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ANALYSIS OF POLYAMINES AND ACETYL DERIVATIVES BY A SINGLE AUTOMATED AMINO ACID ANALYZER TECHNIQUE

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SUMMARY

In a single, rapid and precise analysis, monoacetylputrescine, N^8-acetylspermidine, N^1-acetylspermidine, putrescine, spermidine, and spermine can be separated using a five-buffer system on an automatic amino acid analyzer. This method allows, for the first time, the separation of all the known acetyl derivatives of putrescine and spermidine as well as the parent compounds in urine and tissues with a single automated procedure. The method has been applied to the analysis of biological samples from normal volunteers, cancer patients and a rat liver supernatant. Mass spectral confirmation was obtained for each compound.

INTRODUCTION

We have reported previously a single-step high-performance liquid chromatographic method for the analysis of acetylated polyamines [1]. Because of the possible clinical relevance of polyamines to evaluate the success or failure of cancer chemotherapy [2—6], we have continued to work on a method which would allow us to rapidly analyze multiple samples and which would separate both the acetyl derivatives and the parent compounds in a single, automated amino acid analyzer procedure. Not only are the two acetylspermidine derivatives made in cytoplasmic (N^1-derivative) and nuclear (N^8-derivative) compartments [7, 8], but the extent of conjugation for each polyamine as well as N^1/N^8 ratio may be of importance in characterizing tumor cell kinetics of common malignancies [9, 10]. A recent method reports separation of the urinary acetyl derivative of putrescine in a second procedure but does not separate N^1- and N^8-acetylspermidine derivatives [11].

Therefore, this method allows for the first time the separation of all the known acetyl derivatives of putrescine and spermidine in urine and tissues as

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well as the parent compounds in a single, automated chromatographic method requiring 83 min. Studies using this technique should be useful in evaluating alterations in acetyl derivatives in both urine and tissues.

MATERIALS AND METHODS

Apparatus

A model D-500 automatic amino acid analyzer (Dionex, Sunnyvale, CA, U.S.A.) equipped with a fluorescence detector (Aminco, Silver Spring, MD, U.S.A.) was used for analyses. The D-500 also included a strip chart recorder (Honeywell, Minneapolis, MN, U.S.A.) and a PDP/8M Digital computer (Digital Equipment Corp., Maynard, MA, U.S.A.) for peak retention time recording, integration, and instrument control. The analyzer was equipped with a column heating jacket designed to accommodate 3.2 mm O.D. columns where column temperature was determined by inserting a thermometer directly to the position of the column in the jacket. The D-500 has an automatic injection system capable of holding 80 samples.

The column used for the analyses was 11.5 cm × 1.7 mm I.D. packed with Aminex A-9 cation-exchange resin (> 8% crosslinked styrene—divinylbenzene copolymer, bead diameter 11.5 ± 0.5 μm) (BioRad Labs., Richmond, CA, U.S.A.). The column was packed from a slurry of resin in 0.2 *N* sodium citrate, 2.1 *N* sodium chloride, 2.5% ethanol, and 0.5% thiodiglycol, pH 5.6, at a pressure of 126 bars at ambient temperature.

Samples were prepared for analysis using 1.5-ml polypropylene microcentrifuge tubes (West Coast Scientific, Berkeley, CA, U.S.A.). A Beckman microfuge B (Beckman, Berkeley, CA, U.S.A.) was used for centrifugations.

A Radiometer Copenhagen PHM 83 pH meter (The London Company, Cleveland, OH, U.S.A.) was used in buffer preparation.

Chemicals

Only high purity chemicals were used in reagent preparation. Sodium citrate dihydrate (analytical reagent grade; Mallinckrodt, Paris, KY, U.S.A.), sodium hydroxide, boric acid, concentrated hydrochloric acid, sodium chloride (J.T. Baker, Phillipsburg, NJ, U.S.A.), ethanol (U.S.I., U.S.P., U.S. Industrial Chemicals, New York, NY, U.S.A.), thiodiglycol, and pentachlorophenol preservative (Pierce, Rockford, IL, U.S.A.) were used for the preparation of buffers.

