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 [14C]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 [14C]- and resident [14C]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 [14C] 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.

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 diffuse 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***

**RESULTS**

Compartmentation of Spermidine in *Vivo*—Previous studies have shown that exponentially growing mycelia of *Neurospora crassa* rapidly take up traces of [14C]ornithine from the medium and incorporate it into polyamines (21–23). We wished to use [14C]ornithine to label the spermidine pool and to observe the rate and pattern by which the spermidine 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 [14C]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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* 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 demonstrates 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 x 0.31 = 2.3 nmol of unlabeled spermidine. This is 23/18.2, 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 rather low. Because they are drawn from intervals of label accumulation that permits some mixing of sequestered and spermine-precursor pools, spermine 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, [14C]spermidine was added to cells before cell breakage. In these experiments, only 8% of the total spermidine was sedimentable at 20,000 x g. Sorbitol-sucrose density gradient centrifugation of this organellar pellet revealed that, while spermidine was associated with both mitochondria and the vacuoles, the only vacuoles were relatively free of the added [14C]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 vacular pool, which is nonexchangeable even in vitro.
with added [14C]spermidine. This is a finding directly comparable with that of Seiler and Deckhardt (25) who found nonexchangeable 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 vitro 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 (and putrescine) to cell constituents. The increased rate reflects the emergence of unsaturated polyamines in synaptosomal membranes of Neurospora. 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, a-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.

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Spermidine Compartmentation in Neurospora

**Supplemental Material to**

*Compartmentation of Spermidine in Neurospora crassa*

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This section contains Experimental Procedures, part of the Results.

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**Figures 1 and 2 and Tables 1-11**

**In vivo labelling experiments.** One liter of the gla stage was grown exponentially to about 7 x 10^11 cells/ml. The cultures were split into two portions. (3H)putrescine (47 Ci/mmol) was added to 200 ml of one portion and (3H)sperridine (279 Ci/mmol) was added to 10 ml of the other portion. After 60 min the specific radioactivities of the polyamines were determined using the method of Halperin [10]. 

**Experimental procedures.**

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**Steady-state pool sizes and total radioactivity measurements.** Steady-state polyamine pools were determined using extracts from Neurospora crassa. Glau stage mycelia were washed in 0.6 M sorbitol in buffer containing 0.1 M Tris-HCl (pH 7.5) and 1 mM dithiothreitol. The mycelia were then homogenated in a glass homogenizer and the total polyamine content of the homogenate was determined using the specific radioactivity of 

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**Table 1**

| Cation pool sizes of polyamines | Number of determinations (mol/cell, mg) |
|--------------------------------|---------------------------------------|
| Glutamate | 4.9 |
| Ornithine | 1.1 ± 0.3 |
| Putrescine | 1.0 ± 0.3 |
| Spermidine | 0.3 ± 0.0 |

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**Figure 1.** Radioactivity associated with putrescine (O), ornithine (■) and the total polyamine fraction (△) in the wild type (left) and the mutant (right). Specific radioactivities of putrescine (O) and spermidine (△) were determined in the course of the same experiment.

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**Table 2**

| Cation pool sizes of polyamines | Number of determinations (mol/cell, mg) |
|--------------------------------|---------------------------------------|
| Glutamate | 4.9 |
| Ornithine | 1.1 ± 0.3 |
| Putrescine | 1.0 ± 0.3 |
| Spermidine | 0.3 ± 0.0 |

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**Figure 2.** Radioactivity associated with putrescine (O), ornithine (■) and the total polyamine fraction (△) in the wild type (left) and the mutant (right). Specific radioactivities of putrescine (O) and spermidine (△) were determined in the course of the same experiment.
Spermidine Compartmentation in Neurospora

**Table I**

| Spermidine Fraction | Specific activity | Radioactivity | Total radioactivity | Total spermidine |
|---------------------|------------------|---------------|---------------------|-----------------|
| Cytosol             | 0.5 ng/mol        | 0.5 mCi/mL    | 0.5 mCi/mL          | 0.5 ng/mol      |
| Vacuolar            | 0.5 ng/mol        | 0.5 mCi/mL    | 0.5 mCi/mL          | 0.5 ng/mol      |

**Table II**

| Spermidine Fraction | Specific activity | Radioactivity | Total radioactivity | Total spermidine |
|---------------------|------------------|---------------|---------------------|-----------------|
| Cytosol             | 0.5 ng/mol        | 0.5 mCi/mL    | 0.5 mCi/mL          | 0.5 ng/mol      |
| Vacuolar            | 0.5 ng/mol        | 0.5 mCi/mL    | 0.5 mCi/mL          | 0.5 ng/mol      |

**Figure 2**

Total radioactivity associated with the pools of putrescine (P), spermidine (S), and spermine (M) in the experiment of Fig. 1.

**Figure 3**

This figure shows the analysis of cell fractions in the experiment described in Table II. The asterisk indicates a significant difference between the control and experimental conditions.

**Figure 4**

This figure illustrates the distribution of spermidine in the different cell fractions as indicated by the specific radioactivity and total radioactivity data. The asterisk indicates a significant difference between the control and experimental conditions.

**Figure 5**

This figure depicts the changes in the specific radioactivity of spermidine in the different cell fractions as indicated by the total radioactivity data. The asterisk indicates a significant difference between the control and experimental conditions.

**Figure 6**

This figure shows the changes in the total radioactivity of spermidine in the different cell fractions as indicated by the specific radioactivity data. The asterisk indicates a significant difference between the control and experimental conditions.

**Figure 7**

This figure illustrates the probability of spermidine becoming a putative subpool of polyamines during the first 20 minutes of incubation. The asterisk indicates a significant difference between the control and experimental conditions.