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Compartmentation of Spermidine in *Neurospora crassa**

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The polyamines putrescine, spermidine, and spermine are multivalent cations that bind to anionic cell constituents such as nucleic acids. Their distribution between free and bound states within the cell is not known. Such knowledge would be important in relation to the negative control of polyamine synthesis. We report a tracer experiment in which [^{14}C]ornithine was added to logarithmically growing *Neurospora crassa* mycelia. The amount and the specific radioactivity of the three polyamines thereafter suggested that new molecules of spermidine were made preferentially from new molecules of putrescine, and that new molecules of spermine were made from new molecules of spermidine. The extent of mixing of new [^{14}C]- and resident [^{12}C]spermidine indicated that 70% or more of the resident spermidine was sequestered, and not immediately accessible to spermine synthase. Cell fractionation revealed that about 28% of the cellular spermidine was vacuolar, and nonexchangeable with [^{14}C] spermidine added at the time of cell breakage. We suggest that the remainder of sequestered spermidine is bound strongly to anionic sites in the cell, and is relatively inactive in the control and synthesis of polyamines.

The polyamines (putrescine, spermidine, and spermine) and in initial enzyme of the synthetic pathway, ornithine decarboxylase (EC 4.1.1.17), are widely distributed in nature (1). Elevated activities of ornithine decarboxylase are almost universally associated with rapid cell growth in organisms as diverse as *Escherichia coli*, fungi, and mammals (2-5). The control of ornithine decarboxylase by polyamines is not direct, as a rule, and various indirect mechanisms have been proposed (5-9). Much uncertainty surrounds these mechanisms, not only because the effect of polyamines is indirect, but also because the pool sizes of polyamines are poorly correlated with the manifestation of control. For instance, ornithine decarboxylase activity of *Neurospora* cells with substantial polyamine pools becomes elevated after a brief interruption of polyamine synthesis brought about by ornithine deprivation.[1] Similarly, in other systems, very small amounts of added, exogenous polyamines have major negative effects upon ornithine decarboxylase activity, despite the existence of large endogenous pools in the same cells (10, 11).

Most of the polyamines, which are highly charged cations at cellular pH values, are probably bound to anionic cell constituents such as ribosomes, DNA, and membranes (3, 12, 13), and these sequestered pools may be inactive in control (14). While this possibility is supported by many polyamine-binding studies *in vitro* (*e.g.* Ref. 15), direct evidence that polyamines are sequestered *in vivo* is scarce (16, 17). Because cell disruption alters the ionic environment and leads to rapid equilibration of labeled (exogenous) and unlabeled (endogenous) polyamines among cell fractions (*e.g.* Ref. 18), no definite conclusions can be drawn about the diffusible state or the location of polyamines *in vivo*.

The present work demonstrates through tracer metabolism in living cells that most of the endogenous spermidine, the predominant polyamine in *Neurospora*, is sequestered from the metabolic reactions which produce and use it. Moreover, the vacuole of *Neurospora* is shown to sequester some of the polyamines of *Neurospora* in a nonexchangeable form. This work extends previous work done on putrescine sequestration (19) and vacuolar polyamines (20) of *Neurospora*.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Compartmentation of Spermidine in Vivo—Previous studies have shown that exponentially growing mycelia of *Neurospora crassa* rapidly take up traces of [^{14}C]ornithine from the medium and incorporate it into polyamines (21-23). We wished to use [^{14}C]ornithine to label the spermidine pool and to observe the rate and pattern by which the spermine pool acquired label. Compartmentation of spermidine would be indicated: (i) if the specific radioactivity of new spermine molecules synthesized during the labeling period was significantly greater than the specific radioactivity of the total, acid-extractable spermidine pool; and (ii) if spermine became labeled more quickly than predicted according to isotope dilution by the resident spermidine pool. Such observations would indicate that label flowing from ornithine into spermine was bypassing the resident spermidine pool. Similar experiments have revealed the vacuolar compartmentation of ornithine in *Neurospora* (21) and the sequestration of putrescine (19).

In the experiment, 10 μM [^{14}C]ornithine (4200 cpm/nmol) was added to exponentially growing cells. The radioactivity was wholly taken up by 20 min. The amounts and specific radioactivities of the polyamines were determined at intervals

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[1] R. H. Davis and G. N. Krasner, unpublished observations.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1 and 2, and Tables I-III) are presented in miniprint at the end of this paper. 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. 82M-3265, cite authors, and include a check or money order for $8.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

for 2 h after the addition of labeled ornithine. The basic data underlying the specific radioactivity comparisons to be made (Figs. 1 and 2; Table I) are discussed in the Miniprint.

One objective method of estimating spermidine sequestration is to compare the specific radioactivity of new molecules of spermine (and, thus, of their actual precursor pool) with the specific radioactivity of total, extractable spermidine for a short interval. The results of such a comparison are shown in Fig. 3 for all three polyamines and their extractable precursors. On the *right*, total spermidine is compared to new spermine. Over the interval of 0–10 min, the average specific radioactivity of spermidine was 62.5 cpm/nmol, while that of new spermine was 210. Thus, in this period, only 62.5/210, or 30% of the spermidine, was available as a spermine precursor. This is a maximal estimate, because during 10 min, there is an opportunity for equilibration of the sequestered and the spermine-precursor pools, as we have noted in previous applications of this method (21). Nevertheless, it demonstates that at least 70% of the spermidine pool is sequestered from use as a spermine precursor. The pattern of the new spermine and total spermidine curves in later times (Fig. 3, *right*) is consistent with this interpretation. Label increases in total spermidine, while the new spermine ultimately comes to be made from *less* radioactive molecules. The data suggest that increasing numbers of labeled spermidine molecules are becoming sequestered, and spermine is made, in the 80–120-min interval, from newer, less radioactive molecules of spermidine.