Boric acid, potassium hydroxide, 2-mercaptoethanol, potassium thiocyanate (J.T. Baker), ethanol (U.S. Industrial Chemicals), and *o*-phthalic dicarboxaldehyde (OPA) (Aldrich, Milwaukee, WI, U.S.A.) were used in preparation of the *o*-phthalaldehyde reagent.

Sample preparation involved sulfosalicylic acid (Sigma, St. Louis, MO, U.S.A.) and sodium hydroxide (J.T. Baker).

Calibration standards

Putrescine, spermidine and spermine hydrochloride salts were purchased from Sigma. Acetylputrescine, N^1-acetylspermidine, and N^8-acetylspermidine were synthesized in our laboratory as previously described [1]. The concentration and purity of the acetyl-derivative stock solutions for use as analytical

standards were determined as follows: acetyl-derivative stocks were analyzed for the presence of non-derivatized polyamine. Aliquots of each stock were quantitatively hydrolyzed for 16 h in sealed tubes. An aliquot of each 6 *N* hydrolysate was diluted with distilled water to 0.6 *N* hydrochloric acid and analyzed for polyamine content against an analytical standard. These values were compared to the acetylpolyamine stock solutions and an analytical standard was prepared. The standard contained 200 pmole per 50 μl of acetylputrescine, N^1- and N^8-acetylspermidine, putrescine, cadaverine, spermidine, and spermine. The standard, prepared in 0.1 *N* hydrochloric acid, was found to be stable at the longest time assayed, 4 months, when stored at 4°C or below.

The relative fluorescence responses (RFR) were determined similar to relative weight responses (RWR) as described by Davis et al. [12]. The internal standard (IS) used was 3,3'-iminobispropylamine (IPA). The RFR of each polyamine (PA) and acetylpolyamine based on IPA was calculated as follows:

$$RFR_{PA/IS} = \frac{\text{area PA}}{\text{nmole/ml PA}} \times \frac{\text{nmole/ml IS}}{\text{area IS}}$$

The RFR values were determined by ten analyses of analytical standards of the polyamines and acetyl derivatives. The RFR can be used to control for assay variation.

Buffer preparation

All buffers were prepared and brought to volume prior to pH adjustment. Buffers 2—4 were adjusted to pH 5.6—5.7 using concentrated hydrochloric acid, while buffers 1 and 5 were adjusted using granular boric acid (Table I). Before use, all buffers were filtered and degassed through 0.45-μm filters (Millipore, San Francisco, CA, U.S.A.).

Reagent preparation

o-Phthalaldehyde reagent was prepared as follows: borate buffer was

TABLE I

BUFFER COMPOSITION AND PROCEDURE TIMING

All buffers contain 0.5% thiodiglycol with 4 drops pentachlorophenol per liter.

Buffer	Sodium citrate (*N*)	Sodium chloride (*N*)	Sodium hydroxide (*N*)	Ethanol (%)	pH	Time of procedure (min)
1*	0.125	0.0	0.05	0	10.2	0—38, injection at 25
2	0.2	0.4	0.0	2.5	5.7	38—43
3	0.2	1.2	0.0	2.5	5.65	43—59
4	0.2	2.1	0.0	2.5	5.6	59—73.5
5*	0.2	1.8	0.05	2.5	10.2	73.5—83

*All buffers adjusted to the proper pH with concentrated hydrochloric acid except those indicated with an asterisk were adjusted with boric acid.

prepared by initially adding 25 g potassium hydroxide to 800 ml deionized water; granular boric acid was then added to a final pH of 10.4 ± 0.02. The volume was brought to 1 liter with deionized water. Potassium thiocyanate (5.8 g), 4.5 ml 2-mercaptoethanol, 3 ml of 30% Brij solution, and 800 mg o-phthalic dicarboxaldehyde predissolved in 20 ml ethanol were added to the liter of buffer. The reagent was gently stirred and filtered through a 0.45-μm filter. After transfer to the reagent reservoir, the solution was purged with purified nitrogen to prevent oxidation and degradation. The reagent was stored at 4°C and was found to be stable for at least 7 days.

