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# Metabolism of $N^1$-Acetylspermidine and $N^8$-Acetylspermidine in Rats (41930)

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*Abstract.* Metabolism of $N^1$-acetylspermidine and $N^8$-acetylspermidine in rat liver and kidney was studied *in vivo*. $N^1$-Acetylspermidine was metabolized primarily to putrescine while $N^8$-acetylspermidine underwent deacetylation to yield spermidine. The rate of metabolism of these two compounds was much greater than that of spermidine. The rate of metabolism of $N^8$-acetylspermidine exceeded that of $N^1$-acetylspermidine in both liver and kidney. These rapid rates of metabolism could at least in part account for the low levels of these two compounds found in tissues. The differences in routes of metabolism of $N^1$-acetylspermidine and $N^8$-acetylspermidine may indicate differences in cellular functions of the two compounds. 

Spermidine has been shown to undergo acetylation in mammalian tissues giving rise to two different acetylated products $N^1$-acetylspermidine (acetylated on the propylamine nitrogen) and $N^8$-acetylspermidine (1, 2). $N^8$-Acetylspermidine formation appears to be catalyzed by a nuclear enzyme (3) while $N^1$-acetylspermidine can be produced by an enzyme found in the cytosol (4). Although these enzymatic reactions have been demonstrated *in vitro*, tissue levels of both of these acetylated spermidine derivatives are extremely low (5, 6). In fact, only in human, mouse, and rat urine have appreciable levels of both of these compounds been found (2, 7, 8).

This lack of acetylated spermidine in tissues could be explained by low levels of acetylating activity in tissues, by the rapid excretion of the acetylated products, or by the rapid metabolism of these compounds. While the first two alternatives may also be true, *in vitro* evidence indicates the occurrence of metabolizing activities for both $N^1$-acetylspermidine and $N^8$-acetylspermidine. Interestingly, these two isomers appear to be metabolized *in vitro* by two distinctly different enzyme activities. Blankenship (9) demonstrated the deacetylation of $N^8$-acetylspermidine to yield spermidine catalyzed by an enzyme in rat liver cytosol. $N^1$-Acetylspermidine does not serve as a substrate for this enzyme activity. The enzyme responsible for the metabolism of $N^1$-acetylspermidine appears to be polyamine oxidase, an enzyme found in rat liver cytosol by Höltta (10). Polyamine oxidase catalyzes the conversion of $N^1$-acetylspermidine to putrescine but has virtually no activity with $N^8$-acetylspermidine as substrate (11). Pegg and co-workers (12) have demonstrated the formation of $N^1$-acetylspermidine *in vivo* and have reported that $N^1$-acetylspermidine synthetase can be induced by a variety of treatments. Polyamine oxidase activity does not change with these treatments, and the $N^1$-acetyltransferase appears to be the rate-limiting enzyme in $N^1$-acetylspermidine synthesis and metabolism. There have been no *in vivo* studies of $N^8$-acetylspermidine metabolism and no basis for comparison of $N^1$-acetylspermidine and $N^8$-acetylspermidine metabolism in intact animals.

The purpose of the present study is to establish *in vivo* the primary routes of metabolism of $N^1$-acetylspermidine and $N^8$-acetylspermidine and to compare the relative rates of metabolism of these two compounds.

**Materials and Methods.** *Animals and chemicals.* Male Wistar rats, 160–180 g in weight, were obtained from Simonsen Laboratories, Inc. Spermidine–3HCl ([1,4-$^{14}$C]-tetramethylene, 85.2 mCi/mmole) and spermidine–3HCl (terminal [$^3$H(N)]methylenes, 30.1 Ci/mmole) were obtained from New England Nuclear.

