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Differential Inhibition of Histone and Polyamine Acetylases by Multisubstrate Analogues†

Bradley G. Erwin, Lo Persson, and Anthony E. Pegg*

ABSTRACT: Mammalian cells contain a number of enzymes catalyzing the acetylation of polyamines and histones including an inducible spermidine/spermine N^1-acetyltransferase which may play a key role in regulating the interconversion of polyamines [Matsui, I., Wiegand, L., & Pegg, A. E. (1981) *J. Biol. Chem. 256*, 2454-2459]. The present experiments were carried out in order to provide a method to distinguish this enzyme from other polyamine/histone acetylases and to test whether specific inhibitors of its activity could be obtained. Rabbit antiserum to homogeneous rat liver spermidine/spermine N^1-acetyltransferase had no effect on the activity of a crude nuclear extract from rat liver, indicating that its spermidine acetylating capability is not related to the cytosolic spermidine/spermine N^1-acetyltransferase induced by hepatotoxins. Potential multisubstrate analogues were prepared by attaching various polyamines to coenzyme A via an acetic acid linkage and tested as potential inhibitors of the acetylation of spermidine and histones. There was little difference in the potency of these polyamine derivatives as inhibitors of histone or spermidine acetylation by the crude nuclear extracts which appeared to contain at least two such activities, one inhibited completely by 20-30 μM and the other amounting to 50% of the total being unaffected by 100 μM. Spermidine/spermine N^1-acetyltransferase was also inhibited by all the derivatives, but the potency toward this enzyme differed widely. The derivative from *sym*-norspermidine was a very strong inhibitor, giving 50% inhibition at 0.3 μM, and was more than 1 order of magnitude more active than the others. These results are consistent with *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide acting as a multisubstrate analogue since *sym*-norspermidine is a preferred substrate of spermidine/spermine N^1-acetyltransferase, having a K_m (9 μM) 14 times less than that for spermidine (130 μM). Comparisons of the effects of these inhibitors on cells and nuclear extracts may be valuable in understanding the physiological role of polyamine and histone acetylases.

A number of reports have appeared describing the presence of enzymes acetylating polyamines (Seiler & Al-Therib, 1974; Blankenship & Walle, 1977, 1978; Libby, 1978, 1980; Matsui et al., 1981, 1983; Della Ragione & Pegg, 1982; Cullis et al., 1982). Some but not all of the preparations also acetylate histones, and multiple forms of the enzymes acetylating both polyamines and histones have been described (Libby, 1978, 1980; Sures & Gallwitz, 1980; Garcea & Alberts, 1980). Intact cells and crude cell extracts are, therefore, likely to contain a mixture of proteins capable of catalyzing the formation of acetylated spermidine and spermine using acetyl coenzyme A (acetyl-CoA) as substrate. One of these enzymes, which we have described as spermidine/spermine N^1-acetyltransferase (Matsui et al. 1981; Della Ragione & Pegg, 1982), may play an important role in the regulation of intracellular polyamine concentrations. This enzyme is the rate-limiting step in the interconversion of polyamines which is brought about by the degradation of N^1-acetylspermidine and N^1-acetylspermine by polyamine oxidase, forming 3-acetamidopropanal and either putrescine or spermidine, respectively (Pegg et al., 1981; Seiler et al., 1981). Spermidine/spermine N^1-acetyltransferase is highly inducible in rodent liver in response to treatment with the hepatotoxin carbon tetrachloride (Matsui et al., 1981; Pösö & Pegg, 1982). After such induction, it is the predominant form of polyamine acetylase in cytosolic extracts, but it was not clear to what extent it contributed to the nuclear activity. Also, the importance of the oxidase/acetylase pathway for the interconversion of polyamines in maintaining normal polyamine content is not yet well understood. A specific and potent inhibitor of the spermidine/spermine N^1-acetyltransferase would provide one means by which to investigate these questions.

In the present paper, we describe tests of a number of potential inhibitors based on the synthesis of multisubstrate analogues of CoA with various polyamines. Cullis et al. (1982) have previously reported that such a spermidine derivative was a powerful inhibitor of the histone acetylase A from calf thymus. Since cytosolic spermidine/spermine N^1-acetyltransferase has a much lower K_m for *sym*-norspermidine than for spermidine and the reaction has been shown to occur via an ordered Bi-Bi mechanism (Della Ragione & Pegg, 1982, 1983), we reasoned that *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide should be a potent inhibitor of this enzyme, and this was found to be the case. This inhibitor was less active against nuclear histone/spermidine acetylase and could be used to distinguish between these enzymes. The

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different polyamine acetylases could also be distinguished by the use of a specific antiserum raised against spermidine/spermine N^1-acetyltransferase which completely precipitated this enzyme but had no effect on the nuclear activity.