Sample preparation

Urine samples were collected in 15-ml screw-cap centrifuge tubes and stored at −20°C until preparation for analysis. Samples were then thawed at room temperature and 1 ml was transferred to a 1.5-ml microfuge tube where 100 μl 50% sulfosalicylic acid were added. After vortexing for 5 min, the sample was centrifuged at 8000 *g* for 10 min and 750 μl of the supernatant were transferred to another microfuge tube where 50 μl of a 4% sodium hydroxide solution were added. The sample was then placed on ice to complete precipitation prior to a 10-min centrifugation. After final centrifugation, the supernatant was decanted and stored at −20°C until analysis.

Ion-exchange chromatography

The deproteinized urine sample was thawed and vortexed prior to loading into a sample cartridge. The buffer program was designed as shown in Table I with buffer 1 running 25 min to equilibrate the column for injection. Acetylputrescine was eluted in buffer 1. Buffer 2 was used to lower column pH in preparation for the elution of polyamines in buffers 3 and 4. Both isomers of acetylspermidine and putrescine were eluted in buffer 3 whereas buffer 4 eluted cadaverine and spermidine. Spermine was eluted in buffer 5, which also served as an analytical column cleaning and regeneration buffer due to its high pH and high salt content.

Polyamines were quantitated by their relative fluorescence intensity at 455 nm (emission) with 340-nm excitation. Areas under the polyamine peaks were integrated and compared to areas of 200 pmole standard polyamine peaks. To assure quantitation accuracy, polyamine standards were chromatographed before and after each group of samples.

Peak identification

Polyamines and acetylpolyamines were identified based on retention times compared with analytical standards. Confirmation of identities was accomplished by mass spectrometry using a Finnigan Model 3300 mass spectrometer coupled to a Model 2300 INCOS data system (Finnigan, Sunnyvale, CA, U.S.A.).

RESULTS

Optimization of chromatography

In working with polyamines and ion-exchange chromatography, polyamines generally are separated from other physiological amines with a strategy exploiting their relatively strong cationic charge. The acetylpolyamines are somewhat less cationic than the parent compounds, which makes their chromatographic resolution from other physiological amines and basic amino acids more complicated. Acetylputrescine, in particular, posed this problem; when chromatographed at a moderate pH (5.6), acetylputrescine eluted earlier than arginine from the A-9 cation-exchange column. Another problem with using ionic buffers of low pH was that the two isomers of acetylspermidine, N^1 and N^8, co-eluted in one peak.

Elution with high pH (10.2) buffers yielded completely different results. Not only did arginine elute earlier than acetylputrescine, but N^8-acetylspermidine eluted earlier than N^1-acetylspermidine, as reported previously [1]. In order to resolve an unknown amine peak from N^8-acetylspermidine in urine, pH 5.7 and pH 5.65 buffers were employed. The separation of the acetylspermidine isomers was maintained.

Table I illustrates the 5-buffer separation of the polyamines and acetyl derivatives of putrescine and spermidine. The entire analysis, including equilibration, is completed in less than 85 min. The standard solution contained 200 pmole of each polyamine. A 11.5 cm × 1.7 mm I.D. column of A-9 cation-exchange resin (BioRad) was employed with a flow-rate of 19.2 ml/h. *o*-Phthalaldehyde reagent flow-rate was also maintained at 19.2 ml/h. Column temperature was maintained at 54 + 1°C.

Minimum detection limit

The sensitivity of fluorescence detection allows a low detection limit of about 10—20 pmole for the polyamines and acetylputrescine with the limit for acetylspermidine (both isomers) being slightly higher, 20—30 pmole. The detection limit was calculated as the concentration of polyamine which yielded a fluorescence response equal to twice the noise level.