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*Synthesis of radiolabeled compounds.* Acetylspermidine derivatives were synthesized and purified following previously published procedures (1, 9). For the $^{3}H$-labeled compounds and for $^{14}C$-labeled $N^1$-acetylspermidine, this involved reaction of radiolabeled spermidine (free base) with equimolar quantities of acetic anhydride. The monoacetylspermidine derivatives were isolated as described previously (1). For $^{14}C$-labeled $N^8$-acetylspermidine, synthesis was achieved through a procedure described by Ganem ((13) and personal communication). Spermidine–3HCl ($^{14}C$ labeled) was combined with a 35-fold excess of unlabeled spermidine to yield a specific activity of 2.4 mCi/mmole. The spermidine (free base) in 6.5 ml water was reacted with 5 $\mu$l formaldehyde (37%) in an ice bath to yield a cyclic product (1-(4-aminobutyl)hexahydropyrimidine). This product was extracted with 10 ml methylene chloride (extraction was performed ten times with 1-ml aliquots until no additional radioactivity was extracted). The methylene chloride extract was dried with $Na_2SO_4$ and evaporated under nitrogen to a volume of 1 ml. Acetylation of this compound was achieved by reaction overnight with 7.4 mg *N*, *N'*, *N''*, *N'''*-tetraacetylglycoluril (Aldrich Chemical Co.). The remainder of the methylene chloride was evaporated and the residue was dissolved in 1 ml of 2 *N* HCl in methanol. The solution was heated at reflux for 3 hr to open the cyclic structure. The $N^8$-acetylspermidine was purified by column chromatography using Amberlite CG-50, a procedure used previously in this laboratory (1). A 15% yield of $N^8$-acetylspermidine was obtained in this synthesis. Confirmation that greater than 95% of the $^{14}C$ label was contained in the $N^8$-acetylspermidine product was achieved using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) in comparison with a known standard as described previously (1, 9).

*Administration of radiolabeled compounds to animals.* Rats were injected intraperitoneally with radiolabeled $N^1$-acetylspermidine, $N^8$-acetylspermidine, or spermidine in a volume of less than 1.0 ml of 0.15 *M* NaCl. Each animal that received a $^{3}H$-labeled compound was given 4.5 $\mu$Ci of radioisotope contained in approximately 0.15 nmole of $N^1$-acetylspermidine–3HCl or $N^8$-acetylspermidine–3HCl. Immediately after injection animals were placed in individual metabolic cages. Urine was collected in flasks containing 1 ml of 1 *N* HCl. At 30 min, 2 hr, or 24 hr after injection, animals were sacrificed by decapitation. Tissue samples were quickly removed and immediately placed in 4 vol of ice-cold 0.25 *N* perchloric acid containing 2.5 $\mu M$ 1,7-diaminoheptane (internal standard). Urine was also added to 4 vol of the ice-cold perchloric acid solution. Urine from only the 24-hr-treated animals was saved for analysis.

Each animal that received a $^{14}C$-labeled compound was given 2 $\mu$Ci of radioisotope contained in approximately 25 nmole of spermidine or $N^1$-acetylspermidine or 810 nmole of $N^8$-acetylspermidine. At 2 hr after injection, animals were sacrificed by decapitation, and liver samples were removed and placed in 4 vol of ice-cold 0.25 *N* perchloric acid containing internal standard.

*Preparation of tissue samples for analysis.* Tissue and urine samples were homogenized with a Polytron (Brinkman) homogenizer in 4 vol of 0.25 *N* perchloric acid containing the internal standard (2.5 $\mu M$ 1,7-diaminoheptane). The homogenates were centrifuged at 10,000*g* and the supernatants were filtered through 0.22-$\mu$m cellulose nitrate filters. The filtrate was stored at 4°C for later analysis.

*Analysis of tissue samples by high-performance liquid chromatography.* The filtered tissue extracts were analyzed for polyamine and acetylpolyamine content by the HPLC technique described by Seiler and Knodgen (5). This technique utilizes reverse-phase column chromatography and *n*-octane sulfonate for ion pairing. The polyamines were quantitated by fluorescence measurements following postcolumn derivatization with *o*-phthalaldehyde. The chromatography system consists of a Spectra-Physics (SP8700) solvent delivery system, a guard column (5 cm × 4.6 mm), a Waters C-18 $\mu$Bondapak column (30 cm × 3.9 mm), an Eldex (E 1205) postcolumn derivatization pump, and a Perkin–Elmer (204A) fluorescence spectrophotometer equipped with a flow cell. The results were analyzed and recorded using a Hewlett–Packard 3390A Reporting Integrator. Quantitation was based on comparison with stan-