Materials and Methods

Materials. 1,3-Diaminopropane, 3,3′-diaminodipropylamine (*sym*-norspermidine), and triethylamine were purchased from Eastman Kodak Co., Rochester, NY. N^1- and N^8-acetylspermidine derivatives were generous gifts of Dr. M. M. Abdel-Monem, University of Minnesota, Minneapolis, MN. 1,9-Diamino-5-azanonane (*sym*-homospermidine) was kindly provided by Dr. K. Samejima, Tokyo Biomedical Research Institute, Tokyo, Japan, and by Dr. R. Bergeron, University of Florida, Gainesville, Fl. Thiophenol and bromoacetyl bromide (which was redistilled prior to use) were purchased from Aldrich Chemical Co., Milwaukee, WI. [1-^{14}C]Acetyl coenzyme A (49.8 mCi/mmol) and formula 949 liquid scintillation counting fluid were purchased from New England Nuclear, Boston, MA. Coenzyme A (Li salt), spermidine trihydrochloride, histones (Sigma preparation II-A), and bovine serum albumin (fraction V) (for use as a standard in protein estimations) were purchased from Sigma Chemical Co., St. Louis, MO. Reagents for protein estimation [the method of Bradford (1976) was used] and cellulose phosphate resin (Cellex-P) were purchased from Bio-Rad Laboratories, Richmond, CA. Cellulose phosphate paper (P-81) and cellulose thin-layer chromatography plates (K2F) were purchased from Whatman, Inc., Clifton, NJ.

Extraction and Assay of Cytosolic and Nuclear Acetyltransferase Activities. Cytosolic spermidine acetyltransferase activity was extracted from the livers of male Sprague-Dawley rats (weighing 200–350) 6 h after intraperitoneal injection of carbon tetrachloride (2 mL/kg) as described by Matsui et al. (1981). The extract was kept frozen at −20 °C prior to use, and there was no activity loss after 2 weeks of storage. Spermidine acetyltransferase activity was assayed in a volume of 100 μL containing 100 μg of extract protein, 300 nmol of spermidine, 0.8 nmol (40 nCi) of [1-^{14}C]acetyl coenzyme A, and 10 μmol of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)(pH 7.8). The reaction was allowed to proceed at 30 °C for 10 min and was terminated by the addition of 20 μL of 1 M NH_2OH·HCl. Protein was precipitated by boiling the samples for 3 min and was removed by centrifugation. Fifty microliters of the supernatant was applied to a 2.2 cm diameter disk of cellulose phosphate paper and was allowed to dry. The disks were washed in a beaker 5 times with water and 3 times with ethanol. The disks were then placed in 10 mL of scintillation fluid, and the radioactivity in them was assessed with a Beckman LS 3133T liquid scintillation spectrometer. The efficiency of this counting system was approximately 55%.

A crude nuclear acetyltransferase extract was prepared from the livers of untreated male Sprague-Dawley rats (weighing 200–350 g) by the method of Libby (1980). Approximately 650 μg of extract protein was used per assay. Histone acetyltransferase activity was assessed as in the cytosolic extract except that histones (270 μg per assay) were used as the acetyl acceptor, the reaction time was 5 min, and the terminated reaction mixture was not centrifuged. The procedures for the assay of spermidine acetyltransferase activity in the nuclear extract were the same as in the cytosolic extract except that the reaction time was 5 min.

Nonenzymatic blank values for both histone and spermidine acetylation assays were routinely obtained by incubations of all the reactants except enzyme in the appropriate buffer. These values, which were always less than 7% of those observed when enzyme was present, were subtracted from all experimental values.

