Effect of Expression of Human Spermidine/Spermine N^1-Acetyltransferase in *Escherichia coli*†

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Received October 28, 1994; Revised Manuscript Received December 16, 1994®

ABSTRACT: A plasmid expression vector, pINSAT2, was constructed in order to express spermidine/spermine N^1-acetyltransferase (SSAT) in *Escherichia coli*. Cells transfected with this vector produced large amounts of SSAT, amounting to up to 2% of the soluble protein when isopropyl β-D-thiogalactopyranoside (IPTG) was added and 0.3% of the soluble protein in the absence of inducer. The growth rate of cells expressing SSAT was reduced, and all of the cellular spermidine was converted to N^1-acetylspermidine, much of which was excreted. Putrescine and 1-methylspermidine, which is not a substrate for SSAT, could reverse the effects of SSAT expression on growth, but spermidine was only effective when the amount of SSAT expression was limited by omitting the IPTG inducer. The lack of stimulation of growth by spermidine correlated with its complete conversion to N^1-acetylspermidine. These results show that N^1-acetylspermine is not able to substitute for the unmodified polyamines in supporting growth and suggest that acetylation is a physiological response to convert excess polyamines to a physiologically inert form which is readily excreted. Cells expressing large amounts of SSAT were much more senstive to the growth inhibitory action of the antitumor agent N^1,N^{12}-bis(ethyl)spermine, supporting the hypothesis that the ability of such bis(ethyl) polyamines to induce SSAT contributes to their antiproliferative actions. SSAT was readily purified to homogeneity from extracts of DH5α cells containing pINSAT2. The purified enzyme had a simialr specific activity and K_m values for spermine and spermidine as the enzyme purified from human colon cancer cells, suggesting that posttranslational modifications specific to eukaryotes are not needed for enzymatic activity. The recombinant SSAT was found to acetylate the drugs 15-deoxyspergualin, 2-[(aminopropyl)amino]ethanethiol, and *N*-(*n*-butyl)-1,3-diaminopropane.

Spermidine/spermine N^1-acetyltransferase (SSAT)[1] is a highly inducible cytosolic enzyme that was first identified in extracts from the livers of rats treated with carbon tetrachloride (Matsui et al., 1981). Many subsequent experiments have shown that SSAT is induced in response to a wide range of stimuli including hormones and growth factors, toxic compounds, various drugs, and pathophysiological insults [reviewed by Della Ragione et al. (1984) and Casero and Pegg (1993)]. SSAT is also induced by the polyamines themselves and by a variety of polyamine analogs (Casero & Pegg, 1993; Persson & Pegg, 1984; Pegg & Erwin, 1985; Erwin & Pegg, 1986; Casero et al., 1989; Saab et al., 1993; Shappell et al., 1993). The exact cellular function of SSAT has not been defined precisely, but the enzyme leads to the conversion of spermidine and spermine to N^1-acetylspermidine and N^1-acetylspermine. These acetylated polyamines are then acted upon by polyamine oxidase (PAO)[1] to form putrescine and spermidine, respectively (Pegg, 1986; Seiler, 1987). The overall effect of induction of SSAT is therefore to convert the higher polyamines to putrescine and N^1-acetylspermidine, which are both readily excreted from the cell (Casero & Pegg, 1993; Pegg et al., 1989, 1990; Wallace, 1987), and putrescine may also be degraded by diamine oxidase (Morgan, 1986). The result of these changes is to reduce the cellular content of spermidine and spermine. This fact, coupled with the striking induction of SSAT by exposure to polyamines, has led to the suggestion (Pegg, 1986) that the SSAT/PAO system provides a regulatory mechanism to prevent polyamine levels from rising too high. Excess intracellular accumulation of polyamines results in severe toxicity even in the absence of their conversion to toxic aldehydes (Brunton et al., 1990; Mitchell et al., 1992; Poulin et al., 1993, Byers et al., 1994). A common feature of the myriad of stimuli that induce SSAT may be the ability to raise the intracellular concentration of free polyamines either by increasing their synthesis or by displacing polyamines from bound sites.