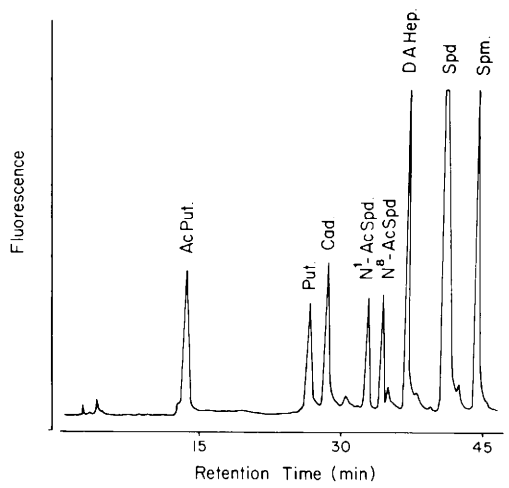


FIG. 1. Separation of standard polyamines and acetyl polyamines by HPLC. A mixture of standard polyamines was separated by HPLC as described in the text. The tracing shows the order of elution as follows: acetylputrescine, putrescine, cadaverine, $N^1$-acetylspermidine, $N^8$-acetylspermidine, 1,7-diaminoheptane (DAH), spermidine, and spermine.

dard polyamines and acetylpolyamines and individual analyses were compared using the internal standard.

Analysis of radioactive metabolites was achieved by hand collection of the fractions of the HPLC effluent containing each of the primary polyamines and acetylated polyamines. The total radioactivity in each fraction was determined by liquid scintillation spectrometry.

**Results.** A tracing illustrating the separation achieved by HPLC of a standard mixture of polyamines and acetylated polyamines is shown in Fig. 1. Except for 1,7-diaminoheptane, each of these peaks represents a naturally occurring polyamine. The polyamine and acetylated polyamine peaks from tissue samples were identified by retention times and the identity was confirmed by cochromatography with known standard compounds. Both HPLC and TLC were used to confirm identity of the compounds. The 1,7-diaminoheptane was added to tissue samples during homogenization and used as an internal standard for quantitative analysis. This assay procedure permits determination of acetylated polyamine levels as low as 0.5 nmole per gram of tissue.

The concentrations of $N^1$-acetylspermidine and $N^8$-acetylspermidine normally occurring in tissues were much lower than that of spermidine (Table I). In fact, $N^8$-acetylspermidine was not detected in liver and kidney and was found in spleen at a concentration of only 1.0 nmole per gram. It is of interest that spleen contained the highest concentration of spermidine of the three tissues studied. One possible explanation for the low tissue levels of acetylated polyamines is that metabolism may occur during the homogenization and analysis procedures. However, this does not appear to be the case since in several experiments known quantities of radiolabeled $N^1$-acetylspermidine or $N^8$-acetylspermidine were added to liver samples immediately before homogenization and greater than 90% of the compounds was recovered unchanged in the HPLC effluent. The absence of detectable levels of $N^8$-acetylspermidine in liver and kidney does not necessarily indicate a total lack of this compound in these organs but is more likely due to a limit in the sensitivity of the analytical procedure. The polyamine concentrations in urine are expressed in nmole excreted per 24 hr (Table I). The results indicate a significant amount of both acetylated compounds with $N^1$-ace-

TABLE I. NORMAL POLYAMINE LEVELS IN RAT TISSUES AND URINE

| Tissue | $N^1$-Acetylspermidine | $N^8$-Acetylspermidine | Spermidine |
|---|---|---|---|
| Liver | 1.78 ± 0.54[a] | n.d.[b] | 898 ± 91 |
| Kidney | 1.44 ± 0.80 | n.d. | 553 ± 91 |
| Spleen | 1.79 ± 0.33 | 1.00 ± 0.16 | 1550 ± 80 |
| Urine | 181 ± 40 | 75 ± 27 | 514 ± 126 |

*Note.* Concentrations are in nmole/g of tissue except for urine which are in nmole excreted/24 hr.
[a] Means ± standard deviation, $N = 8$ for each value.
[b] n.d. represents not detectable.

tylspermidine excreted in greater quantities than $N^8$-acetylspermidine. In comparison with the ratio in tissues, there is a much greater ratio in urine of the acetylated compounds to spermidine. These results are in agreement with values reported by Abdel-Mouem *et al.* (7) and Seiler *et al.* (14).

