmol*	
Polyphosphate-P	1.0		1.0	
Mg^{2+}	0.108	0.216	0.1	0.20
Spermidine	0.057	0.171	0.06	0.18
Ca^{2+}	0.012	0.024	0.01	0.02
Arg bound[c]	0.27		0.29	
Poly-P unavailable for Arg binding[d]	0.46	0.411	0.42	0.40

[a] Values for a representative vacuolar extract from cultures grown on minimal medium were adjusted to a polyphosphate pool of 1.0 μmol.

[b] Cations were added to Na polyphosphate (n = 200) as $MgSO_4$, spermidine 3HCl, and $CaCl_2$ to approximate their vacuolar ratios. The small anions and unbound cations were removed by gel filtration with 50 mM NaCl. The arginine-binding capacity of the polyphosphate was then tested on a second gel filtration column eluted with 3 mM arginine HCl, 3 mM NaCl.

[c] Arginine found in macromolecular fraction of a gel filtration column eluted with equimolar arginine and Na^+.

[d] Calculated as 1 − (2 × arginine bound).

to the arginine-binding capacities of polyphosphate also confirmed a stoichiometric relationship of 1 Mg^{2+} to 2 phosphate residues, and 1 spermidine to 3 phosphate residues.

Evidence that spermidine and Mg^{2+} in vacuolar preparations are not merely adventitiously bound to vacuoles in the course of preparation is presented in the Miniprint and Ref. 12.

The amount of spermidine and Mg^{2+} present *in vivo* within the vacuole may be estimated if we assume no preferential leakage of arginine, Mg^{2+}, or spermidine during isolation. The ratio of arginine to polyphosphate, a macromolecule, remains approximately constant during isolation, suggesting little leakage of arginine. In one experiment, 8.4% of the cellular arginine was recovered in purified vacuoles. In the same preparation, 2.1% of the whole-cell spermidine was recovered. We estimate that 25% (2.1:8.4) of the cellular spermidine pool of 16 nmol/mg dry weight is vacuolar. Similarly, we estimate that approximately 10% of the cellular Mg^{2+} pool of 68 nmol/mg dry weight is vacuolar.

Total Vacuolar Cation Pool—The interaction of basic amino acids with vacuolar polyphosphate depends upon the total cation pool, not merely upon the multivalent cations. Monovalent cations are equivalent to one another in their competition for polyphosphate sites (see Miniprint). An estimate of the total vacuolar cation pool was made by titration of Dowex 50-H^+ (see "Experimental Procedures" in Miniprint), using Na^+ equivalents to determine the remaining H^+ in the resin. The total cationic equivalents measured in this way are compared to the sum of individually measured cationic equivalents in Table II. We have identified 90% of these cations (Table II); the most abundant are arginine and ornithine, followed by lysine and histidine. Vacuolar lysates and trichloroacetic acid extracts of vacuoles gave similar values, suggesting that almost all vacuolar cations are acid-soluble. The total cationic charge exceeded that of polyphosphate-P by 50% in this preparation (Table II). K^+ and Na^+ represent only 4% of the total vacuolar cation pool, while they are the major cations of *Neurospora* cytoplasm (180 and 14 mM, respectively, in normal cultures (13)). The fact that only 1% of the cellular K^+ is vacuolar suggests its substantial exclusion from the vacuole.

TABLE II

Cations of vacuoles isolated from cultures growing on minimal medium

Ions	Concentration in vacuolar extract	Charge equivalents
	μmol/ml	*μeq/ml*
Monovalent cations		
Ornithine	18.1	18.1
Arginine	13.6	13.6
Histidine	1.9	1.1[a]
Lysine	1.8	1.8
Na^+	1.5	1.5
K^+	0.7	0.7
Multivalent cations		
Spermidine	2.5	7.5
Mg^{2+}	2.7	5.4
Ca^{2+}	0.3	0.6
Total monovalents	37.6	36.8
Total multivalents	5.5	13.5
Multivalents + monovalents	43.1	50.3
Total cation (by Dowex titration)		57.2
Total polyphosphate-P	36.7	~36.7

[a] Calculated at intravacuolar pH *in vivo*, 6.1 (22).