Chromatographic Identification of the Acetyltransferase Reaction Products. The reaction conditions were the same as those used in the assay except that the reaction was terminated by the addition of an equal amount of 10% trichloracetic acid. The samples were left at 4 °C for 2 h, and the protein was removed by centrifugation. Trichloroacetic acid was removed by three extractions with 5 volumes of ether. The resultant aqueous solution was applied to a 7 × 15 mm column of cellulose phosphate resin. The column was washed with 8 mL of 10 mM HCl, and the acetylspermidine derivatives were eluted with 2 mL of 500 mM HCl. The eluate was taken to dryness, redissolved in 50 μL of distilled water, and spotted on a strip of Whatman 3MM paper. The paper was developed for 16 h by using 1-propanol/triethylamine/water (85:3:15) as a descending solvent system. The strips were dried, sliced into 3-cm sections, and placed in 20 mL of scintillation fluid. Radioactivity was assessed with a Beckman LS 3133T liquid scintillation spectrometer. Nonradioactive standard compounds were stained with a 0.5% ninhydrin solution in acetone.

Synthesis of N-[2-(S-Coenzyme A)acetyl]polyamine Amides. Derivatives of *sym*-norspermidine, spermidine, *sym*-homospermidine, 1,3-diaminopropane, and putrescine were produced by the procedure developed by Cullis et al. (1982) for the synthesis of *N*-[2-(*S*-coenzyme A)acetyl]spermidine amide. Separation of the amides from the reactants was achieved by applying crude reaction mixture to a 10-mL column of DEAE-Sephadex that had been equilibrated with 50 mM triethylammonium bicarbonate (pH 7.6). The column was eluted with an ascending 200-mL gradient (50–500 mM) of the same buffer. Eluted ultraviolet light absorbing peaks were assayed for inhibitory activity. The active peaks were concentrated by lyophilization. The purity of the derivatives was assessed by thin-layer chromatography on cellulose plates (Whatman K2F) developed in acetonitrile/2% acetic acid (1:1) or isobutyric acid/sodium isobutyrate, pH 4.3. Compounds were detected by reaction with ninhydrin and by UV absorbance. The R_f value of 2-(*S*-coenzyme A)acetic acid thiophenyl ester in the former solvent was 0.73, the R_f value of *sym*-norspermidine amide was 0.59, and R_f values of the two spermidine derivatives were 0.51 and 0.59.

Immunological Techniques. Antiserum to rat liver spermidine/spermine N^1-acetyltransferase was obtained by immunizing a female New Zealand white rabbit with a homogeneous enzyme preparation isolated as described by Della Ragione & Pegg (1982). The rabbit was immunized by multiple-site intradermal injections using 45 μg of the protein emulsified in Freund's complete adjuvant. A booster injection of 25 μg in incomplete adjuvant was given at 6 weeks, and antiserum was collected 2 weeks later. Immunoprecipitation of acetylase activity was carried out by incubation of the sample in a total volume of 0.11 mL containing 50 mM Tris-HCl, pH 7.5, 1% bovine serum albumin, 1 mM spermidine, 0.02% Brij 35, and the antiserum at 4 °C overnight. After this incubation, 25 μL of bacterial protein A adsorbant (Miles Laboratories, Elkhart, IN) was added, and after a further incubation of 90 min at 4 °C, the solution was centrifuged at 15000*g* for 1 min. The supernatant was then tested for acetylase activitiy.

Results

The method used by Cullis et al. (1982) for synthesis of the spermidine derivative was found to be satisfactory for the

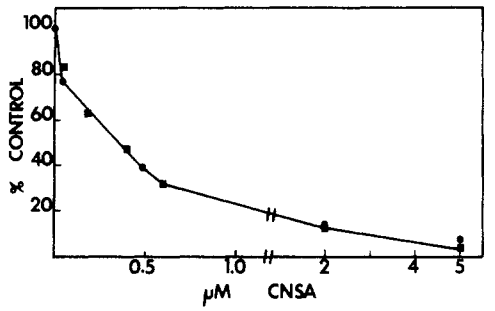

FIGURE 1: Inhibition of spermidine/spermine N^1-acetyltransferase by *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide (CNSA). The acetylation of spermdine in the absence of inhibitor was set at 100%, and the results shown are the amount of inhbition in the presence of the concentration of CNSA indicated using either purified spermidine/spermine N^1-acetyltransferase (■) or a cytosolic extract (●) prepared from rat liver 6 h after administration of carbon tetrachloride (2 mL/kg).