A second method of estimating spermidine sequestration is to compare the specific radioactivities of *new* spermidine (Fig. 3, *middle*) and new spermine (Fig. 3, *right*). The first point of the curves (1750 and 210 cpm/nmol for spermidine and spermine, respectively) show that, as radioactivity moves from new spermidine to new spermine, it is diluted 8.3-fold by unlabeled, endogenous spermidine. The amount of spermine made each generation is very small: 0.31 nmol/mg dry weight. To dilute this 8.3-fold requires only $7.3 \times 0.31 = 2.3$ nmol of unlabeled spermidine. This is 2.3/18.2, or 12.6% of the total spermidine pool. Thus, on this basis, about 87% of the spermidine pool fails to participate in spermine synthesis.

Thus, according to these calculations, over 70% of the cellular spermidine pool is sequestered from the enzyme which uses it in spermine synthesis. Both methods use single initial values of specific radioactivity, one of which (spermine) is

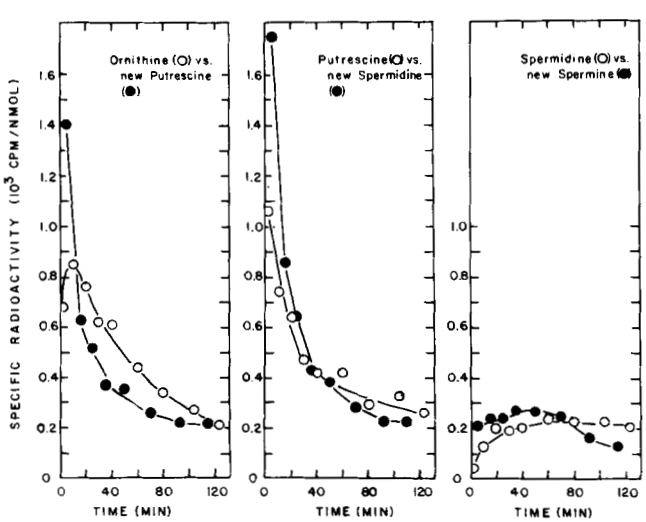

FIG. 3. **Comparison of the specific radioactivity of total pools of precursors (○) and the specific radioactivity of new molecules of their metabolic products (●).** *Left*, ornithine *versus* new putrescine. *Middle*, putrescine *versus* new spermidine. *Right*, spermidine *versus* new spermine.

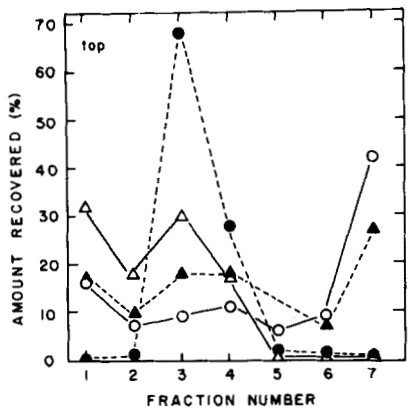

FIG. 4. **Sucrose-sorbitol density gradient centrifugation of crude cell homogenate to which [^{14}C]spermidine had been added.** Succinate dehydrogenase (mitochondrial marker) (●); arginine (vacuolar marker) (○); total spermidine (endogenous) (▲); [^{14}C] spermidine (exogenous) (△). The top of the gradient is at the *left*; fraction 7 is the pellet.

rather low. Because they are drawn from intervals of label accumulation that permits some mixing of sequestered and spermine-precursor pools, spermidine sequestration as we have defined may be in the 80–90% range, if not higher. The data are consistent with a previous experiment using a somewhat different tracer rationale (19). Further discussion of Fig. 3 is found in the Miniprint.

Spermidine Compartmentation in Cell Fractions—We wished to associate at least some of the sequestered spermidine with one or more cell fractions. To do so, we disrupted cells in conditions which preserve many organelles, and isolated organelles by differential and gradient centrifugation. In order to control for redistribution of spermidine during cell breakage and fractionation, [^{14}C]spermidine was added to cells before cell breakage. In these experiments, only 8% of the total spermidine was sedimentable at 20,000 × *g*. Sorbitol-sucrose density gradient centrifugation of this organellar pellet revealed that, while spermidine was associated with both mitochondria and the vacuoles, only the vacuoles were relatively free of the added [^{14}C]spermidine tracer (Fig. 4). About 2% of cellular spermidine was found in vacuoles; the recovery of vacuoles was estimated to be 7%, judging from the arginine content of this fraction. Assuming proportionate loss of vacuolar arginine and vacuolar spermidine, and knowing that 98% of cellular arginine is in vacuoles (24), we consider 28% of cellular spermidine to reside in (or bound in nonexchangeable form to) vacuoles. This is only a minor portion of the 70–87% of spermidine judged to be sequestered by the metabolic experiments.

The analysis of other cell fractions (see Miniprint) reveals that spermidine associated with mitochondria and ribosomes has the same specific radioactivity as the spermidine in supernatants from which it came. Therefore, no conclusion can be drawn regarding specific associations of spermidine with these organelles *in vivo*.