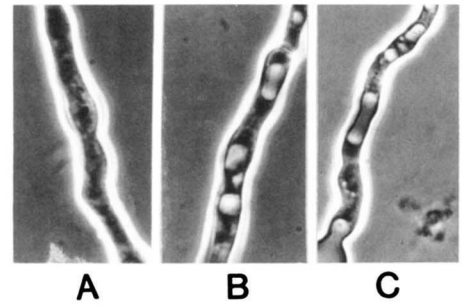

FIG. 3. **Mycelial vacuolation in different growth conditions.** *A*, normal culture during exponential growth (0.6 mg dry weight/ml) on minimal medium. *B*, phosphate-starved culture, 2.5 h after transfer to phosphate-free medium. *C*, culture during exponential growth (0.4 mg dry weight/ml) on 20 mM L-arginine HCl as sole nitrogen source. Samples were observed using a Zeiss phase-contrast microscope within 2 min of placing mycelia on the slide. Magnification, × 1500.

Osmotic Effects of Polyphosphate—Macromolecular polyanions may reduce the osmotic activity of low molecular weight cations. This role has been ascribed to polyphosphates of the yeast vacuole (2) and to ATP of chromaffin granules (4, 14). The polyphosphates of the *Neurospora* vacuole are mainly in the range of 15 to 45 in chain length (9). Solutions of arginine and polyphosphate of chain length 45, whose total solute concentration was 230 mM, was 94 mosM (Table III, Miniprint). This finding suggests that the vacuolar polyphosphate not occupied with multivalent cations can reduce the osmotic activity of univalent cations by as much as 50%. However, in normal vacuoles, the total cationic charge exceeds polyphosphate-P by at least 1.5, and this excess is greatly exaggerated in cells depleted of polyphosphate. In the latter case, because there is no alternative polyanion, the osmotic pressure of the small anions required for charge neutralization must be significantly greater than in the normal cell. In fact, both mycelia grown on arginine as a sole nitrogen source and mycelia starved for phosphate have enormous vacuoles when inspected by light microscopy. By contrast, vacuoles are not visible at this level in cells grown in minimal medium (Fig. 3).

DISCUSSION

Our results show that polyphosphate is the only major macromolecular anion in the vacuoles of normal or polyphosphate-depleted *Neurospora* cells. Even in normal conditions, polyphosphate cannot neutralize all cations of the vacuole, of which we have identified 90% of the charge equivalents. The cations found are arginine, ornithine, lysine, histidine, spermidine, and Mg^{2+}, with small amounts of Ca^{2+}, Na^+, and K^+. In isolated vacuoles of normal cells, these components exceed by about 50% the charge which could be neutralized by polyphosphate-P. The excess is at least 13:1 in conditions of extreme polyphosphate depletion (8) and 3:1 in cultures in which arginine is the sole nitrogen source. Clearly, small anions must normally be imported into the vacuole in order to preserve electroneutrality.

It should be stressed that the cations listed are those of purified vacuoles, not vacuoles *in vivo*. However, cations remain in constant ratio with polyphosphate during purification of vacuoles and, therefore, leakage of the cations does not appear to take place (8). On the other hand, our methods do not exclude the loss of zwitterions and other neutral species in the course of vacuole isolation. Because we have not identified neutral molecules or small anions, no definitive statement can be made regarding the osmotically active contents of vacuoles in the living cell (see below).