Table I: Effect of Substrate Concentration on the Inhibition of Spermidine/Spermine N^1-Acetyltransferase by *N*-[2-(*S*-Coenzyme A)acetyl]-*sym*-norspermidine Amide[a]

[acetyl-CoA] (μM)	[spermidine] (μM)	% inhibn of acetylase activity
8	0.06	94 ± 1
8	0.30	85 ± 1
8	3.00	58 ± 0.2
20	3.00	42 ± 2
40	3.00	33 ± 2

[a] Results are shown for the percent inhibition of the activity of homogeneous spermidine/spermine N^1-acetyltransferase by 0.5 μM *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide.

preparation of *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide. The norspermidine derivative was obtained in 36% yield based on the amount of 2-(*S*-coenzyme A)acetic acid thiophenyl ester used and gave a single UV-absorbing and ninhydrin-positive spot on thin-layer chromatography in two solvent systems (see Materials and Methods). This compound was a very potent inhibitor of the acetylation of spermidine by crude cytosolic extracts from livers of carbon tetrachloride treated rats and by highly purified spermidine/spermine N^1-acetyltransferase (Figure 1). There was little difference in the sensitivity of both preparations, and 50% inhibition required 0.3 μM when the assays were carried out in the standard assay mix (Figure 1). This contains 3 mM spermidine and 8 μM acetyl-CoA. A full kinetic analysis was not carried out, but the inhibition was greater when the assays were carried out at low concentrations of either substrate (Table I). This trend is compatible with the inhibitor acting as a multisubstrate analogue.

Similar derivatives were prepared from spermidine, *sym*-homospermidine, 1,3-diaminopropane, and putrescine and tested for inhibitory activity toward spermidine/spermine N^1-acetyltransferase (Figure 2). The product from the reaction of 2-(*S*-coenzyme A)acetic acid thiophenyl ester with spermidine was separated into two peaks on DEAE-Sephadex chromatography. These derivatives could also be separated by thin-layer chromatography and were designated I and II in accordance with their retention on DEAE-Sephadex. The two derivatives differed slightly in their ability to inhibit the spermidine/spermine N^1-acetyltransferase (Figure 2B). It is likely that they represent amides at the N^1- and N^8-position of spermidine, respectively, although they were not fully characterized. The spermidine amides were strong inhibitors of the N^1-acetyltransferase but were more than 1 order of magnitude less potent than the *sym*-norspermidine derivative.

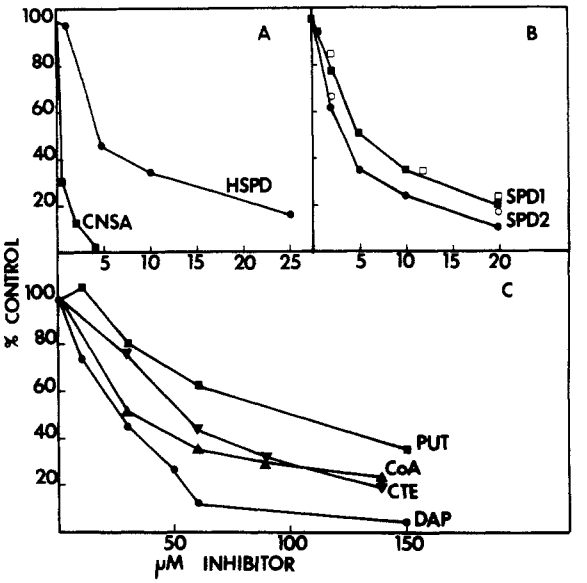

FIGURE 2: Inhibition of spermidine/spermine N^1-acetyltransferase by *N*-[2-(*S*-coenzyme A)acetyl]polyamine amides. Results are shown in panel A for the derivatives from *sym*-homospermidine (HSPD, ●) and *sym*-norspermidine (CNSA, ■), in panel B for spermidine derivatives (SPD1, ■ or □, and SPD 2, ● or ○), and in panel C for derivatives from putrescine (PUT, ■) and 1,3-diaminopropane DAP, ●), and for CoA itself (CoA, ▲), and for 2-(*S*-coenzyme A)acetic acid thiophenyl ester (CTE, ▼). Solid symbols indicate results for the inhibition of the activity of cytosolic extracts from carbon tetrachloride treated rats and open symbols results for the purified spermidine/spermine N^1-acetyltransferase. Other details are in Figure 1.

Table II: Comparison of Polyamines as Substrates and as Potential Multisubstrate Analogues of Spermidine/Spermine N^1-Acetyltransferase

polyamine	K_m as substrate[a] (μM)	I_{50} of inhibitor or CoA derivative[b] (μM)
spermidine	130	3 (derivative 2) 5 (derivative 1)
sym-norspermidine	9	0.3
sym-homospermidine	inactive	4.5
putrescine	inactive	100
1,3-diaminopropane	460	25
coenzyme A	*c*	33
2-(*S*-coenzyme A)acetic acid thiophenyl ester	*c*	55

[a] From Della Ragione & Pegg (1983). [b] From Figure 2 of this paper. [c] Not appropriate.