The results of the metabolism studies with $^{14}$C-labeled $N^1$-acetylspermidine, $N^8$-acetylspermidine, and spermidine are shown in Fig. 2. Animals were sacrificed at 2 hr after injection and liver and kidney samples were analyzed. Only the liver data are shown in Table I. For each gram of liver analyzed, the total radioactivity collected in the HPLC fractions was 189,000 dpm for spermidine, 51,000 dpm $N^1$-acetylspermidine, and 5500 for $N^8$-acetylspermidine. Since each animal received an injection of 2 $\mu$Ci of the $^{14}$C-labeled compound, the differences in total radioactivity probably reflect both differences in rate of uptake into liver and differences in specific activity of the labeled compounds injected. For each of the three compounds studied, greater than 77% of the total radioactivity in the tissue sample was collected in the five polyamine fractions under study. Thus it would appear that these polyamines do represent the major metabolic products. Variability of the data among the animals was relatively low with percentages of each specific fraction from individual animals lying within 5% of the average values reported in Fig. 2.

The results indicate that at 2 hr after administration no appreciable amounts of $N^1$-acetylspermidine or $N^8$-acetylspermidine remain in liver unmetabolized. Spermidine on the other hand has undergone very little metabolism. Most of the radioactivity in the animals which had received $N^1$-acetylspermidine is found in the putrescine fraction with a smaller but significant amount in spermidine. The pattern is quite different in $N^8$-acetylspermidine-treated animals with almost 80% of the total radioactivity in spermidine and a small amount in the spermine fraction. Thus, the greatest differences observed between the results of $N^1$- and $N^8$-acetylspermidine were the large proportion of radioactivity in the putrescine fraction for $N^1$-acetylspermidine and the lack of radioactivity in the putrescine fraction for $N^8$-acetylspermidine.

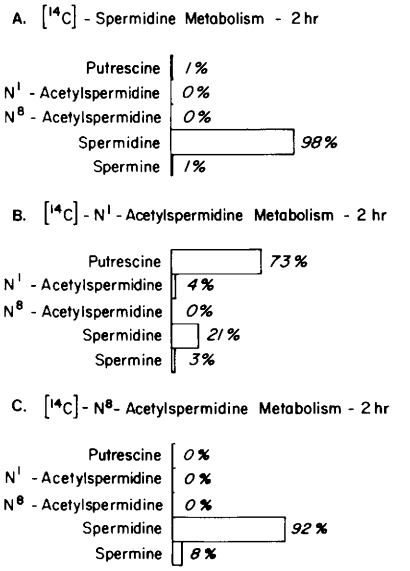


FIG. 2. Products of metabolism of $^{14}$C-labeled spermidine, $N^1$-acetylspermidine, and $N^8$-acetylspermidine in rat liver at 2 hr after administration. Rats received by ip injection spermidine (Experiment A), $N^1$-acetylspermidine (Experiment B), or $N^8$-acetylspermidine [1,4-$^{14}$C]tetramethylene (Experiment C). At 2 hr after injection, rats were sacrificed, liver samples were taken, and extracted polyamines were analyzed by HPLC to determine distribution of radioactivity among the five fractions shown. Each bar graph represents an average of results from three animals and the range of values from each individual animal was within less than 5% of the average values shown.