Our data contradict the notion that basic amino acid accumulation in *Neurospora* vacuoles might be a passive process, governed by selective permeability of the vacuolar membrane and driven by charge neutralization of polyphosphate-P. They further contradict the notion that polyphosphate charge is required to retain basic amino acids in the isolated vacuole and instead point to an almost complete impermeability of the membranes of isolated vacuoles to the cations inside. Using an assumption about vacuolar volume, we can make a rough estimate of the gradient of arginine, for example, across the vacuolar membrane. Arginine is approximately 30 μmol/g dry weight in normal growth conditions, or approximately 12 mM in cell water. If the arginine were confined to an assumed 10% of the cell water in the vacuole, the intravacuolar concentration would be 120 mM. Independent estimates of arginine concentration in the cytosol yield values in the range of 0.1 mM (15). The nominal transmembrane gradient would therefore be 1200-fold or so. Assuming that association of half the arginine with polyphosphate would diminish its activity coefficient by one-half, one is still left with a large nominal concentration gradient (~900-fold). Thus, it appears that basic amino acid sequestration requires an active transport mechanism. In the living cell, metabolic energy production has proved necessary not only for arginine accumulation into vacuoles, but also for its mobilization, despite the downhill arginine gradient in the latter case (16, 17). Recently, a specific, proton-pumping ATPase has been found in the vacuolar membranes of yeast (18) and *Neurospora* (19), and arginine uptake can be shown to be coupled to ATP consumption *in vitro* with vacuolar membranes of these fungi (20).[2] In view of the earlier findings and those reported here, the efflux of arginine from vacuoles *in vivo* may be limited to reversibility of an ATP-dependent uptake process.

Spermidine and Mg^{2+} in vacuoles are associated with polyphosphate. It is likely that binding of basic amino acids to polyphosphate is excluded to the extent that spermidine and Mg^{2+} occupy anionic equivalents. This conclusion has two implications. First, additional small anions are required to neutralize cationic amino acids not associated with the polyphosphate. Second, both the excess amino acids and their small counterions will add substantial osmotic activity to the vacuoles. Polyphosphates reduce the osmotic activity of monovalent cations by as much as one-half; it is likely that they reduce the osmotic activity of multivalent cations even more. Evidence for an arginine-polyphosphate interaction has been offered by Kanamori *et al.* (21), who showed with ^{15}N nuclear magnetic resonance spectroscopy that the guanidino-N of vacuolar arginine has a greatly reduced relaxation time *in vivo*. While vacuolar viscosity may have contributed to this result, a more likely factor was the association of arginine with polyphosphate, a phenomenon directly observable *in vitro* (22). We cannot yet estimate the osmotic concentration of vacuolar ions *in vivo*, owing to our ignorance of water distribution in cytosolic and vacuolar compartments and of the total solute content of vacuoles. Nevertheless, despite the large amounts of basic amino acids in vacuoles, the latter remain iso-osmotic with the cytosol (~580 mosM (13)) in part because of the interaction of basic amino acids and the available polyphosphate. Without this counterion, basic amino acids cannot be stored without great enlargement of vacuoles. It is known, moreover, that excessive polyphosphate synthesis, seen in recovery from phosphate starvation in yeast (10) and in phosphate control mutants of *Neurospora* (23),[3] allows unusually high basic amino acid pools to develop in vacuoles, possibly by reducing further the chemical activity of basic amino acids.

We conclude that polyphosphate has no obligate role in the accumulation, retention, and neutralization of basic amino acids in the vacuole. Polyphosphate is simply a phosphate reserve which can relieve somewhat the osmotic burden of both phosphate and basic amino acid storage. This allows vacuoles to remain small, and thus to pass easily through the narrow septal pores of hyphae during cytoplasmic streaming, a process required for elongation of filamentous fungal cells. From this and previous work, we can characterize the *Neurospora* vacuole as an amine- and (poly)phosphate-storage organelle, as well as a lysosomal compartment (2, 9, 24). It contributes significantly to the control of cytosolic basic amino acid concentrations (5, 25) through its transport activities and permeability properties.