Similarly, the *sym*-homospermidine derivative was 10 times less active than the *sym*-norspermidine compound (Figure 2A). The amides derived from 1,3-diaminopropane and putrescine were much less active, and only that derived from 1,3-diaminopropane was more inhibitory than coenzyme A itself (Figure 2C). These results are consistent with the known properties of spermidine/spermine N^1-acetyltransferase and the concept that these inhibitors act as multisubstrate analogues. Table II gives the I_{50} values for these derivatives and the K_m values for those amines which are substrates for the enzyme (Della Ragione & Pegg, 1983).

A similar extent of inhibition by the *sym*-norspermidine derivative (Figure 1) and the spermidine derivatives (Figure 2) was produced with both highly purified spermidine/spermine N^1-acetyltransferase and thee crude cytosolic extracts. This suggests that the majority of the acetylase activity in these extracts from carbon tetrachloride treated rats is due to the spermidine/spermine N^1-acetyltransferase. Confirmation of

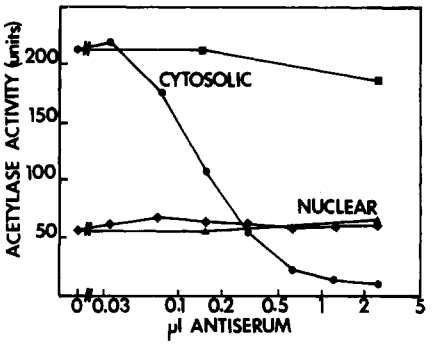

FIGURE 3: Immunotitration of acetylase activity from rat liver extracts. Results are shown for the precipitation of enzyme activity by exposure to the amount of antiserum shown followed by protein A as described under Materials and Methods. The remaining acetylase activity assayed with spermidine as a substrate was then determined. Results are shown for cytosolic extracts from carbon tetrachloride treated rats (●, ■) and for a nuclear extract (♦, ▲). Each extract was exposed to either control rabbit serum (■, ▲) or antiserum from a rabbit immunized with spermidine/spermine N^1-acetyltransferase (●, ♦).

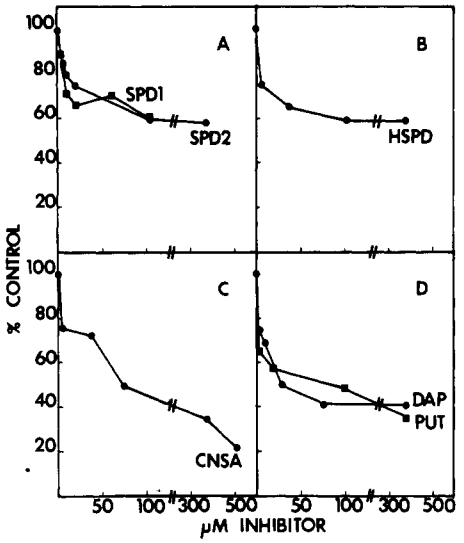

FIGURE 4: Inhibition of histone acetylation by *N*-[2-(*S*-coenzyme A)acetyl]polyamine amides. The acetylation of histones by crude rat liver nuclear extracts was assayed as the percentage of the activity found in the absence of inhibitor. Results are shown in panel A for the derivatives from spermidine (SPD1, ■, and SD2, ●), in panel B for the derivative from *sym*-homospermidine (HSPD, ●), in panel C for the derivative from *sym*-norspermidine (CNSA, ●), and in panel D for the derivatives from 1,3-diaminopropane (DAP, ●) and putrescine (PUT, ■).

this was obtained by the use of a specific rabbit antiserum obtained from a rabbit immunized with a homogeneous preparation of the spermidine/spermine N^1-acetyltransferase. This antiserum followed by addition of protein A led to the complete precipitation of the purified enzyme. When the crude cytosolic extract from carbon tetrachloride treated rats was substituted for the pure enzyme, more than 95% was precipitated by the antibody (Figure 3), and the amount of antiserum needed for 50% inhibition of 1 unit of enzyme activity was identical. In contrast a nuclear acetylase extract which acetylated both histones and spermidine as described below was not precipitated at all by the antiserum (Figure 3).