The initial studies (Fig. 2) examined the metabolic profile of the polyamines at one time point, 2 hr after injection. A second series of studies were undertaken to look more closely at the time course for metabolism of the acetylated polyamines. Since initial studies indicate a rapid disappearance of the acetylated polyamines, a shorter time period (30 min) was of interest to examine the levels of unmetabolized compounds. In addition, a much longer time period (24 hr) was chosen to help identify the ultimate product of acetylated spermidine metabolism. The second series of experiments also differed from the initial studies in the type of isotopic labeling used since a much greater specific activity could be obtained with $^3$H-labeled compared to the $^{14}$C-labeled compounds. With these $^3$H-labeled compounds, we were able to administer more radioactivity while using less of each compound.

The percentages of total radioactivity in the various polyamine fractions at 30 min, 2 hr, and 24 hr after injection of $^3$H-labeled acetylspermidine are shown in Fig. 3. Percentages obtained from tissues from individual animals varied no more than 5% from the average reported in Fig. 3. The average recovery for radioactivity for all liver and kidney samples was 104%. Total radioactivity in tissues of animals receiving $N^1$-[$^3$H]acetylspermidine declined from a peak at 30 min to a minimum at 24 hr. In these animals, the values for total radioactivity (dpm/g of tissue) in liver were 142,100 at 30 min, 46,600 at 2 hr, and 33,300 at 24 hr; in kidney these values were 235,200 at 30 min, 24,600 at 2 hr, and 11,000 at 24 hr. Total radioactivity in the tissues of animals receiving $N^8$-[$^3$H]acetylspermidine was highest at 2 hr after injection with lower levels at 30 min and 24 hr. In these animals, the values for total radioactivity (dpm/g of tissue) in liver

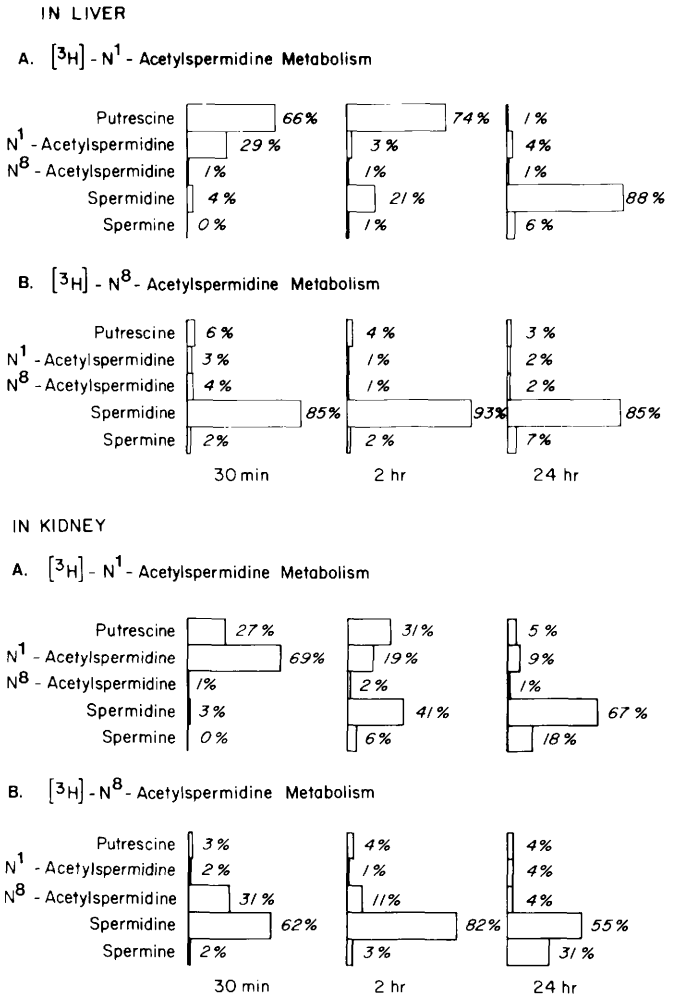


FIG. 3. Products of metabolism of $^3$H-labeled $N^1$-acetylspermidine and $N^8$-acetylspermidine in rat liver and kidney at 30 min, 2 hr, and 24 hr after administration. Rats received by ip injection $N^1$-acetylspermidine or $N^8$-acetylspermidine (terminal [$^3$H]methylenes). At 30 min, 2 hr, and 24 hr after injection, rats were sacrificed, liver and kidney samples were taken, and samples were analyzed by HPLC to determine distribution of radioactivity among the 5 fractions shown. Each bar graph represents the average of results of two or three animals except for $N^8$-acetylspermidine at 2 hr which represents results from only one animal. The percentage values obtained with each individual animal were within 5% of the average values shown.