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REFERENCES

1. Matile, P. (1971) *Cytobiologie* **3,** 324–330
2. Matile, P. (1978) *Annu. Rev. Plant Physiol.* **29,** 193–213
3. Winkler, H., Fischer-Colbrie, and Weber, A. (1981) in *Chemical Neurotransmission: 75 years* (Stjärne, L., Hedquist, P., Lagercrantz, H., and Wennmalm, A., eds) pp.57–68, Academic Press, New York
4. Njus, D., Knoth, J., and Zallakian, M. (1981) *Curr. Top. Bioenerg.* **11,** 107–147
5. Weiss, R. L. (1976) *J. Bacteriol.* **126,** 1173–1179
6. Karlin, J. N., Bowman, B. J., and Davis, R. H. (1976) *J. Biol. Chem.* **251,** 3948–3955
7. Subramanian, K. N., Weiss, R. L., and Davis, R. H. (1973) *J. Bacteriol.* **115,** 284–290
8. Cramer, C. L., Vaughn, L. E., and Davis, R. H. (1980) *J. Bacteriol.* **142,** 945–952
9. Vaughn, L. E., and Davis, R. H. (1981) *Mol. Cell. Biol.* **1,** 797–806
10. Durr, M., Urech, K., Boller, T., Weimken, A., Schwenke, J., and Nagy, M. (1979) *Arch. Microbiol.* **121,** 169–175

[2] B. J. Bowman and E. J. Bowman, personal communication.

[3] C. L. Cramer, unpublished observations.

11. Hummel, J. P., and Dreyer, W. J. (1962) *Biochim. Biophys. Acta* **63,** 530–532
12. Paulus, T. P., Cramer, C. L., and Davis, R. H. (1983) *J. Biol. Chem.* **258,** 8608–8612
13. Slayman, C. W., and Tatum, E. L. (1964) *Biochim. Biophys. Acta* **88,** 578–592
14. Kopell, W. N., and Westhead, E. W. (1982) *J. Biol. Chem.* **257,** 5707–5710
15. Davis, R. H., Weiss, R. L., and Bowman, B. J. (1979) in *Microenvironments and Metabolic Compartmentation* (Srere, P. A., and Estabrook, R. W., eds) pp. 197–210, Academic Press, New York
16. Drainas, C., and Weiss, R. L. (1982) *J. Bacteriol.* **150,** 770–778
17. Drainas, C., and Weiss, R. L. (1982) *J. Bacteriol.* **150,** 779–784
18. Kakinuma, Y., Ohsumi, Y., and Anraku, Y. (1981) *J. Biol. Chem.* **256,** 10859–10863
19. Bowman, E. J., and Bowman, B. J. (1982) *J. Bacteriol.* **151,** 1326–1337
20. Ohsumi, Y., and Anraku, Y. (1981) *J. Biol. Chem.* **256,** 2079–2082
21. Kanamori, K., Legerton, T. L., Weiss, R. L., and Roberts, J. D. (1982) *Biochemistry* **21,** 4916–4920
22. Legerton, T. L., Kanamori, K., Weiss, R. L., and Roberts, J. D. (1983) *Biochemistry* **22,** 899–903
23. Littlewood, B. S., Chia, W., and Metzenberg, R. L. (1975) *Genetics* **79,** 419–434
24. Wiemken, A., Schellenberg, M., and Urech, K. (1983) *Arch. Microbiol.* **123,** 23–35
25. Weiss, R. L., and Davis, R. H. (1977) *J. Bacteriol.* **129,** 866–873
26. Vogel, H. J. (1964) *Am. Nat.* **98,** 435–446
27. Cramer, C. L., Ristow, J. L., Paulus, T. J., and Davis, R. H. (1983) *Anal. Biochem.* **128,** 384–392
28. Seiler, N. (1975) in *Research Methods in Neurochemistry* (Marks, N., and Rodnight, R., eds) Vol. 3, pp. 409–441, Plenum Press, New York
29. Paulus, T. J., and Davis, R. H. (1981) *J. Bacteriol.* **145,** 14–20
30. Felix, A. M., and Jimenez, M. H. (1974) *J. Chromatogr.* **89,** 361–364
31. Manning, G. S. (1978). *Q. Rev. Biophys.* **11,** 179–246
32. Silberberg, A. (1974) in *Polyelectrolytes* (Selegny, E., ed) pp. 1–7, D. Reidel Publishing Co., Dordrecht, Holland

SUPPLEMENTAL MATERIAL TO

Polyphosphate-Cation Interaction in the Amino Acid Containing Vacuole of Neurospora crassa

Carole L. Cramer and Rowland H. Davis

This section contains Experimental Procedures, part of the Results, Figure 1 and Table III.