The multisubstrate analogues were tested as potential inhibitors of the nuclear acetylase(s) acting on histones as a substrate (Figure 4) and spermidine as a substrate (Figure 5). The crude nuclear extract used as a source of enzyme for this experiment did not contain any spermidine/spermine N^1-acetyltransferase since its activity was not reduced by the antiserum to this enzyme but may contain a mixture of en-

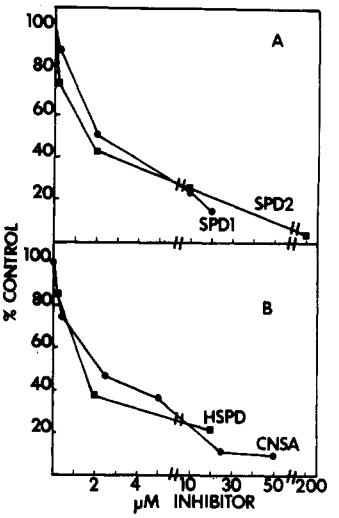

FIGURE 5: Inhibition of spermidine acetylation catalyzed by nuclear extracts in the presence of *N*-[2-(*S*-coenzyme A)acetyl]polyamine amides. The inhibition of spermidine acetylase activity of the same nuclear extract used in Figure 4 was determined in the presence of the derivatives from spermidine [panel A, SPD1 (●) and SPD2 (■)] and from *sym*-homospermidine [panel B, HSPD (■)] and *sym*-norspermidine [panel B, CNSA (●)].

zymes with polyamine/histone acetylating activity. Inhibition of histone acetylation by these derivatives showed two distinct phases (Figure 4). A portion (40–50%) was quite strongly inhibited, requiring about a 20 μM concentration of the inhibitor, whereas the remainder was relatively resistant so that more than a 300 μM concentration of the drug was needed for 70% inhibition. There was little difference in the potency of the inhibitors, and the putrescine derivative was as active as that derived from *sym*-norspermidine. When spermidine rather than histones was used as a substrate with the crude nuclear extracts, the reaction was more sensitive to inhibition, but there was again no discrimination between inhibition by the spermidine, *sym*-norspermidine, and *sym*-homospermidine derivatives (Figure 5). About 1–2 μM was needed for 50% inhibition.

The product of the acetylation of spermidine by the crude nuclear extract and the crude cytosolic extract from carbon tetrachloride treated rats was investigated by using a paper chromatography system which clearly separates N^1- and N^8-acetylspermidine (Figure 6). The product of the nuclear extract was predominantly N^8-acetylspermidine with about 15% N^1-acetylspermidine. Formation of both products was inhibited to about the same extent by *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine amide. The product of the cytosolic extract was exclusively N^1-acetylspermidine (Figure 6). This is consistent with the other data indicating that the great majority of the activity in this extract was due to the spermidine/spermine N^1-acetyltransferase.

Discussion

The results obtained with the antiserum to spermidine/spermine N^1-acetyltransferase and the inhibitors show clearly that the inducible enzyme (which forms exclusively N^1-acetylspermidine and does not use histones as a substrate) is entirely distinct from the nuclear activities which acetylate histones and will also act on spermidine, forming predominantly N^8-acetylspermidine. The most potent inhibitor of the spermidine/spermine N^1-acetyltransferase was *N*-[2-(*S*-coenzyme A)acetyl]-*sym*-norspermidine, which was at least 1 order of magnitude more active than the other derivatives. This is in agreement with the concept that the compounds act

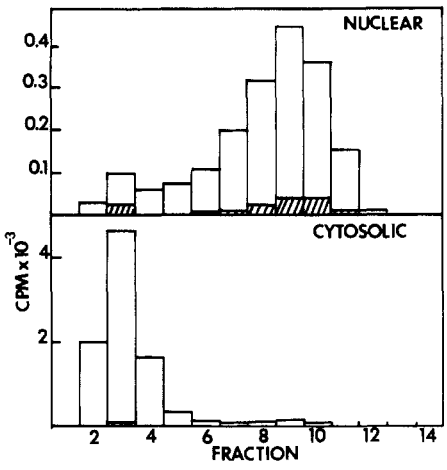

FIGURE 6: Product of acetylation of spermidine by rat liver extracts. Spermidine was acetylated by the action of rat liver nuclear extract (upper panel) or cytosolic extract from rats treated with carbon tetrachloride (lower panel). The labeled product was separated by paper chromatography as described under Materials and Methods, the paper was cut into 3-cm sections, and each fraction was assayed for radioactivity. Authentic markers of N^1-acetylspermidine and N^8-acetylspermidine were run in parallel and detected by reaction with ninhydrin. N^1-Acetylspermidine was present in fractions 2–4 and N^8-acetylspermidine in fractions 7–11. The hatched regions represent the distribution of labeled product formed in the presence of 5 μM CNSA in the reaction mixture.