DISCUSSION

According to our tracer experiments, both spermidine and its precursor putrescine (19) are sequestered *in vivo* in some fashion. The degree of sequestration is on the order of 70–87%. This figure is based on the extent to which resident polyamines fail to mix with newly labeled molecules of polyamine before the latter are used in a subsequent reaction. To our knowledge, these are the first demonstrations of polyamine sequestration with tracers in living cells. We can account for about one-third of the sequestered spermidine as the vacuolar pool, which is nonexchangeable even *in vitro*

with added [^{14}C]spermidine. This is a finding directly comparable with that of Seiler and Deckhardt (25) who found nonexchangable polyamines in synaptosomal membranes of the rat brain. The remainder of the polyamine sequestration cannot be accounted for by our cell fractionation experiments. Two mechanisms besides organellar compartmentation could account for the *in vivo* tracer data. The first is that enzymes of polyamine synthesis are aggregated such that products of one reaction tend not to diffuse before becoming substrates of the subsequent reaction (26–28). This would prevent new molecules from mixing fully with resident polyamines. However, in all systems investigated to date, spermidine and spermine synthetases are distinct enzymes (29, 30). Nevertheless, aggregation of polyamine enzymes may prevail, and must be retained as a hypothesis to explain our data.

The second possible mechanism of sequestration is that binding of resident spermidine (and putrescine) to cell constituents is sufficiently strong to prevent extensive mixing of new, freely diffusing molecules with the bound fraction. This phenomenon may be magnified by a distribution of spermidine to the nucleus and to ribosomes, if they offer sites which are less accessible than others to free diffusion. (Even less basic molecules have been found to be disposed in gradients in bacteria (34, 35).) Ribosomes bind spermidine and spermine well, particularly at low ionic strength (15, 31–33). Spermine appears to bind to ribosomes at sites that cannot be displaced by acridine orange; rRNA does not have such sites (15). The nucleus also may, owing to its membrane, offer barriers to exchange between DNA-bound and cytosolic polyamines (16, 17). Thus "sequestered," nonvacuolar polyamines may be those bound to nucleic acids.

The detection of "bound" and "free" pools of spermidine and putrescine may explain several unusual features of polyamine metabolism in various organisms. Cells treated with the ornithine decarboxylase inhibitor, α-difluoromethylornithine, become depleted of polyamines and develop a greatly increased rate of polyamine transport into the cell (36, 37). It may well be, as Seppanen *et al.* (37) recognized, that the increased rate reflects the emergence of unsaturated polyamine-binding sites during polyamine depletion. A second phenomenon which is widely observed (5, 38–41) is that treatment of cells of various species with hypotonic medium is sufficient to induce elevation of ornithine decarboxylase activity. This may reflect a normal response of the enzyme's negative control system to the greater binding of polyamines (and, thus, their withdrawal) brought about by lowering the ionic strength. Finally, in diverse cell types, treatment with low levels of putrescine or spermidine have large regulatory effects despite far larger internal pools of these molecules (10, 11). In this case, the added polyamines may be more freely diffusible (and, thus, more effective in control) as they enter the cell than the resident, bound polyamines.