Although the presence of acetylated polyamines in *Escherichia coli* has been known to occur for many years, the pioneering studies of C. W. Tabor showed that much of this acetylation occurred as a result of the accumulation of monoacetylspermidines during harvesting at 4 °C (Tabor,

† This research was supported by Grant GM-26290 from the National Institutes of Health. Some equipment was provided by a grant from the Alcoa Foundation. Protein sequencing was made possible by NSF Biological Facility Center Grant DIR 8804758.

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® Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Abbreviations: SSAT, spermidine/spermine N^1-acetyltransferase; PAO, FAD-dependent polyamine oxidase; BESM, N^1,N^{12}-bis(ethyl)-spermine; BENSM, N^1,N^{11}-bis(ethyl)norspermine; IPTG, isopropyl β-D-thiogalactopyranoside; WR-1065, 2-[(aminopropyl)amino]ethanethiol; WR-2721, (*S*)-2-[(3-aminopropyl)amino]ethylphosphoric acid; WR-44923, (*S*)-2-[(3-aminopropyl)amino]propylphosphoric acid; WR-2822, (*S*)-2-[(2-aminoethyl)amino]butylphosphoric acid; BDAP, *N*-(*n*-butyl)-1,3-diaminopropane; HPLC, high-pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

0006-2960/95/0434-2701$09.00/0

1968; Tabor & Dobbs, 1970). Addition of polyamines to the culture medium or other conditions in which large amounts of endogenous polyamines are formed also lead to the production of acetylated polyamines in *E. coli* (Tabor & Dobbs, 1970; Tabor & Tabor, 1985). More recently, a variety of other stresses have been shown to stimulate the accumulation of monoacetylspermidines (Carper et al., 1991). A low level of an enzyme acetylating spermidine has been detected in *E. coli* (Carper et al., 1991; Matsui et al., 1982; Fukuchi et al., 1994). Mutant strains lacking this enzyme grow normally, showing that it is not needed for growth but may be involved in the response to stress (Carper et al., 1991).

The massive induction of SSAT in mammalian cells by polyamine analogs such as N^1,N^{12}-bis(ethyl)spermine (BESM)[1] and N^1,N^{11}-bis(ethyl)norspermine (BENSM)[1] leads to the very rapid depletion of the cellular polyamines and their replacement by these polyamine analogs (Casero et al., 1989; Pegg et al., 1989; Libby et al., 1989; Bergeron et al., 1989; Shappell et al., 1992). This induction may therefore be critical for the inhibition of tumor cell growth by these analogs, some of which have strong antiproliferative effects and are currently undergoing development as antitumor agents. However, the consequences of SSAT induction on cell growth in the absence of perturbations in the cellular polyamines have not been investigated. In the present experiments, we have expressed human SSAT in *E. coli* and have observed changes in cell growth and polyamine levels that provide new information on the functions of polyamine acetylation.

SSAT has been purified to homogeneity from rat liver (Della Ragione & Pegg, 1982), chicken duodenum (Shinki & Suda, 1989), human lung H157 cells (Casero et al., 1990), and human melanoma cells (Libby et al., 1991) following induction with calcitrol, carbon tetrachloride, BESM, and BENSM, respectively. Only minute amounts of protein were obtained from these sources which were limited by either the low specific activity of the starting material or the difficulty of growing large numbers of these mammalian cells.

The availability of recombinant SSAT has now allowed the production of large amounts of the enzyme protein. This was used to investigate the substrate specificity of SSAT. Previous work has shown that SSAT has a striking specificity for acetylation of aminopropyl groups (Della Ragione et al., 1983). In general, all of the compounds which have been found to be acetylated by SSAT have the structure R−NH-$(CH_2)_3NH_2$. These findings raise the possibility that SSAT might also act on some other nonphysiological compounds and drugs that have this structure. We therefore tested a group of such compounds including the antitumor and immunosuppressive agent 15-deoxyspergualin (Muindi et all, 1991; Yu & Morris, 1993), the radioprotective and chemoprotective agents WR-1065 and WR-2721 (Smoluk et al., 1988; Snyder & Schroeder, 1994; Green et al., 1994), and the spermine synthase inhibitor *N*-(*n*-butyl)-1,3-diaminopropane (BDAP)[1] (Baillon et al., 1989) as possible substrates and report here that all of these compounds can be acetylated by SSAT. Such acetylation may be an important metabolic fate of these drugs, and their possible combination with agents inducing SSAT could thus have a marked effect on their duration of action.