as multisubstrate analogues (Cullis et al., 1982) since *sym*-norspermidine is a preferred substrate (see Table II). The *sym*-norspermidine derivative may be a useful compound with which to study the physiological role of the acetylase/oxidase pathway for the interconversion of polyamines since the spermidine/spermine N^1-acetyltransferase has been postulated as the rate-limiting step in this interconversion (Pegg et al., 1981; Matsui et al., 1981; Seiler et al., 1981). Obviously, this would require the uptake of the inhibitor into the cell and its subsequent resistance to intracellular degradation, and these factors are currently under investigation. Another problem with the use of inhibitors in vivo is the question of specificity, and it is clear from the results in Figures 4 and 5 that the *sym*-norspermidine derivative does inhibit the other acetylases albeit at considerably higher concentrations. However, the comparison of the effects of this inhibitor with those of one of the other polyamine derivatives, e.g., the *sym*-homospermidine or putrescine derivatives, which are much less active against the spermidine/spermine N^1-acetyltransferase but have similar activities against the nuclear histone/spermidine acetylases should provide a valuable control. There has been a great deal of recent interest and investigation of the pharmacological possibilities of substances interfering with polyamine metabolism since polyamine content is linked to growth of mammalian cells and of various protozoal pathogens [reviewed by Heby (1981), Pegg & McCann (1982), Sjoerdsma (1981), and Sjoerdsma & Schecter (1984)]. The effect of combining the spermidine/spermine N^1-acetyltransferase inhibitor with these drugs which inhibit ornithine or *S*-adenosylmethionine decarboxylase would be of considerable interest.

The results in Figures 4 and 5 suggest heterogeneity in the nuclear enzymes acetylating histones which is in agreement with some previous studies (Libby, 1978, 1980; Sures & Gallwitz, 1980; Garcea & Alberts, 1980), but it is not clear how many distinct proteins there are with the capability of acetylating spermidine. The nuclear extract produces predominantly N^8-acetylspermidine, but some N^1-acetylspermidine was seen. Since none of the nuclear activity was precipitated by the antibodies, this N^1-acetylspermidine could not have been derived from the presence in the extracts of the cytosolic spermidine/spermine N^1-acetyltransferase, but it is possible that the single enzyme can attack both ends of the spermidine molecule as well as histones. Indeed, both Blankenship & Walle (1977, 1978) and Libby (1978, 1980) report that nuclear preparations could acetylate putrescine, spermidine, spermine, and histones. If this activity resides in a single protein, it is perhaps not surprising that all of the derivatives tested in Figures 4 and 5 inhibit the nuclear acetylase to about the same extent.

The original concept of the synthesis of these multisubstrate analogues was based on the pioneering work of Cullis et al. (1982), who synthesized *N*-[2-(*S*-coenzyme A)acetyl]spermidine amide and found that this compound (presumably a mixture of isomers) was an extremely potent inhibitor of the acetylation of either spermidine or histones by purified histone *N*-acetyltransferase A from calf thymus. Our results with rat liver extracts differ from those of Cullis et al. (1982) in two respects. First, they found that the spermidine derivative was equally potent whether the substrate was histones or spermidine whereas we found that the coenzyme A polyamine amides were much more inhibitory to spermidine acetylation. This discrepancy could be due to the presence of multiple histone acetylases in the crude nuclear extract we employed since part of the histone acetylation was quite sensitive and this could represent the same enzyme which also attacks spermidine. Second, even the more sensitive activity required 1–2 μM for 50% inhibition whereas Cullis et al. (1982) obtained 50% inhibition at 5–15 nM. Although the assay conditions were somewhat different, they are unlikely to be responsible for this difference. A more likely possibility is that the rat liver histone acetylase used here has properties different from the calf thymus histone acetylase A. There is already evidence that this is the case since Wong & Wong (1983) reported that calf thymus histone acetyltransferase A acts via a ping-pong pathway with formation of an acetyl-enzyme intermediate and is not inhibited by N^{ϵ}-acetyllysine whereas Wiktorowicz et al. (1981) reported that the reaction of the histone acetyltransferase which they purified from rat liver proceeded via a rapid equilibrium ordered bireactant mechanism and was strongly inhibited by N^{ϵ}-acetyllysine. Therefore, the potency of these inhibitors may be species specific. In any case, it should be noted that the coenzyme A spermidine amides are not specific inhibitors of histone acetylation and that their potential effect on the spermidine/spermine N^1-acetyltransferase should also be considered in any experiments in which these compounds are used with intact cells.