EXPERIMENTAL PROCEDURES

Experimental organism and growth. Wild type strain 74A was used for all experiments. Cultures were grown from a conidial inoculum (1 x 10^6 conidia/ml for 15-20 hr with aeration at 25°C in Vogel's minimal medium (26) supplemented with 1.5% sucrose. For phosphate-starved cultures, cells from a culture at 0.6 - 0.8 mg dcy weight/ml were filtered through Miracloth, rinsed with distilled water, then rinsed with Vogel's minimal medium with the KH_2PO_4 omitted. The cells were resuspended in phosphate-free Vogel's medium and grown with aeration for another 2 -3 hr.

Vacuole isolation. Vacuoles were purified from cultures grown on minimal medium as described (27). Briefly, 1 - 4 g dcy weight equivalents of mycelia were harvested and disrupted at 4°C in buffered 1M sorbitol by 1.5 min homogenization with glass beads in a Bead Beater (Biospec Products, Bartlesville, OK). All isolation and fractionation solutions contained 10 mM TES, pH 7.5 and 1mM EDTA. Unbroken cells, debris and beads were removed by filtration through cheesecloth, followed by centrifugation for 5 min at 600 x g. This supernatant (S600) was filtered through glass fiber filters (934-AH, Whatman) and centrifuged at 20,000 x g for 20 min. The crude organellar pellet (P20,000) was gently resuspended in buffered 1M sorbitol and layered onto a gradient consisting of 6 ml continuous 1 M sorbitol to 1.8 M sucrose, and a 4 ml 1.8 M sucrose cushion. The tube was centrifuged at 43,000 x g for 2 hr. The vacuolar pellet was resuspended in distilled water, 0.5 M perchloric acid or buffered 1.8 M sucrose, depending upon the experiment. Recovery of intact vacuoles required gentle resuspension in equivalent osmoticum (1.8 M) and was accompanied by about 40% loss due to breakage. The same procedure was also used for isolating vacuoles from cultures grown on 20 mM L-arginine as the sole nitrogen source.

Isolation of vacuoles from phosphate-starved cultures required several changes in the above procedure because of the increased fragility and decreased density of polyphosphate-depleted vacuoles. Cultures, starved for phosphate for 2 hr, were harvested and homogenized with the Bead-Beater for 30 sec in buffer containing 1.5 M sorbitol as osmoticum. After differential centrifugation, the vacuoles of the P20,000 were purified from the other organelles by flotation. The P20,000 (in buffered 1.5M sorbitol) was diluted 1:2 with 1.54 M sucrose. The discontinuous gradient consisted of layers of 1.5 M sorbitol and 1.54 M sucrose in differing proportions: from bottom to top these were 2 ml of 1:1.4 (d = 1.16 g/ml), organellar sample zone at 1:1 (d = 1.15 g/ml), 4 ml of 2.5:1 (d = 1.12 g/ml) and 2 ml of 1:0 (d = 1.08 g/ml) The gradient was centrifuged for 2 hr at 43,000 x g. Vacuoles accumulated at the 1.08 - 1.12 g/ml interface; mitochondria pelletted.

