METABOLIC SEQUESTRATION OF PUTRESCINE IN NEUROSPORA CRASSA

Thomas J. Paulus and Rowland H. Davis

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

SUMMARY: The metabolic fate of putrescine labelled in vivo was investigated after administration of a trace ($10^{-7}$ M) of L-[$^{14}$C]ornithine to exponentially growing mycelia of Neurospora crassa, followed by a large chase (2 mM) of L-[$^{12}$C]ornithine. The specific radioactivities of putrescine and spermidine were determined during the chase period by reaction with [$^{3}$H]dansyl chloride of known specific radioactivity and isolation of the dansyl-derivatives by thin-layer chromatography. Radioactivity remained in the putrescine pool for over 2 h during the chase period. This suggests that putrescine is largely sequestered (80% or more) in vivo. The metabolic sequestration of polyamines may be a significant factor in the regulation of polyamine synthesis.

The polyamines (putrescine, spermidine, and spermine) and a key enzyme of their synthesis, ornithine decarboxylase (E.C. 4.1.1.17), have been widely studied in connection with growth, neoplasia, and differentiation. In most organisms, it is not clear how changes in polyamine pools exert control over ornithine decarboxylase. Our interest lies in the physical state of the polyamines within the cell--whether they are free or bound. If a large fraction of polyamines is "bound", or metabolically sequestered, then the state of polyamines, rather than the total amount of polyamines per cell, may be the important factor in control. This idea is consistent with the observed tendency of the very basic polyamines to bind to ribosomes, nucleic acids and membranes in vitro (1-4). We report here direct evidence for metabolic sequestration of putrescine, using tracer methodology.

EXPERIMENTAL PROCEDURES

Growth and Extraction. Wild-type Neurospora crassa, 74A, was grown as described previously (5,6). Experiments were initiated by addition of L-[U-$^{14}$C]-ornithine (0.1 µM, 266 mCi/mmol) to cultures which had achieved a dry weight of approximately 0.4 mg per ml. After 10 min, non-radioactive ornithine was added to a final concentration of 2 mM. Culture samples were taken at the

end of the labelling period and at intervals during the chase period. Samples for pool analysis were collected by filtration of 25 ml of culture and washing with distilled water (6). Mycelial pads were extracted three times in 1.0 ml of 0.4 M perchloric acid at 4°C. The combined supernatants were analyzed for polyamines.

Specific radioactivities and pools. Polyamines were partially purified from 2 ml of the perchloric acid extract by ion-exchange chromatography (7). 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 perchloric acid. A 0.1 ml aliquot of this solution was added to a tube containing 30 mg $Na_2CO_3$. A 0.1 ml aliquot of [$^3$H]dansyl chloride (1.5 mg/ml acetone, 437 cpm per nmol) was added; the reaction contents were mixed, sealed and incubated overnight in the dark at room temperature. The reaction mixture was extracted with 0.5 ml benzene. The benzene layer was removed and evaporated in a fume hood. The sample was redissolved in 50 µl of benzene and 25 µl were spotted on a silica gel thin-layer chromatogram. The chromatogram was developed in the first dimension with ethyl acetate:cyclohexane (2:3, v/v), air dried, and then developed in the second dimension with chloroform:n-butanol:dioxane (48:1:1). The chromatogram was air-dried after development and sprayed with triethanolamine: isopropanol (1:4). The fluorescent spots, located with a low energy UV lamp, were scraped from the chromatogram and eluted from the silica gel with acetone. The acetone extract was evaporated in a scintillation vial and 5 ml of toluene containing 5 g 2,5-diphenyloxazole per liter was added. All samples were counted in a Beckman LS-230 scintillation counter to 0.2% error or for 100 min. The specific radioactivity of each polyamine was calculated by dividing the $^{14}$C cpm by the number of nmol 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, and four per spermine was assumed for the calculations. The $^3$H cpm were corrected for the amount of $^{14}$C cpm in the $^3$H channel. The specific radioactivity of ornithine in the original perchloric acid extract was determined as previously described (8).

Steady-state putrescine, spermidine, and spermine pools were determined by a double-isotope dilution technique (5) on a control culture grown in minimal medium. Polygram Sil G thin-layer chromatograms (20 x 20 cm, 0.25mm) were purchased from Brinkman Instruments, Inc. Radioactive isotopes were purchased from New England Nuclear.