Retention times and RFR

The retention times and RFR, compared to the internal standard IPA, for the polyamines and the acetyl derivatives are shown in Table II. Retention times were determined for each fresh lot of buffers. Precision of retention times exhibited a 1% variability over a three-day period of analysis.

Precision of chromatographic analysis

Repeated injections of analytical standards gave an average relative standard deviation (R.S.D., %) for the acetyl derivatives of 3.2% or less. Table III shows the precision of analysis of polyamines and acetylpolyamine derivatives from eight independent analyses of a pooled urine sample. Reproducibility is good when the sample analyzed contains more than 20 pmole of the amine or its derivative. Precision of retention times in an analysis of ten different urine samples analyzed over a three-day period was less than 1% R.S.D. in all cases

TABLE II

RELATIVE FLUORESCENCE RESPONSE

Retention time (min)	Compound*	RFR	area/200 pmole peak**
44.43	IPA	1.0	1,309,000
9.48	AcPut	0.63	825,000
27.70	N^8AcSpd	0.74	968,000
30.77	N^1AcSpd	0.87	1,140,000
35.25	Put	1.13	1,475,000
41.08	Cad	1.48	1,940,000
47.75	Spd	1.53	2,000,000
53.90	Spm	0.79	1,030,000

*Abbreviations: IPA = 3,3'-iminobispropylamine; AcPut = acetylputrescine; N^8AcSpd = N^8-acetylspermidine; N^1AcSpd = N^1-acetylspermidine; Put = putrescine; Cad = cadaverine; Spd = spermidine; Spm = spermine.
**Arbitrary fluorescence units.

TABLE III

PRECISION OF ANALYSIS FOR POLYAMINES IN HUMAN URINE

Data are compiled from eight independent analyses on two different days of a pooled urine sample. Values in nmole/mg creatinine.

	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd
	15.5	2.3	5.6	0.4	<0.2
	15.2	2.3	5.6	0.4	<0.2
	15.0	2.2	5.5	0.4	<0.2
	15.0	2.3	5.6	0.4	<0.2
	14.1	2.3	5.7	0.4	<0.2
	15.4	2.2	5.6	0.3	<0.2
	15.4	2.3	5.8	0.4	<0.2
	15.7	2.4	5.9	0.3	<0.2
Mean	15.2	2.3	5.7	0.4	<0.2
S.D.	0.49	0.06	0.13	0.05	
R.S.D. (%)	3.2	2.8	2.3	12	

(Table IV). In other studies, variation in the composition of the sample did not alter the retention time. Regular comparison of the sample to the standard prevented any alteration from affecting accuracy.

Recovery of polyamines from pooled urine

Since no extraction is necessary for urine sample preparation, recovery is essentially 100%.

Linearity

All polyamines and acetyl derivatives of polyamines responded linearly at quantities ranging from 25 pmole to 1.2 nmole injected onto the column (Fig. 1). Polyamine concentrations were sufficient for detection without concen-

TABLE IV

PRECISION OF RETENTION TIMES OF POLYAMINE AND ACETYLPOLYAMINE DERIVATIVES IN HUMAN URINE

Data represent the mean of eight or more independent analyses that were carried out over a three-day period.

	Retention time (min)					
	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
$\bar{x}$	9.53	27.63	30.72	35.20	47.68	53.73
σ	0.04	0.06	0.03	0.03	0.03	0.02
R.S.D. (%)	0.41	0.21	0.10	0.08	0.06	0.03