were 87,700 at 30 min, 127,300 at 2 hr, and 71,600 at 24 hr; in kidney these values were 73,900 at 30 min, 77,300 at 2 hr, and 36,900 at 24 hr. The results of $N^1$-acetylspermidine metabolism in both liver and kidney indicate an increase in radioactivity in the putrescine fraction at 30 min and 2 hr after injection with an increase in the spermidine fraction at 2 and 24 hr. The radioactivity in the $N^1$-acetylspermidine fraction declined in both tissues but the rate of decline was much greater in liver than in kidney. $N^8$-Acetylspermidine metabolism showed a different pattern with the largest proportion of radioactivity appearing in the spermidine fraction for each of the time periods studied. There was no significant increase in radioactivity in the putrescine fraction at any time for either tissue. The decline in radioactivity in the $N^8$-acetylspermidine fraction appeared to be greater for liver than kidney in that only 4% of total radioactivity remained in this fraction at 30 min.

Urine samples were collected only for those animals receiving radiolabeled $N^1$- or $N^8$-acetylspermidine 24 hr before sacrifice. For $N^1$-acetylspermidine-treated rats, an average of 90% of the radioactivity excreted in urine was in the form $N^1$-acetylspermidine, and this represented approximately 40% of total amount of radioactivity injected. For $N^8$-acetylspermidine-treated rats an average of 70% of the radioactivity excreted in urine was in the form of $N^8$-acetylspermidine, and this represented approximately 14% of total radioactivity injected. While these data represent excretion over a 24-hr period, it is likely that most of the radioactivity was excreted soon after injection or before the compounds were taken up into tissues and metabolized. These data are preliminary but suggest there may be significant differences in urinary excretion of $N^1$-acetylspermidine and $N^8$-acetylspermidine.

**Discussion.** This study was undertaken to determine routes of $N^1$- and $N^8$-acetylspermidine metabolism in intact animals. These *in vivo* results indicate two distinctly different routes of metabolism for the two compounds (Fig. 4) which is consistent with results of *in vitro* studies from several laboratories (11, 12). The results shown in both Figs. 2 and 3 indicate that $N^1$-acetylspermidine is rapidly converted to putrescine followed by the slower conversion of putrescine to spermidine. $N^8$-Acetylspermidine on the other hand is rapidly deacetylated to yield spermidine with no apparent conversion to putrescine. The $N^1$-acetylspermidine metabolism results are consistent with oxidation to putrescine and *N*-acetyl-3-aminopropionaldehyde by polyamine oxidase as described by Pegg *et al.* (12) and by Bolkenius and Seiler (11). The $N^8$-acetylspermidine metabolism results are consistent with deacetylation to yield spermidine, a reaction first observed *in vitro* in our laboratory (9).

There are several questions that can be raised regarding this type of study. First, this study focuses only on the five polyamine fractions collected from HPLC, we cannot rule out the possibility of other routes of metabolism for acetylated spermidine derivatives. Second, the metabolism of exogenously administered compounds in this study may be different from that of endogenously produced acetylpolyamines. Neither of these concerns can be fully answered. However, since the levels of radioactivity ultimately appearing in the putrescine and spermidine fractions represent the major fraction (greater than 77%) of the radioactivity in that tissue, these pathways do represent the major routes of metabolism of the exogenously administered compounds. Given the results of *in vitro* studies (11, 12) and evidence for occurrence of $N^1$-acetylspermidine metabolism *in vivo* (12), it can be assumed from these studies that the polyamine oxidase and the deacetylase reactions are major routes of metabolism for endogenous compounds as well. Comparison of the rates of metabolism suggests a much slower metabolism of spermidine than either of the two acetylated derivatives. Even though data are available for comparison at only a single time point, the results in Fig. 2 indicate that little metabolism occurs with spermidine over a 2-hr period while both $N^1$- and $N^8$-acetylspermidine have undergone almost complete conversion to putrescine and spermidine, respectively. While there are differences in specific activity of the radiolabeled spermidine and acetylated spermidine derivatives, the differences are not great enough to account for the differences in the rates of metabolism observed. Comparison of the rates of metabolism of $N^1$-acetylspermidine and $N^8$-acetyl-