MATERIALS AND METHODS

Materials. Protein A bacterial adsorbent and [1-^{14}C]acetyl-CoA (40−55 Ci/mol) were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. Biochemical reagents including isopropyl β-D-thiogalactopyranoside (IPTG)[1] and coenzyme A were purchased from Sigma Chemical Co., St. Louis, MO. DEAE-cellulose was purchased from Whatman Biosystems, Ltd., Maidstone, Kent, England. Cibacron blue−agarose (Affi-Gel) was obtained from Bio-Rad Laboratories, Richmond, CA. Concentrator units were purchased from Amicon, Inc., Beverly, MA. An enhanced chemiluminescence detection kit (ECL no. RPM 2106) was purchased from Amersham International, Little Chalfont, Buckinghamshire, England. Econo-fluor scintillation fluid was purchased from DuPont New England Nuclear, Boston, MA. The antiserum to rat SSAT was prepared in rabbits as previously described (Persson & Pegg, 1984). 1-Methylspermidine was generously synthesized (Lakanen et al., 1992) and provided by Dr. James Coward, University of Michigan, Ann Arbor, MI. Deoxyspergualin was obtained from the Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer treatment, National Cancer Institute, Bethesda, MD, and from Bristol-Myers Squibb, Wallingford, CT. WR-1065 and related compounds were obtained from the Division of Experimental Therapeutics, Walter Reed Army Medical Center, Washington, DC.

Construction of Plasmids Expressing SSAT. The pIN-III-A2 and pIN-III(lpp^{P-5})-A3 *E. coli* expression vectors (Duffaud et al., 1987; Inouye & Inouye, 1985) were obtained from Dr. S. Inouye, Department of Biochemistry, Rutgers University, Piscataway, NJ. The human pSAThl plasmid (Casero et al., 1991) was cut with *Eco*RI and *Hin*dIII, giving a 636 bp fragment which contains all of the coding region of human SSAT. This fragment was then inserted into pIN-III-A2 which had been cut with *Eco*RI and *Hin*dIII. The resulting construct, pINSAT1, was used for some experiments involving SSAT expression, but in order to get a higher level of SSAT synthesis, the promoter was changed to the stronger *lpp*$^{P-5}$ promoter. This was accomplished by cutting pINSAT1 with *Xba*I and *Sal*I and inserting the resulting 1400 bp fragment into the equivalent sites in the pIN-III(lpp^{P-5})-A3 vector to generate pINSAT2. The method of insertion into the vector and the relevant vector sequences should result in the addition of eight amino acids at the amino terminus of the derived SSAT which is changed from M− to MKGKEFPKM−. The M_r of the SSAT subunit is increased from 20 010 to 20 956. The plasmid pINSAT3, which contains only five bases of the 3′ untranslated region from the human SSAT, was constructed by taking the 529 bp fragment liberated by *Eco*RI and *Bam*HI from pEUK-SAT4 (Parry et al., 1995) and using it to replace the insert at these sites in pINSAT2.

The plasmids were transferred to *E. coli* strain DH5α and overnight cultures prepared by inoculating a single colony into 10 mL of Luria−Bertani (LB) media (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.0) containing 50 μg/mL ampicillin. Cells were grown for 12−16 h. The cultures were then diluted into glucose minimal media (M9) (47.7 mM $Na_2HPO_4{\cdot}7H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 2g/L glucose, and 0.2 g/L casamino acids) supplemented with 0.1 mM thiamin and 50 μg/mL ampicillin and grown at 37 °C with vigorous aeration.