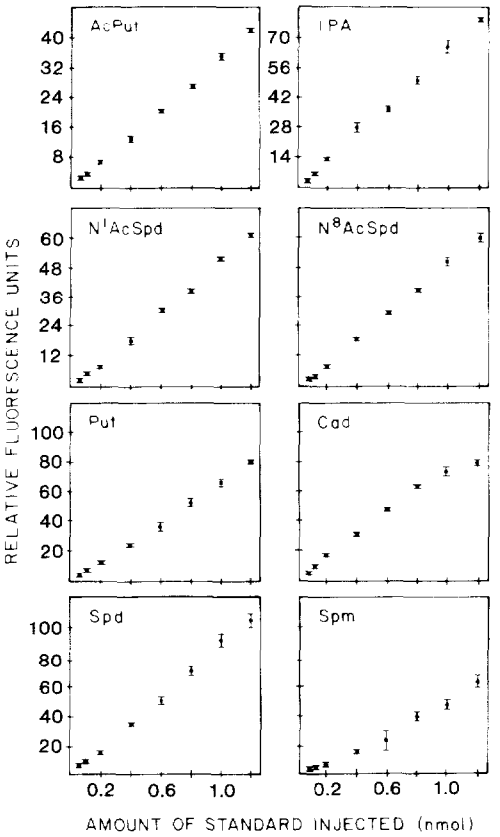

Fig. 1. Linearity of polyamines and acetylpolyamine derivatives over a range from 25 pmole to 1.2 nmole. Abbreviations: AcPut = acetylputrescine; IPA = 3,3'-iminobispropylamine; N^1AcSpd = N^1-acetylspermidine; N^8AcSpd = N^8-acetylspermidine; Put = putrescine; Cad = cadaverine; Spd = spermidine; and Spm = spermine.

tration of the urinary or tissue specimen, and in some cancer patients, urinary acetylputrescine was present in very high concentrations.

Analysis of polyamines and acetylpolyamines in human urine

The described ion-exchange chromatography method has been applied to the analysis of various human urine samples. A chromatogram illustrating standard solutions of polyamines and acetylpolyamines, a chromatogram of a normal human urine, and a rat liver analysis after carbon tetrachloride injection are shown in Fig. 2. Samples of eight different human urines were analyzed for polyamine and acetylpolyamine content and quantitated per mg creatinine. The values are shown in Table V. Table VI illustrates the urinary values of three patients with ovarian carcinoma before and after surgery.

TABLE V

POLYAMINE AND ACETYLPOLYAMINE CONTENT OF EIGHT NORMAL HUMAN URINE SPECIMENS

Data in nmole/mg creatinine.

Subject	Sex	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
SH	F	20	2.0	3.0	2.8	N.D.*	<0.2
CI	F	19	1.6	4.7	0.8	N.D.	N.D.
GS	F	14	1.4	3.7	0.7	<0.2	<0.2
SC	M	15	1.5	3.0	0.4	N.D.	<0.2
RS	M	16	1.9	3.8	1.2	N.D.	N.D.
CP	M	19	1.1	2.7	0.8	N.D.	<0.2
JR	M	12	1.1	2.0	0.7	N.D.	<0.2
JE	M	10	1.6	2.6	0.5	<0.2	<0.2
Range		10—20	1.1—2.0	2.0—4.7	0.4—2.8		
$\bar{x}$		15.6	1.5	3.2	1.0		
S.E.M.		0.45	0.2	0.3	0.1		

*N.D. = nondetectable.

TABLE VI

ACETYLPOLYAMINE AND POLYAMINE CONTENT OF THREE PATIENTS WITH OVARIAN CARCINOMA BEFORE AND AFTER SURGERY

Data in nmole/mg creatinine.

Subject	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
ZH presurgery	26	1.0	9.8	1.4	N.D.*	N.D.
ZH postsurgery	40	3.2	43	5.2	N.D.	N.D.
ZH postsurgery	42	1.3	18	5.8	N.D.	N.D.
MA presurgery	16	0.7	9.8	0.4	N.D.	N.D.
MA postsurgery	16	2.3	21	1.7	N.D.	N.D.
EM presurgery	20	1.8	7.9	N.D.	N.D.	N.D.

*N.D. = nondetectable.