spermidine (Fig. 3) suggests more rapid metabolism of the $N^8$-derivative than the $N^1$-derivative. In fact, in liver the conversion of $N^8$-acetylspermidine to spermidine was virtually complete within 30 min after administration. This occurs in spite of the fact that there is a greater accumulation of radioactivity at 2 hr. In kidney this metabolism of $N^8$-acetylspermidine is slower than in liver but still exceeds that of $N^1$-acetylspermidine.

The rapid rate of metabolism of both $N^1$-acetylspermidine and $N^8$-acetylspermidine may at least in part account for the low levels of these compounds found in tissues. For example, as shown in Table I, levels of spermidine compared to levels of acetylated spermidine in tissues are quite high and this corresponds to a slower rate of metabolism of spermidine compared to acetylated spermidine. The rate of synthesis of these compounds probably plays an equal if not more important role in regulating tissue levels of these compounds. From our radioactivity studies, it can be seen that negligible amounts of radioactive spermidine are converted to the acetylated spermidine derivatives while large proportions of both of the radioactive acetylated derivatives end up in the putrescine or spermidine fractions. These results are consistent with the finding of Pegg *et al.* (12) that $N^1$-acetylspermidine levels are regulated by the activity of the acetyltransferase and not of polyamine oxidase.

As shown in Fig. 4, $N^1$-acetylspermidine and $N^8$-acetylspermidine are formed by different enzymatic reactions and subsequently undergo different routes of metabolism. These differences point to the possibility that the two compounds may play different roles in cellular processes. Several groups of workers have shown that $N^1$-acetylspermidine serves as a metabolic intermediate in the conversion of spermidine to putrescine (11, 12). The metabolism of $N^8$-acetylspermidine via a deacetylation reaction would appear to exclude the possibility that it serves as an intermediate in conversion of spermidine to some secondary product. These acetylation and deacetylation reactions could serve as a means of regulating spermidine or acetylated spermidine levels in tissues. In fact, the localization of the $N^8$-acetyltransferase activity in chromatin suggests that this process may be involved in regulating activity of these compounds in the cell nucleus. An analogous regulatory system exists in the nucleus for the histones (15). Acetylation and deacetylation of amine groups on histones influence the binding of histones to DNA and hence the structure and function of chromatin. Acetylation of histones has been shown to be correlated with increased transcriptional activity of chromatin (16). A similar role has been proposed for the polyamine acetylation reactions (1). An acetylation–deacetylation process is consistent with such a proposal. From the present study, $N^8$-acetylspermidine would appear to be a more likely candidate for this nuclear regulatory process than would $N^1$-acetylspermidine. Thus the present study indicates the occurrence of major differences in the metabolism of the two acetylated derivatives and points to a need for future studies on differences in cellular functions of $N^1$-acetylspermidine and $N^8$-acetylspermidine.

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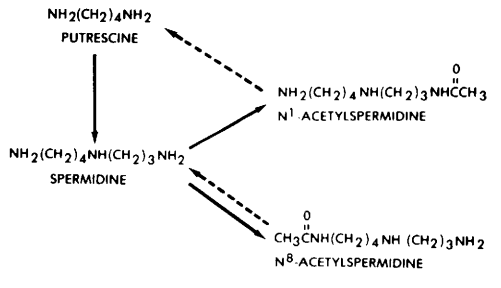


FIG. 4. Routes of synthesis and metabolism of $N^1$-acetylspermidine and $N^8$-acetylspermidine in rats.

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