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New Synthetic Cluster Ligands for Galactose/*N*-Acetylgalactosamine-Specific Lectin of Mammalian Liver†

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ABSTRACT: Synthetic ligands containing up to six residues of nonreducing terminal galactose were prepared. The synthesis involved coupling of carboxyl groups of *N*-benzyloxycarbonylaspartic acid or of *N*-benzyloxycarbonyltyrosyl-γ-glutamylglutamic acid to the ω-amino group of the aglycon of a glycoside that contained up to three lactosyl residues. The benzyloxycarbonyl group was removed by hydrogenolysis before these ligands were tested as inhibitors to the binding of ^{125}I-asialoorosomucoid to the galactose/*N*-acetylgalactosamine lectin, both soluble and on the surface of freshly isolated mammalian hepatocytes. Each addition of a galactosyl residue to an existing ligand structure invariably increased the binding affinity of such a ligand. However, at each level of galactose valency, the binding constant varied as much as 1000-fold depending on the structure of the ligand. At a given level of valency, the binding strength of a cluster ligand depended mainly on two factors: (1) the maximum spatial inter-galactose distances and (2) the flexibility of the arm connecting galactosyl residues and the branch points. It has been postulated that the three galactose-combining sites of the lectin are arranged in space at the vertexes of a triangle whose sides are 15, 22, and 25 Å [Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., & Bock, K. (1984) in *Biochemical and Biophysical Studies of Proteins and Nucleic Acids* (Lo, T. B., Liu, T. Y., & Li, C. H., Eds.) pp 349–360, Elsevier, New York]. Ligands having inter-galactose distances shorter than these lengths were invariably poor ligands at their respective level of valency. Among the ligands having sufficiently long inter-galactose distances, those with the most flexible structure were the best inhibitors. The 50% inhibition of ^{125}I-asialoorosomucoid binding was achieved by 3×10^{-7} and 5×10^{-8} M, respectively, of the best synthetic bi- and trivalent ligands, and inhibitory power of these ligands was comparable to that of the most inhibitory bi- and triantennary oligosaccharide structures of natural origin [Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202].

The mammalian hepatic lectin specific for Gal[1] and GalNAc has a strong affinity (K_d = ca. 10^{-9} M) for desialylated serum glycoproteins such as ASOR[2] [e.g., Weigel (1980), Baenziger & Maynard (1980), and Connolly et al. (1983)] that bear multiple oligosaccharide chains of the complex type and thus can remove such glycoproteins rapidly from the circulation (Morell et al., 1971). The binding site of the lectin appears to be rather small, binding only the nonreducing terminal Gal (or GalNAc) and a portion of the penultimate sugar (Sarkar et al., 1979; Lee et al., 1982). Although galactosides will compete for the lectin site occupied by ASOR, the binding of monovalent galactosides to the lectin is much weaker, with K_d in the range of 10^{-4} M (Connolly et al., 1982). However, the binding strength is highly dependent on the number of galactosyl residues in a ligand, with K_d increasing exponentially with the increasing number of clustered Gal (Lee, 1982). The most spectacular cluster effect (an increase in the binding strength beyond that expected from the increase in Gal concentration) was observed (Lee et al., 1983) with a bivalent oligosaccharide, PENTA-2,4 with a K_d of 3×10^{-7} M, and a trivalent oligosaccharide, NONA I wtih a K_d of 8×10^{-9}

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[1] All sugars are of D configuration and in pyranose form. All amino acids are of L configuration.

[2] Abbreviations: ASOR, asialoorosomucoid; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; BSA, bovine serum albumin; AH, aminohexyl; LacAH, 6-aminohexyl β-lactopyranoside; AHT, (6-aminohexanamido)tris(hydroxymethyl)methane; Lac-BSA, BSA that has been modified with Lac-$SCH_2CONHCH_2CHO$ via reductive alkylation (Lee & Lee, 1980); Cbz, carbobenzyloxy; TLC, thin-layer chromatography.

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