Calculations. The specific radioactivity of newly synthesized polyamine (called "new putrescine" or "new spermidine") was calculated from changes in the amount of polyamine as the culture grew and from the specific radioactivity of the total pool of the given polyamine. First, the amount of polyamines made in a given interval, $\Delta X$, 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 163 min, 0.0043 min$^{-1}$. Second, the total radioactivity in the entire putrescine pool of a given sample was calculated by multiplying the known concentration of putrescine (nmol per mg dry wt) by the specific radioactivity of the extracted polyamine ($^{14}$C cpm per nmol). To determine the specific radioactivity of newly synthesized polyamine, the change in radioactivity ($\Delta$ cpm) of the polyamine was divided by the change in nanomoles of the polyamine ($\Delta X$). Similar calculations have been described in detail in previous publications from this laboratory in connection with the vacuolar compartmentation of basic amino acids in N. crassa (8,9).

RESULTS AND DISCUSSION

In N.crassa, ornithine decarboxylase is a cytosolic enzyme (10). Putrescine, the product of the ornithine decarboxylase reaction, is converted to

TABLE 1.  Pool sizes and radioactivity of ornithine and polyamines at the
end of the pulse period.

| COMPOUND | POOL SIZE nmol/mg dry wt | SPECIFIC RADIOACTIVITY[a] cpm/nmol | RADIOACTIVITY cpm/mg dry wt |
|---|---|---|---|
| ORNITHINE | 36.2 | 1,410 | 51,042 |
| PUTRESCINE | 1.2 | 606 | 727 |
| SPERMIDINE | 19.2 | 264 | 5,069 |
| SPERMINE | 0.5 | 26 | 13 |

[a]To compare ornithine with the polyamines, the specific radioactivity and
the total radioactivity of the ornithine pool must be multiplied by 0.8.
This compensates for the loss of $^{14}CO_2$ from L-[U-$^{14}C$]ornithine during putre-
scine formation.

spermidine by addition of an aminopropyl group; spermidine is transformed to
spermine by addition of a second aminopropyl group.  We wished to know
whether labelled putrescine, formed from [$^{14}C$]ornithine, turned over as a
single pool, or whether a fraction of the labelled putrescine would behave
as a long-lived, sequestered pool.  A small amount of highly labelled [$^{14}C$]-
ornithine was given to a logarithmically growing mycelium, followed by a
chase of [$^{12}C$]ornithine after 10 min.  Over 90% of the [$^{14}C$]ornithine enters
cells within 5 min (8,11,12).  During this time, the cytosolic ornithine is
highly labelled because little mixing with vacuolar ornithine takes place.
In the pulse period, therefore, a wave of radioactivity labels the products
of ornithine decarboxylase and other enzymes.  The [$^{14}C$]ornithine not used
in these reactions is sequestered in the vacuole (8,11,12). A chase of [$^{12}C$]-
ornithine minimizes continued use of radioactivity from the vacuolar pool over
the long term.  At the end of the 10 min pulse period, putrescine, spermidine,
and spermine had acquired label in our experiment as shown in Table 1.

The extractable putrescine pool is about one-sixteenth the size of the
spermidine plus spermine pools, and little increase is seen upon addition of
ornithine in the chase period (5).  In order to sustain the level of the sper-
midine and spermine pools, the putrescine pool must be regenerated 17 times
every doubling time of 163 min if all molecules are used randomly.  Thus,
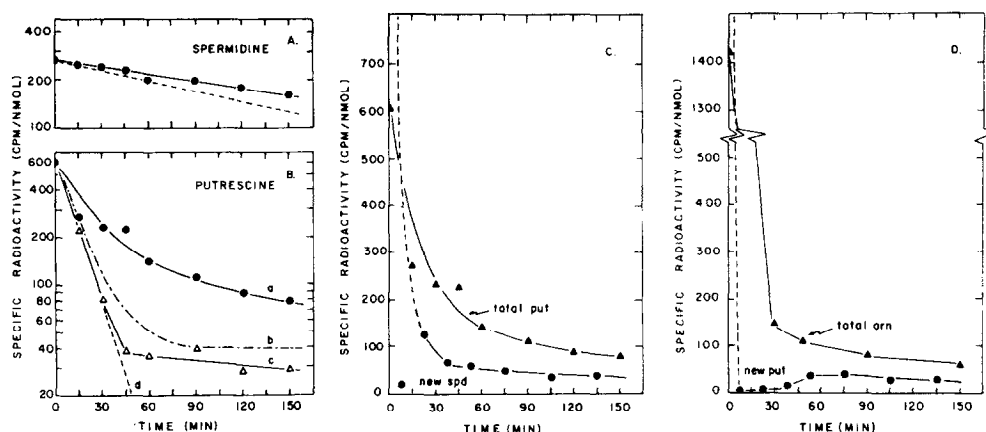
FIGURE 1. Panel A: Spermidine specific radioactivity observed during chase period (●); the dashed line represents the theoretical result based on a doubling time of 163 min and the premise that no further radioactivity enters spermidine. Panel B: Putrescine specific radioactivity observed during chase period (curve a); the theoretical prediction based on a turnover rate of 10 min with no further entry of radioactivity from ornithine (curve d); the theoretical results based on a constant influx of "new putrescine" at 40 cpm/nmol and a turnover rate of 10 min (curve b); the calculated prediction based on a turnover rate of 10 min and the calculated specific radioactivity of "new putrescine" from Fig. 2 (bottom) (curve c). Panel C: The specific radioactivity of the total putrescine pool and "new spermidine". Panel D: The specific radioactivity of the total ornithine pool and "new putrescine". To compare total ornithine with polyamines, the specific radioactivity of ornithine should be multiplied by 0.8. Dashed lines extrapolate to the average specific radioactivities of "new polyamines" during the pulse period. These values were 6560 cpm per nmol for new putrescine and 6090 cpm per nmol for "new spermidine". orn, ornithine; put, putrescine; spd, spermidine.