REFERENCES

1. Cohen, S. S. (1971) *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs, NJ
2. Tabor, C.W., and Tabor, H. (1976) *Annu. Rev. Biochem.* **45,** 285–306
3. Stevens, L., and Winther, M. D. (1979) *Adv. Microbiol Physiol.* **19,** 63–148
4. McCann, P. (1980) in *Polyamines in Biomedical Research* (Gaugas, J. M., ed) pp. 109–123, John Wiley & Sons, New York
5. Canellakis, E. S., Viceps-Madore, D., Kyriakidis, D. A., and Heller, J. S. (1979) *Curr. Top. Cell. Regul.* **15,** 155–202
6. McCann, P. P., Tardif, C., Hornsperger, J.-M., and Böhlen, P. (1979) *J. Cell. Physiol.* **99,** 183–190
7. Costa, M., de Mars, M., and Lin, S. N. (1982) *Biochem. Biophys. Res. Commun.* **107,** 109–116
8. Atmar, V. J., and Kuehn, G. D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 5518–5522
9. Sekar, V., Atmar, V. J., Krim, M., and Kuehn, G. D. (1982) *Biochem. Biophys. Res. Commun.* **106,** 305–311
10. Mitchell, J. L. A., Carter, D. D., and Rybski, J. A. (1978) *Eur. J. Biochem.* **92,** 325–331
11. Heller, J. S., Chen, K. Y., Kyriakidis, D. A., Fong, W. F., and Canellakis, E. S. (1978) *J. Cell. Physiol.* **96,** 225–234
12. Viotti, A., Bagni, N., Sturani, E., and Alberghina, F. A. M. (1971) *Biochim. Biophys. Acta* **244,** 329–337
13. Cohen, S. S. (1978) *Adv. Polyamine Res.* **1,** 1–10
14. Paulus, T. J., and Davis, R. H. (1981) *J. Bacteriol.* **145,** 14–20
15. Stevens, L., and McCann, L. M. (1970) *Ann. N. Y. Acad. Sci.* **171,** 827–837
16. McCormick, F. (1977) *J. Cell. Physiol.* **93,** 285–292
17. Mach, M., Ebert, P., Popp, R., and Ogilvie, A. (1982) *Biochem. Biophys. Res. Commun.* **104,** 1327–1337
18. Tabor, C. W., and Kellogg, P. D. (1967) *J. Biol. Chem.* **242,** 1044–1052
19. Paulus, T. J., and R. H. Davis (1982) *Biochem. Biophys. Res. Commun.* **104,** 228–233
20. Vaughn, L. E., and Davis, R. H. (1981) *Mol. Cell. Biol.* **1,** 797–806
21. Karlin, J. N., Bowman, B. J., and Davis, R. H. (1976) *J. Biol. Chem.* **251,** 3948–3955
22. Bowman, B. J., and Davis, R. H. (1977) *J. Bacteriol.* **130,** 274–284
23. Bowman, B. J., and Davis, R. H. (1977) *J. Bacteriol.* **130,** 285–291
24. Cramer, C. L., Vaughn, L. E., and Davis, R. H. (1980) *J. Bacteriol.* **142,** 945–952
25. Seiler, N., and Deckhardt, K. (1978) *Adv. Polyamine Res.* **2,** 145–159
26. Davis, R. H. (1967) in *Organizational Biosynthesis* (Vogel, H. J., Lampen, J. O., and Bryson, V., eds) pp. 303–322, Academic Press, New York
27. Matchett, W. H. (1974) *J. Biol. Chem.* **249,** 4041–4049
28. Traut, T. W. (1982) *Trends Biochem. Sci.* **7,** 255–257
29. Cohn, M. S., Tabor, C. W., and Tabor, H. (1980) *J. Bacteriol.* **142,** 791–799
30. Zappia, V., Cacciapuoti, G., Pontoni, G., Della Ragione, F., and Carteni-Farina, M. (1981) *Adv. Polyamine Res.* **3,** 39–53
31. Nakamoto, T., and Hamel, E. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **59,** 238–245
32. Silman, N., Artman, M., and Engleberg, H. (1965) *Biochim. Biophys. Acta* **103,** 231–240
33. Cohen, S. S., and Lichtenstein, J. (1960) *J. Biol. Chem.* **235,** 2112–2116
34. Leive, L., and Davis, B. D. (1965) *J. Biol. Chem.* **240,** 4370–4376
35. Tabor, H., and Tabor, C. W. (1969) *J. Biol. Chem.* **244,** 6383–6387
36. Alhonen-Hongisto, L., Seppanen, P., and Janne, J. (1980) *Biochem. J.* **192,** 941–945
37. Seppanen, P., Alhonen-Hongisto, L., and Janne, J. (1981) *Eur. J. Biochem.* **118,** 571–576
38. Mitchell, J. L. A., and Kottas, G. E. (1979) *FEBS Lett.* **102,** 265–268
39. Perry, J. W., and Oka, T. (1980) *Biochim. Biophys. Acta* **629,** 24–35
40. Kapyaho, K., and Janne, J. (1982) *Biochim. Biophys. Acta* **714,** 93–100
41. Harris, W. A., and North, M. J. (1982) *J. Bacteriol.* **150,** 716–721
42. Davis, R. H., and Mora, J. (1968) *J. Bacteriol.* **96,** 383–388
43. Vogel, H. J. (1964) *Am. Naturalist* **98,** 435–446
44. Davis, R. H., and deSerres, F. J. (1970) *Methods Enzymol.* **17A,** 79–143
45. Paulus, T. J., and Davis, R. H. (1983) *Methods Enzymol.* **94,** in press
46. Chinard, F. P (1952) *J. Biol. Chem.* **199,** 91–95
47. Inoue, H., and Mizutani, A. (1973) *Anal. Biochem.* **56,** 408–416
48. Davis, R. H., Bowman, B. J., and Weiss, R. L. (1978) *J. Supramol. Struct.* **9,** 473–488
49. Cramer, C. L., Ristow, J. L., Paulus, T. J., and Davis, R. H. (1983) *Anal. Biochem.* **128,** 384–392
50. Pennington, R. J. (1961) *Biochem. J.* **80,** 649–654
51. Stewart, P. R. (1975) *Methods Cell. Biol.* **12,** 111–147
52. Russell, P. J., and Wilkerson, W. M. (1980) *Exp. Mycol.* **4,** 281–337

SUPPLEMENTAL MATERIAL TO

Compartmentation of Spermidine in Neurospora crassa

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This section contains Experimental Procedures, part of the Results, Figures 1 and 2 and Tables I-III.

EXPERIMENTAL PROCEDURES

Strains and growth - Wild-type Neurospora crassa (74A) and the mutant strain ota (UM-728) were from the collection of R.H. Davis. The ota strain lacks a functional ornithine transaminase (42). Vogel medium N (43) was used for growth. Mycelia were grown from conidial inocula (10^6 conidia per ml) with forced air at 25°C. Dry weights were determined by harvesting 15 to 25 ml of culture on Whatman no. 540 filter circles and then acetone-drying the mycelial pad (44).

In vivo labelling experiment - One liter of the ota strain was grown exponentially to a dry weight of approx. 0.6 mg/ml. The culture was split into two portions. [^{14}C]ornithine (0.01 mM, 4200 cpm/nmol) was added to 300 ml of culture. Non-radioactive ornithine (0.01 mM) was added to the remainder; this culture was used as a control for continued dry weight measurement and polyamine pool analysis.

The radioactive culture was sampled at 10 and 20 min intervals (as indicated) by filtering 20 ml of culture on Gelman cellulose acetate membrane filters (5.0 μ pore size). The pad was washed with 5.0 ml of water and quickly placed in 1 ml of cold 0.4 M $HClO_4$. The insoluble portion was removed by centrifugation, and extracted twice more with 1 ml cold 0.4 M $HClO_4$. The supernatants of each extraction were combined (3 ml total) and fractionated to determine specific radioactivities of ornithine, putrescine, spermidine and spermine; total radioactivity in ornithine, arginine and polyamine fractions; and ornithine pool size. Samples were periodically harvested from the control culture (non-radioactive) and extracted by an identical procedure. Radioactivity remaining in the medium was determined by counting an aliquot after filtration through Whatman #540 filters.