Gel filtration assay for cation binding to macromolecules. The gel filtration method of Hummel and Dreyer (11) was used to study the binding of arginine to polyphosphate or other possible macromolecular polyanions of the vacuoles. The method avoids problems of dissociation of bound ligand during elution by using a constant ligand concentration in the eluant. Sephadex G-25 (fine) columns (0.9 x 10 cm) gave adequate separation of polyphosphates (chain length > 5 in the void volume) and small molecules. Columns were equilibrated at 4°C with 12 - 15 ml eluant, which was 3 mM L-arginine-HCl and 3 mM NaCl except where noted. Samples (0.1 -0.6 ml) were brought to 3 mM arginine, applied to the column, and eluted with 9.5 - 10 ml of elution buffer. The eluate was collected with a fraction collector (approximately 0.35 ml/fraction) and the concentrations of arginine, polyphosphate and other compounds in the fractions were determined. Bound arginine was defined as the amount of arginine in excess of the 3 mM baseline in the eluate. The ionic binding of arginine by the Sephadex matrix was minimized by including 3 mM NaCl in the eluate. Columns were washed with 100 mM NaCl and distilled water between experiments.

Binding of arginine to the polyphosphate added to the column was expressed as an arginine:polyphosphate-P ratio. This ratio was 0.5 for synthetic polyphosphate, owing to the presence of [Na^+] equivalent to the [arginine$^+$]. Several controls were done to assure ourselves that expectations of the technique were met. First, by varying the Na^+:arginine ratio of the eluant, it was shown that Na^+ and arginine were equivalent in their relation to the polyphosphate. Polyphosphate eluted with 3 mM arginine and 6 mM NaCl yielded an arginine:polyphosphate-P ratio of 0.33. Second, a neutral amino acid, alanine, showed no binding to polyphosphate. Third, arginine, ornithine and lysine were equivalent and competitive in their relation to polyphosphate, while at the same pH (6.8, unbuffered), histidine competed less well. The latter behavior would be expected, because a portion of the histidine is uncharged at this pH.

Thin layer chromatography. Vacuolar cationic amines or their dansyl derivatives were identified by their mobilities on one-dimensional thin layer silica plates (Brinkman Polygram Sil G, 20 x 20 cm, 0.25 mm thick). Polyamines were identified by the dansylation and chromatographic procedures described by Seiler (28). Compounds strongly bound to polyphosphates were purified by gel filtration on Sephadex G-25 columns. (Purified vacuoles were extracted with cold 0.5 N perchloric acid for 1 hr and centrifuged. The perchloric-acid-soluble supernatant was neutralized with 1 N NaOH and run on gel filtration columns eluted with 50 mM NaCl. Because only a very small sample (ca. 200 µl) was put on the column, the high cation concentration was immediately reduced, and the elution of the macromolecular fraction and associated, strongly-bound cations was carried out, effectively, in 50 mM NaCl. "Strongly bound" cations emerged with the polyphosphates in the void volume.) The amines eluting with the macromolecules and appropriate standards were reacted with dansyl-Cl, extracted with benzene, and developed on silica thin layer plates in cyclohexane-ethyl acetate (3:2, v/v). Quantitative determinations of spermidine and putrescine were done with a double-isotope dilution assay (29).

For determination of peptides or polyaminoglycans, non-derivatized macromolecular components were treated by desalting on Dowex AG-50X8 (0.7 x 3 cm, H^+ form), which removed polyphosphate as unbound material. The cations were removed from the column by successive washes of 2 ml 0.1 N HCl wash), 4 ml 1.5 N HCl, and 2 ml 6 N HCl. Na^+ emerged in the 1.5 N HCl fraction; the more basic amines were recovered in the 6 N HCl fraction. HCl was removed from fractions by evaporation.

Aliquots of the basic "macromolecular" vacuolar components were hydrolzyed in 4 N HCl for 8 hr at 100°C to reduce polymers to their constituent amino acids or amino sugars. More than 70% of glucosamine and galactosamine standards survived this hydrolysis. Samples (1 - 10 µl) were spotted on silica plates, developed in butanol : acetic acid : ethyl acetate : water (1:1:1:1, v/v/v/v) for approximately 4 h (30). Plates were dried at 110C for 10 min, sprayed with triethanolamine in 2-propanol (1:4, v/v), air dried, sprayed with 0.05% fluorescamine in acetone, air dried and sprayed again with triethanolamine. Plates were viewed using a long-wave UV lamp.