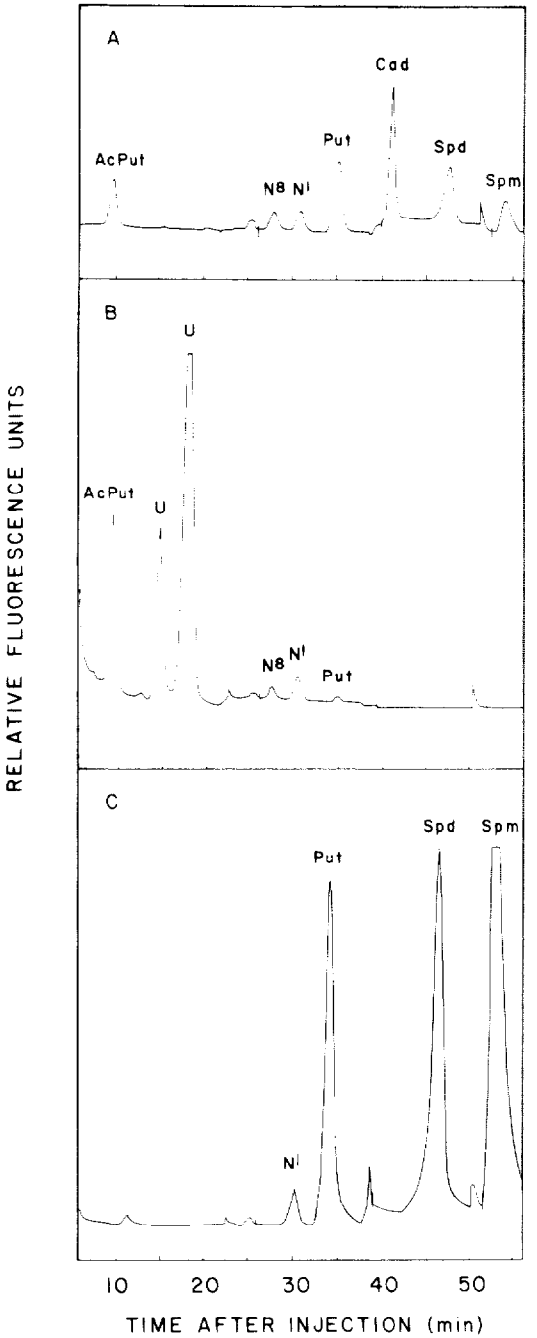

Fig. 2. (A) Chromatogram of calibration standards of acetyl derivatives and polyamines. A standard containing 200 pmole of each amine in 50 μl of 0.1 *N* hydrochloric acid was analyzed as described in Materials and Methods. (B) Chromatogram of a normal urine. Note the almost complete absence of nonconjugated polyamines. (C) Chromatogram of rat liver 6 h after carbon tetrachloride injection (2 mg/kg, intraperitoneally). This procedure results in N^1-acetylspermidine accumulation [13]. Abbreviations as in the legend to Fig. 1.

DISCUSSION

This is the first reported automated method that allows for the separation of both acetyl derivatives of polyamines found in urine and tissues and the free polyamines in a single chromatographic procedure. This is of importance because the urinary excretion of polyamines appears to reflect accurately disease activity in cancer patients as well as a partial or complete response to cancer therapy [3]. Further studies of possible specific patterns of N^1- and N^8-acetylspermidine excretion compared to total excretory polyamines now can be conducted with this methodology.