Steady-state pool sizes and total radioactivity measurements - Steady-state polyamine pools were determined using extracts from the control culture using the double-isotope derivative assay as previously described (14, 45). No derivatives of polyamines would be detected by this method. No derivatives of polyamines were seen in Neurospora cultures when sought, where 5% or more of the normal pools would have been detected.

The ornithine pool size and total radioactivity in ornithine, arginine, and a polyamine fraction were determined from 1 ml of the radioactive extracts by the procedure of Karlin et al. (21); 1 μmol of non-radioactive spermidine was added to each sample prior to loading on the Dowex-50 (Na^+) columns to assure good recovery of labelled polyamines from the column. Total radioactivities were determined in the various fractions by counting 0.5 ml aliquots (plus 0.5 ml H_2O) in 10 ml of 1 part triton X-100:2 parts toluene (v/v), the latter with 0.5% (w/v) 2,5-diphenyloxazole.

Total ornithine was determined colorimetrically by the method of Chinard (46). The specific radioactivity of ornithine was determined from the pool size and total radioactivity.

Specific radioactivities of the polyamines - For the tracer experiment, polyamines were purified and concentrated from 2 ml of the radioactive samples using the Dowex-50 (H^+) procedure of Inoue and Mizutani (47). This step was necessary because of very low levels of radioactivity in spermine. The polyamine fraction, in 6 N HCl, was evaporated to dryness in vacuo at 55°C and resuspended in 0.5 ml of 0.4 M $HClO_4$. The specific radioactivities of putrescine and spermidine were determined from 0.1 ml of this solution using [^{3}H]dansyl chloride as previously described (19, 45). The specific radioactivity of spermine was expected to be low; therefore it was important to dansylate and purify as much spermine as possible. The remaining 0.4 ml of the radioactive $HClO_4$ extract was reacted with 0.4 ml of [^{3}H]dansyl chloride (1.5 mg per ml acetone, 473 cpm per nmol) in the presence of excess Na_2CO_3 for 6 h at 37°C. The reaction mixture was extracted with 1 ml of reagent grade benzene. The benzene layer was removed and allowed to evaporate in a fume hood. The sample was resuspended in 30 ul of benzene and 20 μl were spotted on a silica gel thin layer chromatogram. The chromatogram was developed, using the same solvents as used for putrescine and spermidine, as previously described (45).

The specific radioactivity of each polyamine was calculated by dividing the ^{14}C cpm (recovered as dansyl-polyamine) by the number of nmol of polyamine calculated from the ^{3}H cpm (using the known specific radioactivity of the [^{3}H]dansyl chloride). A stoichiometry of two dansyl groups per putrescine adduct, three per spermidine adduct, and four per spermine adduct was assumed for the calculations. All samples were counted in a Beckman LS-230 scintillation counter to 0.2% error or for 100 min. The ^{3}H cpm were corrected for the amount of ^{14}C cpm in the ^{3}H channel (approx. 16 to 20%). This was determined for every experiment using non-radioactive dansyl chloride and [^{14}C]polyamine standards. When very low ratios of ^{14}C cpm to ^{3}H cpm were encountered the ^{14}C cpm were corrected for the amount of ^{3}H cpm in the ^{14}C channel (1.2%, determined using [^{3}H]dansyl chloride and non-radioactive polyamines). The counting efficiencies were about 20% for ^{3}H and > 90% for ^{14}C.

Calculations applying to tracer experiment - The double-isotope method gives the specific radioactivities of the total polyamine pools at the time of extraction. The specific radioactivity of newly synthesized polyamine (e.g., "new spermine") was calculated from changes in the amount of polyamine as the culture grew and from the specific radioactivity. First, the amount of polyamines made in a given interval, ΔX, can be found by the formula; $\Delta X = X_0(e^{kt} - 1)$, where X_0 is the amount at the beginning of the interval, t is the time of the interval in minutes, and k is, for a culture doubling time of 126 min, 0.0055 min^{-1}. Second, the total radioactivity in the entire polyamine pool of a given sample was calculated by multiplying the known concentrations of polyamine (nmol per mg dry weight) by the specific radioactivity of the extracted polyamine (cpm per nmol). To determine the specific radioactivity of newly synthesized polyamine the change in radioactivity (Δ cpm) of the polyamine was divided by the change in nanomoles of the polyamine (ΔX). Similar calculations have been described in detail in previous publications from this laboratory in connection with the vacuolar compartmentation of basic amino acids (21, 48).

The calculations are not compromised by serious turnover or metabolism of polyamines in the course of the experiment. First, no turnover of bulk polyamines was found in previous work (14). Second, an ornithine decarboxylase mutant, grown on spermine-supplemented medium and transferred to minimal medium, showed little loss (no more than 4% per hour) over a seven-hr period. Third, as noted above, no derivatives of polyamines have been detected in our cultures. Thus we use spermine accumulation as a measure of spermine synthesis in our calculations.