Dowex titration. The total vacuolar cation content was measured by the decrease in titratable H^+ equivalents of Dowex-50 (X8, 200-400 mesh). Small Dowex columns (0.06 ml bed volume) were constructed in Tygon tubing (ID=1.6 mm), and eluted slowly, using a peristaltic pump. The total H^+ equivalents of the Dowex resin were determined by titration with 1 mM NaOH. The amount of Na^+ required to displace all of the H^+ from the resin was monitored either as an increase in eluate pH to greater than pH 7, or as the appearance of Na^+ in the eluate. The latter was measured by atomic absorption spectrophotometry, which was much more accurate than direct pH measurements in the small volumes available. Vacuolar cations were measured by applying vacuoles lysed in 0.1% Triton X-100 in distilled water or 0.5 M perchloric-acid extracts of vacuoles to the columns. Neutral and acidic compounds were washed through with 3 ml distilled water. The H^+ equivalents remaining on the column were determined by the appearance of Na^+ during titration, as described above. The vacuolar cationic equivalents were determined as the difference between the total H^+ of the column before sample application and the H^+ remaining afterward. Trials of the method gave quite reproducible results, and the expected displacement of H^+ by mono-, di- and trivalent cations was observed (Fig. 1).

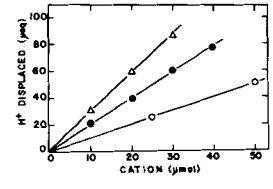

Fig. 1. Dowex-50H^+ titration with various cations. After application of NaCl, $MgCl_2$ or spermidine-3HCl, the column was washed and the remaining H^+ equivalents were determined by titration with NaOH. Symbols: (○) Na^+; (●) Mg^{++}; (△) spermidine^{3+}.

Analytical procedures. Arginine, phosphate, and polyphosphate were extracted and quantified as described previously (8). Amino acids of vacuolar trichloroacetic-acid extracts, after ether extraction, were determined by Ms. Gloria Turner in the laboratory of Dr. R. L. Weiss, Department of Chemistry and Biochemistry, University of California, Los Angeles, using a Beckman amino acid analyzer. Mg^{2+}, Ca^{2+}, Na^+ and K^+ were determined with a Varian AA-275 atomic absorption spectrophotometer. An air-acetylene flame was used. The wavelength (and specific band width) for each compound was as follows: Mg^{2+}, 285.2 nm (0.5); Ca^{2+}, 422.7 nm (0.5); Na^+, 589.6 nm (1.0); K^+, 769.9 nm (1.0). Quenching agents, added to suppress ionization or to minimize interference, were 10 mg La^{3+} per ml for Mg^{2+} and Ca^{2+}, 2 mg K^+ per ml for Na^+, and 1 mg Cs^+ per ml for K^+.

Na^+ and K^+ were determined in vacuolar preparations made in the presence of [^{14}C]sucrose, which allowed calculation of extravacuolar water and subtraction of extravacuolar Na^+ and K^+. In addition, in determination of Na^+, the standard buffer concentration in the final gradient was decreased to 10% of normal; under these conditions, only about 10% of the Na^+ in the final vacuolar pellet was extravacuolar. Finally, Na^+ and K^+ concentrations were determined in preparations made in a buffer with the bulk of the Na^+ replaced with K^+. The determined vacuolar concentrations were not sensitive to this change.

Osmolarity was measured by freezing point depression with an automatic osmometer (Model 4002 Osmette S, Precision Systems, Inc., Waltham, MA).

Chemicals. All chemicals were reagent grade; most were purchased from Sigma Chemical Co. All isotopes were obtained from New England Nuclear Corp. Sephadex G-25 (fine) was from Pharmacia Fine Chemicals. Dowex resins were from Bio-Rad Laboratories. Miracloth was purchased from Calbiochem-Behring.