the nominal turnover time (half-life) of the putrescine pool is approximately 10 min at steady-state. Fig. 1B compares the behavior of the extractable putrescine pool with this expectation. After 120 min (12 half-lives), the putrescine pool retains significant radioactivity, where almost none [(0.5)^{12}] would be expected on the simple exponential loss model. This comparison is compromised by a small, continued entry of labelled ornithine into the putrescine pool later in the experiment (Fig. 1D). "Wash-out" curves have been constructed which take this influx into account, using either the data points in Fig. 1D or the maximal specific radioactivity (40 cpm/nmol) attained by "new putrescine" during the chase period. Even with the most conservative assumption of a steady influx of radioactivity during the chase period (Fig. 1B, curve b), the observed specific radioactivity of putrescine is over 2-fold the theoretical value at the end of the experiment. Thus, the putrescine pool does not appear to be freely metabolizable, but instead appears

to have a significantly sequestered fraction.  In the same experiment, the specific radioactivity of the spermidine pool decreases at approximately the growth rate (Fig. 1A).  This is consistent with a very small influx of radioactivity from the putrescine pool and a very low rate of spermidine use for spermine synthesis.  No degradation of polyamines is detectable under the conditions used (5).

A second method of demonstrating sequestration of putrescine is to compare the specific radioactivities of the extractable putrescine pool with the molecules of "new spermidine" being made at the same time (see Experimental Procedures).  The "new spermidine" molecules are always less radioactive than the total, extractable putrescine pool (Fig. 1C).  Thus, the latter has a sequestered radioactive fraction, labelled in the pulse period, which does not participate thereafter as an intermediate in spermidine synthesis.  A similar comparison of extractable ornithine and "new putrescine" shows the expected (vacuolar) compartmentation of ornithine (Fig. 1D).  Significantly, the "new putrescine" and "new spermidine" come to have similar specific radioactivities, as though "new putrescine" might be destined for immediate use in the formation of spermidine (of Fig. 1C, 1D, 60-150 min).  The same conclusion emerges from a comparison of the specific radioactivity of "new putrescine" (6560 cpm per nmol) with "new spermidine" (6090 cpm per nmol) in the pulse period (cf. Fig. 1, legend).  These figures suggest that pre-existing putrescine dilutes "new putrescine" only 10% prior to conversion to spermidine instead of the 50% dilution expected on the basis of one 10-min replacement of the pool.

We have not eliminated the vacuole as a possible location of "bound" putrescine.  Previous tests indicate that at least 60% of the cellular spermidine is non-vacuolar (13), and this may be true of putrescine as well.  However, the detection of "bound" putrescine in vivo with tracers demonstrates sequestration in the ionic environment of the cell.  If sequestration of putrescine reflects ionic binding to cell constituents, sequestration of spermidine, a more basic molecule is likely to be even more extreme.  Therefore,

the negative effector(s) of the ornithine decarboxylase reaction, by whatever

mechanism they work, may be minor diffusible fractions of the putrescine and/

or spermidine pool (5).  It is very likely that the mechanism(s) by which

polyamines control the ornithine decarboxylase reaction can be triggered

indirectly by changes in ionic or osmotic conditions which alter the ratio

of bound to free polyamines (14-16).

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