Cell fractionation experiments - Wild-type (74A) cultures were grown exponentially to a dry weight of approximately 1 mg per ml. Cells were disrupted and subcellular fractions obtained as described elsewhere (20, 49). Briefly, buffer-washed mycelia were homogenized for 1.5 min with glass beads in a commercially available instrument, the "Bead-Beater" (Biospec Products, Bartlesville, OK). Unless noted, the fractionation buffer consisted of 10 mM TES-NaOH (pH 7.5), 1 mM EDTA and ½ M sorbitol. Breakage, and all subsequent steps, were carried out at 0-4°C. Glass beads were removed by filtering thru cheesecloth. The broken cell filtrate was centrifuged at 600 X g for 5 min. The supernatant was filtered through glass fiber filters (934-AH, Whatman, Ltd.) to remove remaining cell wall fragments. This cell free filtrate (S600) was centrifuged at 20,000 X g for 20 min. The supernatant (S20,000) was centrifuged at 100,000 X g for 2 h in a SW41-Ti Beckman rotor to obtain a pellet enriched in ribosomal RNA. The crude organellar pellet (P20,000) was gently resuspended in buffered 1 M sorbitol and layered onto a density gradient of 6 ml. continuous from 1 M sorbitol to 1.8 M sucrose, on top of a 4 ml cushion of 1.8 M sucrose. The gradient was centrifuged at 43,000 X g for 2 h in a Sorvall SS-34 rotor. Mitochondria band approximately one-third the way into the gradient; vacuoles pellet (49). Fractions were collected from the top using a pump and automatic fraction collecter.

Approximately 8 μCi of [^{14}C]spermidine was added with (8 μmol) and without carrier spermidine directly to the "Bead-Beater" chamber prior to cell breakage. The specific radioactivities of spermidine in $HClO_4$ extracts of the resulting fractions were determined using [^{3}H]dansyl chloride as described for the tracer experiments. Pellet fractions were resuspended in 1 or 2 ml of fractionation buffer. A 0.5 ml aliquot of each fraction was mixed with 0.5 ml of cold 0.8 M $HClO_4$. After standing for 30 min in an ice bath, the samples were centrifuged to remove insoluble material. In experiments involving vacuole purification the supernatants were processed through the column procedure of Inoue and Mizutani (47). The final dried sample was resuspended in 0.4 M $HClO_4$ to achieve a concentration of radioactivity of about 10^4 to 10^5 cpm per ml. Either 0.1 or 0.2 ml was dansylated and processed as described above. In experiments not requiring isolation of vacuoles, the high recovery of spermidine obviated the need for concentration via the column procedure. In these experiments, 0.1 ml of the $HClO_4$ extract was dansylated and processed directly.

Total radioactivity in each fraction was determined by counting an aliquot (in 1 ml H_2O) added to 10 ml of the scintillation fluid described above. The extraction and determination of polyphosphate and arginine have been described previously (24). Succinate dehydrogenase was estimated by the method of Pennington (50). RNA was determined using the orcinol assay of Stewart (51).

Isotopes - Radioactive isotopes, [1, 4-^{14}C]putrescine dihydrochloride (90.2 mCi/mmol), [tetramethylene-1, 4-^{14}C]spermidine trihydrochloride (98.7 mCi/mmol), [tetramethylene-1, 4-^{14}C]spermine tetrahydrochloride (74.0 mCi/mmol), 5-[methyl-^{3}H]dimethylaminonaphthalenesulfonyl chloride (26.08 Ci/mmol) and L-[U-^{14}C]ornithine (279 mCi/mmol) were purchased from New England Nuclear.

RESULTS

Polyamines formed from labelled ornithine - In this experiment, 10 uM [^{14}C]ornithine (4,200 cpm per nmol) was added to exponentially growing mycelia of Neurospora and the specific radioactivities of ornithine and the polyamines were determined thereafter. The steady-state pool sizes of ornithine, determined in the test culture, and of the polyamines, determined in a parallel control culture, are shown in Table I. The concentration of [^{14}C]ornithine was higher in this experiment than in previous experiments of this type (19, 21) in order to extend the labeling period. This was necessary because of the extremely low rate of spermine synthesis (≤ 0.005 nmol per min per mg dry weight). The ota mutation in the strain confined the use of [^{14}C]ornithine to the arginine and polyamine pathways. The pattern of labeling of acid-extractable ornithine, arginine and polyamines is shown in Fig. 1 (left). Label disappeared from the culture medium within 20 min. A significant portion of the [^{14}C]ornithine entered the cell in the first minute (first point). Arginine and polyamines became labelled quickly in the first 20 min. Accumulation of label into free arginine ceased after this period. This was due largely to the balance of arginine synthesis and its use in protein synthesis in the last three-quarters of the experiment. The total polyamine fraction, however, continued to accumulate label, as expected of "dead-end" products of ornithine metabolism.

The specific radioactivities of the polyamines determined with the [^{3}H]dansyl chloride technique are shown in Fig. 1 (right). This figure depicts the raw data from which, with the pool sizes of Table I, subsequent calculations were made. The initial specific radioactivity of putrescine was high and decreased thereafter. The specific radioactivity of spermidine was initially low because of the large resident spermidine pool; however, it quickly increased, reaching a steady value after 30 min. The specific radioactivity of spermine was always low, but it increased steadily in the course of the experiment.

TABLE I

Cellular pool sizes of ornithine and the polyamines.