RESULTS

Spermidine and Mg^{2+} are vacuolar. Multivalent cations tend to bind to many cell constituents. It was therefore necessary to show that the Mg^{2+} and spermidine associated with polyphosphate in our preparations were actually vacuolar. It could be that they co-purified with vacuoles after cell breakage, then bound to polyphosphate when vacuoles were lysed. To distinguish these possibilities, we exploited the high affinity of polyphosphate for Mg^{2+} and spermidine. Various amounts of polyphosphate were added to intact vacuoles. When they were repelleted, less than 1% of the added polyphosphate was associated with the vacuoles. The ratio of Mg^{2+}:arginine (0.18) and of spermidine:arginine (0.15) remained the same, even when external polyphosphate-P exceeded internal polyphosphate-P by 60:1. The arginine:polyphosphate-P ratio in pelleted vacuoles also remained the same, suggesting no leakage of vacuolar arginine. Because Mg^{2+} and spermidine are not removed, the experiment suggests they are within the vacuoles of the living cell. The vacuolar localization of spermidine was also confirmed by the lack of exchange between added [^{14}C]spermidine and vacuolar [^{14}C]spermidine during cell breakage and vacuole purification (12).

Osmometry of phosphate and polyphosphate salts. The osmolarity of phosphate or polyphosphate salt solutions containing similar total solute concentrations was determined (Table 3). For ideal solutions, the osmolarity reflects the concentration of total solute molecules, assuming 100% dissociation. We have compared the osmolarity and solute concentrations (as the mOsM:mM ratio) as a measure of non-ideal behavior (i.e., deviation from 1.0). In comparing phosphate solutions with those of polymer solutions, the ratio of cations to the phosphates was varied to maintain a total solute concentration of about 200 mM. At this concentration, monophosphate salts gave an osmolarity:concentration ratio of about 0.87. Solutions containing polyphosphates had significantly lower osmotic activities than solutions having the same number of monomeric phosphate equivalents (Table 4). Larger polymers were more effective in reducing the osmotic activity of counterions. The effect was also more pronounced at lower concentrations, as predicted by theories of polyelectrolyte condensation (31, 32).

Table III

Osmometry of phosphate and polyphosphate salts.[a]

Sample	Concentration (mM) Cation	Poly-P	P	Total solute	Osmolarity (mOsM)	mOsM/mM
1) Effect of polymer size						
NaH_2PO_4	100	0	103	203	177	0.87
$NaPP_{45}$	186	4	16	206	92	0.45
$NaPP_{200}$	220	1	1	222	56	0.25
2) Effect of concentration						
$NaPP_{45}$	360	8	32	400	170	0.43
$NaPP_{45}$	180	4	16	200	86	0.43
$NaPP_{45}$	90	2	8	100	44	0.44
$NaPP_{45}$	45	1	4	50	16	0.32
3) Effect of counterion						
NaH_2PO_4	100	0	103	203	177	0.87
$NaPP_{45}$	186	4	16	206	92	0.45
KH_2PO_4	91	0	109	200	183	0.92
KPP_{45}	170	4	21	195	103	0.53
$ArgPO_4$	96	0	103	200	159	0.80
$ArgPP_{45}$	204	4	22	230	94	0.41
$OrnPO_4$	96	0	103	199	169	0.84
$OrnPP_{45}$	196	4	21	221	98	0.44

[a] Phosphate determinations were made before and after acid hydrolysis to determine phosphate and polyphosphate-P. The molarity of polyphosphate is given as the acid labile phosphate divided by the average polymer length. As noted, small amounts of phosphate were present in the polyphosphate standards. Counterion coversions were accomplished by passing Na polyphosphate through Dowex-50 columns in the K^+, arginine$^+$ or ornithine$^+$ forms.

Polyphosphate-cation interaction in the amino acid-containing vacuole of Neurospora crassa.

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