Determination of SSAT Activity and Substrate Specificity. Samples were removed from the growing cell cultures and centrifuged at 4000*g* for 10 min at 4 °C. The pellet was washed in ice-cold M9 medium and resuspended at one-quarter of the original volume in 10 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 0.02% Brij, and the cells were disrupted by sonication. Cell debris was removed by centrifugation at 15000*g* for 30 min at 4 °C, and the supernatant was removed and used for assay of SSAT activity either immediately or after storage at −70 °C, at which temperature the activity was stable for several weeks. SSAT activity was measured by incubation at 30 °C in a total volume of 0.1 mL of assay buffer containing 50 mM Tris-HCl, pH 7.8, 3 mM spermidine, and 15 μM [1-^{14}C]-acetyl-CoA (53 μCi/μmol) for 10 min at 30 °C. The reaction was terminated with 20 μL of 1 M hydroxylamine and the formation of [^{14}C]acetylspermidine determined using binding to cellulose phosphate disks (Matsui et al., 1981). Results were expressed as nanomoles of acetylspermidine formed per milligram of protein per minute. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

The substrate specificity of SSAT was determined by replacing the spermidine substrate with the compound being tested and measuring the formation of ^{14}C-labeled derivatives by separating the reaction mixture using high-pressure liquid chromatography (HPLC)[1] with the same column and gradient used for polyamine samples and a radioactivity monitor (Pegg et al., 1990; Seiler, 1983). Where WR-1065 and related compounds were used, the dithiothreitol concentration in the assay medium was increased to 10 mM. The elution times of the acetylated derivatives found to be substrates were 17.3 min for WR-1065, 20.0 min for WR-2721, 22.1 min for WR-44923, 25.4 min for BDAP, 27.5 min for spermidine, 32.5 min for deoxyspergualin, and 36.9 min for spermine.

Immunoprecipitation of SSAT Activity and Western Blotting of SSAT. Cellular lysates were incubated with rotation in a total volume of 0.1 mL with 10% rabbit antiserum to SSAT (Persson & Pegg, 1984) and 1% bovine serum albumin overnight at 4 °C. Protein bound to the antibody was then precipitated by the addition of one-fifth volume of 10% washed protein A bacterial adsorbent, incubation with rotation at 4 °C for 1 h, and centrifugation for 5 min in a microcentrifuge. Portions of the supernatant were removed and assayed for SSAT activity. Preimmune serum was used in control samples in place of the specific antiserum. For immunoblotting of SSAT, samples were separated by PAGE using 12% (w/v) acrylamide in the presence of sodium dodecyl sulfate (Laemmli, 1970). Gels were transferred to a nitrocellulose membrane using a transblot apparatus at 30 V overnight at 4 °C in 192 mM glycine, 25 mM Tris-HCl, pH 8.3, and 20% methanol. Immunodetection was then performed at room temperature using the Amersham enhanced chemiluminescence detection system.

Determination of Polyamines. Samples were removed from the cell cultures and centrifuged at 12000*g* for 5 min. The pellets were washed twice in minimal media and then resuspended in 0.2 N perchloric acid overnight. Samples were then placed in a water bath, sonicated for 30 s, and then centrifuged at 12000*g* for 5 min. The supernatant was removed and used for assay of polyamines using ion-pair, reversed-phase HPLC and postcolumn derivative formation with *o*-phthalaldehyde (Seiler, 1983; Seiler & Knödgen, 1985). The protein was resuspended overnight in an equal volume of 0.1 N NaOH and protein determined (Bradford, 1976). Results were expressed as nanomoles of polyamine per milligram of protein. For the determination of polyamines in media, a portion of medium was treated with one-tenth the volume of 50% perchloric acid overnight. Protein was then removed by centrifugation at 12000*g* for 5 min and the supernatant used for polyamine analysis.