The major excretory polyamines have been identified as acetylpolyamine derivatives [9, 11, 14—32]. Spermine appears to be excreted mainly in a free, nonconjugated form [22], although a trace amount of N^1-acetylspermine has been detected in a mouse tissue [24]. Abdel-Monem and Ohno [9] were the first to suggest that the N^1- to N^8-acetylspermidine ratio might be altered in cancer patients. In a study of patients with leukemia, 13 of 15 patients had an elevated N^1- to N^8-acetylspermidine ratio when compared to normal volunteers. This suggested that quantitation of isomeric monoacetylspermidine derivatives might provide more information about disease activity and response to therapy. The two isomers were found in a nearly 1:1 ratio in the urine of healthy human volunteers [19, 20]. Seiler et al. [22] studied urinary acetylpolyamine excretion in two male melanoma patients and found that in one patient the acetylputrescine excretion was normal, N^8-acetylspermidine excretion was slightly elevated, and N^1-acetylspermidine excretion was several-fold higher than in healthy male controls. In the other patient, monoacetylputrescine, N^1- and N^8-acetylspermidine were excreted in elevated amounts as compared to controls. Prussak and Russell [1] found elevated monoacetylputrescine in the urine of three cancer patients and elevated N^8-acetylspermidine compared to N^1-acetylspermidine in two of the three patients. Therefore, it appears that extensive studies of intracellular and extracellular acetylpolyamine derivatives are required in order to establish whether specific functions exist for the two acetyl isomers of spermidine related to pathology. This should be possible with the instrumentation of the method reported in this communication.

Utilizing this method we found that acetylated derivatives of putrescine and spermidine comprised 90—95% of the total excreted polyamines in a series of urine specimens collected from normal controls (Table V). Of interest, the excretion of N^1-acetylspermidine in three ovarian carcinoma patients before and after surgery was higher in every instance than its excretion in normal controls (Table VI). After surgery there appeared to be an elevation also in unconjugated putrescine.

Follow-ups of studies of specific acetyl excretory patterns in pathology may be important since the N^1- and N^8-acetylspermidine derivatives are formed in different compartments of the cell [22]. Substrates for the nuclear N-acetyltransferase reaction are histones and polyamines including putrescine [7, 25, 26]. In the presence of spermidine, N^8-acetylspermidine is the only spermidine derivative formed by the nuclear enzyme [22]. The substrates of the cytosolic enzyme are spermine and spermidine, forming the N^1-monoacetyl

derivatives of these compounds [24, 27]. Putrescine and histone are not substrates for this enzyme. Pretreatment of a rat with carbon tetrachloride increases the cytosolic enzyme activity and the formation of N^1-acetylspermidine [13, 24, 27, 28]. The nuclear N-acetyltransferase activity, apparently not induced by carbon tetrachloride, can be stimulated in the kidneys of rats after administration of growth hormone and ACTH [29].

The importance of the formation of N^1-acetylspermidine in the cytoplasm may relate to its ability to be converted to putrescine through the action of polyamine oxidase. N^1-Acetylspermidine can be metabolized to acetylputrescine in transformed chick embryo fibroblasts [30], and acetylputrescine also can be deacetylated in the cytosol [31]. A recent report from Matsui and Pegg [32] indicates that carcinogens, such as dimethylnitrosamine, induce N^1-acetyltransferase, the formation of N^1-acetylspermidine, and ultimately, large increases in the concentration of putrescine, presumably by a conversion of spermidine to putrescine.

The concentration of acetylated polyamines excreted in urine will depend on the rate of formation and breakdown of these compounds in tissues. The treatment of rats with agents which cause considerable cell damage, such as epidermal UV irradiation or cyclophosphamide injection causes both N^1- and N^8-acetylspermidine excretion to increase [22]. Studies of urinary acetylspermidine excretion in hepatoma-bearing rats [21] indicated that urinary N^1-acetylspermidine excretion increased exponentially during the time of linear increase in tumor mass whereas the excretion of N^8-acetylspermidine increased only when the tumor mass was 35 g, shortly before the period of observed necrosis.

Because of the importance of polyamines as markers of growth kinetics, both normal and neoplastic, it is important now to generate information related to the regulation of the formation and excretion of acetylputrescine, N^1-acetylspermidine, and N^8-acetylspermidine. These studies may add to the usefulness of polyamines as markers of tumor cell kinetics as well as to our understanding of possible differences in acetylation patterns of normal and neoplastic cells.

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