Metabolite	Content (± std.dev.)	Number of determinations
	(nmol/mg dry wt.)	
Ornithine	40 ± 5	7
Putrescine	1.1 ± 0.3	5
Spermidine	18.2 ± 0.9	5
Spermine	0.31 ± 0.03	5

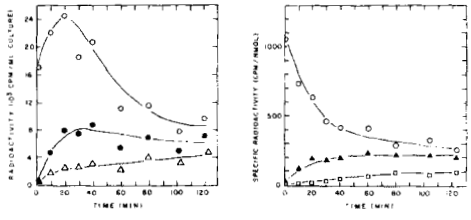

Figure 1. Left: Radioactivity associated with ornithine (○), arginine (●) and the total polyamine fraction (△) after addition of [^{14}C]ornithine at time zero. Right: Specific radioactivities of putrescine (○), spermidine (▲) and spermine (□) in the course of the same experiment.

The total radioactivity in putrescine, spermidine and spermine (Fig. 2) was calculated by multiplying the data in Fig. 1 (left) times the steady state pool sizes (Table I) and the dry weight per ml culture at each sample point. (The growth curve is not shown; the culture had a doubling time of 126 min.). The sum of the radioactivity in the three polyamine pools determined in this way yielded a curve indistinguishable from the one for total polyamines in Fig. 1 (left). The latter was obtained from direct measurement of the bulk polyamine fraction from the Dowex 50 (Na^+) column.

Patterns of change in metabolism of labelled intermediates - The appearance of label in spermine (Fig. 2) begins before 10 minutes, and reaches a constant rate by 20 minutes. Unfortunately, the curve is not quantitatively interpretable because the rate of entry of label into spermidine is not constant. Thus, while new spermine molecules achieve constant specific radioactivity quickly, the time it takes to do so cannot be used (as it has been previously (21)) to calculate the size of the dynamic subpool of spermidine which gives rise to it. The constant rate of appearance of radioactive spermine, in fact, obscures the compensatory changes during the first 40 minutes in the entry of label into the spermidine pool (diminishing with time; see below) and the number of turnovers of this pool (increasing with time). Nevertheless, the time required for label to appear in spermine is very short, given the large spermidine pool and the low rate of synthesis of its product (Table I).

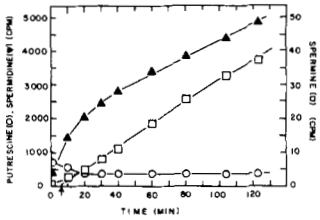

Figure 2. Total radioactivity associated with the pools of putrescine (O), spermidine (▲) and spermine (□) in the experiment of Fig. 1.

It is useful to analyze Fig. 3 more fully in order to test the adequacy of the data, and to suggest possible models of polyamine-intermediate channeling. In the left panel, new putrescine is more radioactive than ornithine initially, because the cytosol is being preferentially labelled by [^{14}C]ornithine uptake; most of the cellular ornithine is unlabelled and sequestered in the vacuole. After 10 min, however, new putrescine becomes less radioactive than ornithine because the cytosol has been flushed with endogenously synthesized ornithine, and the remaining labelled ornithine is now largely in the vacuole (25, 33, 34).

In the middle panel, the new spermidine curve is very similar to the new putrescine curve of the left panel. In fact, it is slightly higher. (This cannot actually be the case, and points to limitations of our specific radioactivity data.) Nevertheless, the similar patterns of decline in the specific radioactivities of new putrescine and new spermidine suggest that new putrescine molecules are destined with high probability to become new spermidine molecules, a conclusion we reached on the basis of a pulse-chase experiment previously (19). Finally, while considerable dilution of new spermidine molecules takes place as they are used for spermine synthesis, it is clear that the tiny flux toward spermine selects new spermidine molecules at a higher probability (1 of 8; see main text) than their representation among spermidine molecules of the cell as a whole (1 of 59, based on the 0.31:18.2 ratio of pool sizes). Thus the synthesis of polyamines is "channeled", with new molecules destined for immediate use in later reactions without mixing fully with the resident, cognate pools.

Analysis of cell fractions - In the cell fractionation experiments, the criterion for the existence of a sequestered, endogenous spermidine pool was the failure of the added [^{14}C]spermidine to equilibrate with the endogenous spermidine in a given fraction; that is, the detection of a spermidine specific radioactivity significantly lower than in soluble fractions. The spermidine content of each fraction was estimated by dividing the total radioactivity by the specific radioactivity. When only trace levels of [^{14}C]spermidine were added, the nmol of spermidine determined in this way was representative of the endogenous spermidine in each fraction. This was verified with the standard isotope-dilution assay in parallel samples to which no radioactivity had been added.

During fractionation most of the endogenous and exogenous spermidine remained in the soluble fractions (Table II, Exp. 1). Only 8% of the total spermidine was found in the organellar pellet (20,000 x g pellet). However, the organellar spermidine had a specific radioactivity only 37% that of spermidine in the 20,000 x g supernatant. Subsequent fractionation of the organellar pellet by density gradient centrifugation is shown in Table II and Fig. 4. The vacuolar pellet contained 25% of the endogenous organellar spermidine, but less than 1% of the added [^{14}C]spermidine. The specific radioactivity of vacuolar spermidine was only 1.7% the specific radioactivity of spermidine at the top of the gradient. Mitochondria were located in this gradient by assay for succinic dehydrogenase activity (Fig. 4). The mitochondrial fractions contained a substantial amount of spermidine but it had equilibrated to a large extent with exogenous spermidine. The peak mitochondrial fraction had spermidine of a specific radioactivity comparable to the sample zone.

In a second experiment (Table II, Exp. 2) enough cold carrier spermidine was added with the [^{14}C]spermidine to double the endogenous spermidine content in an attempt to force redistribution. The difference between the specific radioactivity of spermidine in the organellar pellet and the 20,000 x g supernatant was muted in this case; however, the specific radioactivity of vacuolar spermidine was still less than 10% of the spermidine in the sample zone. The absolute amount of spermidine associated with the vacuoles increased only slightly (approximately 20%) as a result of the addition of an equal amount of spermidine to the original preparation (the amount of [^{14}C]spermidine was the same in each case). The 3-fold increase in the specific radioactivity of vacuolar spermidine in Exp. 2 over Exp. 1 is not readily explained. However, because of the very low specific radioactivity of spermidine in the vacuoles and the complex nature of the experiment, precise calculations are difficult. In general, the effect of added carrier spermidine was to increase the spermidine content of all fractions. In other experiments, [^{14}C]spermidine was added after cell breakage to the 600 x g supernatant and to the 20,000 x g pellet during vacuolar purification. In each case, added label failed to equilibrate with vacuolar spermidine (data not shown).

TABLE II

Distribution of exogenous (^{14}C) and endogenous (^{12}C) spermidine in organellar fractions without (Exp. 1) and with (Exp. 2) carrier spermidine.

Fraction	Exp. 1[a] specific radioactivity	Exp. 1[a] total cpm	Exp. 1[a] total nmol	Exp. 2[b] specific radioactivity	Exp. 2[b] total cpm	Exp. 2[b] total nmol
	cpm/nmol	%[c]	%[c]	cpm/nmol	%[c]	%[c]
Broken cell	2540	(100)	(100)	1170	(100)	(100)
600 X g supernat.	2090	75.1	91.2	1180	74.6	74.1
600 X g pellet	2400	17.5	19	1100	19.8	21
20,000 X g supernat.	2700	70.1	66	1240	68.5	64.7
20,000 X g pellet	980	3.1	8.0	740	3.7	5.9
100,000 X g supernat.	2700	63.7	60	1230	60.2	57.4
100,000 X g pellet	2500	4.2	4.3	1200	8.7	8.5
Gradient fractions:						
1. (sample zone)	1800	0.96	1.4	990	0.74	0.88
2.	1800	0.56	0.78	860	0.89	1.2
3.	1600	0.90	1.4	810	0.95	1.4
4.	970	0.52	1.4	810	0.55	0.80
5.		0.03		620	0.06	0.1
6.	40	0.01	0.6	150	0.03	0.2
7. (vacuoles)	30	0.02	2	92	0.090	1.1

[a] 8 μCi of [^{14}C]spermidine (98.7 mCi/mmol) added to mycelia prior to cell breakage.

[b] 8 μCi of [^{14}C]spermidine (98.7 mCi/mmol) and 8 μmol of carrier spermidine added prior to cell breakage.

[c] expressed as percent of broken cell; 100% values are, for Exp. 1: total radioactivity, 1.15 X 10^7 cpm; total spermidine, 4530 nmol; for Exp. 2: total radioactivity, 1.10 X 10^7 cpm; total spermidine, 9380 nmol. The nmol of spermidine were calculated from the specific radioactivity and the total radioactivity. The number of significant digits retained reflects the number of significant digits in the original data.

Vacuolar spermidine cannot account for all of the sequestered spermidine, as indicated by the tracer experiment. Numerous reports suggest that a likely site for spermidine is the ribosome (12, 15, 18). The spermidine content of ribosomes and its equilibration with added [^{14}C]spermidine was examined (Table III). More than 40% of the total cellular RNA was found in the 100,000 x g pellet, whereas only 20% of the spermidine was associated with this fraction. In sharp contrast to the results observed for the vacuole, complete equilibration of endogenous and exogenous spermidine occurred. The specific radioactivies of the spermidine of the supernatant and pellet of the 20,000 x g centrifugation step were also similar. More spermidine was associated with the organellar pellet in this experiment than in Exp. 1 of Table II. This reflects the lower osmotic strength of the fractionation buffer in the experiment of Table III which led to lysis and loss of many vacuoles (20). Another difference between the fractionation schemes of Table II and III was the inclusion of 2 mM Mg^{2+} for the experiment in Table III. This was necessary for efficient ribosome isolation (52). In fact, less than 3% of the total RNA was pelletable at 100,000 x g in Exp. 1 of Table II (6% in Exp. 2), compared to 42% in Table 3. The data in Table II also indicated complete equilibration of spermidine in the 100,000 x g supernatant and pellet fractions. Spermidine thus cannot be shown to be sequestered on ribosomes in vivo on the basis of these data.

TABLE III

Distribution of endogenous (^{14}C) and exogenous (^{12}C) spermidine in isolated RNA.

Cell fraction	Specific radioactivity	Total radioactivity	Spermidine	RNA
	cpm/nmol	% cpm[a]	% nmol[a]	% mg[a]
broken cells	2480	100	100	100
600 X g supernatant	2500	73.2	72.7	76
600 X g pellet	2220	13.3	14.9	12
20,000 X g supernatant	2670	60.3	56.1	61
20,000 X g pellet	2110	13.5	15.9	9.2
100,000 X g supernatant	2790	31.1	27.7	16
100,000 X g pellet	2710	24.6	22.5	42

[a] Expressed as percent of broken cells, 100% values are: total radioactivity, 1.14 X 10^7 cpm; total nmol, 4590 nmol; total RNA, 21 mg. The nmol of spermidine were calculated from the specific radioactivity and the total radioactivity. The number of significant digits retained reflects the number of significant digits in the original data.