Purification of Recombinant SSAT. Cultures were grown to a density of 2.5 at 37 °C in M9 media supplemented with 0.15 mM IPTG, 20 mM putrescine, and 50 μg/mL ampicillin in a 5-L capacity fermenter (New Brunswick Scientific, Edison, NJ). Cells were collected by centrifugation for 15 min at 4000*g*, washed once in minimal media, resuspended in 10 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, and 0.1 mM EDTA, and broken by pulse sonication for 8 at 4 °C. All subsequent operations were carried out at 4 °C. The extract was centrifuged at 15000*g* for 30 min. The supernatant was taken, and nucleic acids were precipitated by the addition of streptomycin sulfate (10 mg/mL). The pellet was then removed by centrifugation at 15000*g* for 30 min. The supernatant was made 50% in ammonium sulfate and left for at least 1 h and the pellet collected by centrifugation at 15000*g* for 30 min dissolved in buffer A (50 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA, 0.02% Brij 35) and dialyzed against 2 changes of 1 L of the same buffer overnight. The dialyzed solution was loaded onto a DEAE-cellulose column (2.5 cm × 8 cm) and washed with 200 mL of buffer A + 100 mM NaCl at a flow rate of 78 mL/h. The column was then eluted with 1.5 L of buffer A containing 1.5 M NaCl at a flow rate of 78 mL/h. Fractions (10.4 mL) were collected, and those containing SSAT activity were pooled and added to an equal volume of buffer A to reduce the salt concentration to 0.75 M NaCl. This material was then loaded at a flow rate of 50 mL/h onto a column (2.5 cm × 14 cm) of *sym*-norspermidine−Sepharose [prepared as described (Della Ragione & Pegg, 1982)] at a flow rate of 50 mL/h. The column was washed with 400 mL of buffer A containing 0.75 M NaCl. It was then eluted at a flow rate of 20 mL/h with 40 mM *sym*-norspermidine in buffer A containing 0.75 M NaCl. Fractions of 12 mL were collected, and those containing activity were concentrated in a 50-mL concentrator unit using an Amicon YM10 membrane. Five successive cycles of 10-fold dilution with buffer A containing 1 mM spermidine were used to remove the NaCl and *sym*-norspermidine. Further purification was attempted using an Affi-Gel Blue column eluted with coenzyme A (Della Ragione & Pegg, 1982). The purified SSAT protein was also separated on a Spherogel TSK-G3000-SW HPLC size-exclusion column (2.15 cm × 60 cm) (Beckman Instruments, Inc., Fullerton, CA) eluted with 100 mM sodium phosphate, pH 7.5, 0.2 M $(NH_4)_2SO_4$, 1 mM EDTA, and 1 mM dithiothreitol at a flow rate of 6 mL/min.

For the determination of the amino-terminal sequence, the purified SSAT protein was separated by SDS−PAGE, transferred to PVDF membranes (Bio-Rad), and sequenced with an Applied Biosystems 477A protein sequencer (Speicher, 1989).

Table 1: Expression of Human SSAT in *E. coli*[a]

plasmid	time (h)	SSAT activity [nmol min^{-1} (mg of protein)$^{-1}$] −IPTG	SSAT activity [nmol min^{-1} (mg of protein)$^{-1}$] +IPTG	% precipitated by anti-SSAT
none	6	<1	<1	ND
pINSAT1	6	ND	130	ND
pINSAT2	4	80	460	ND
pINSAT2	5	ND	730	99.7
pINSAT2	5 (+polyamines)	ND	680	99.4
pINSAT2	6	100	880	99.5
pINSAT2	6 (+polyamines)	ND	830	99.8
pINSAT2	8	120	900	ND
pINSAT2	9	ND	1000	ND

[a] Cell lysates were prepared from *E. coli* DH5α cells transformed with pINSAT1 or pINSAT2 and grown with no IPTG or 0.15 mM IPTG as indicated. SSAT activity was then determined. Selected samples from the cultures treated with IPTG were immunoprecipitated with anti-rat SSAT antiserum plus protein A and centrifuged, and the percentage of the activity which remained soluble was determined. Some cultures had polyamines (25 mM putrescine and 2.5 mM spermidine) added to the medium. ND, not determined. Results are duplicate estimations from a single experiment. Additional experiments gave similar levels of SSAT induction, but the time course of SSAT induction varied slightly.

RESULTS

Expression of Human SSAT in E. coli. The cDNA coding for the human SSAT was inserted into the pIN-III vector which can be used to express proteins from the lipoprotein (*lpp*) promoter and *lac* promoter operator (Duffaud, 1987). Both pINSAT1, which contains the *lpp* promoter, and pINSAT2, which contains the stronger $lpp^{P\text{-}5}$ promoter, were constructed. These vectors also contain the *lacI* gene so that the *lac* repressor is made and the synthesis of the recombinant protein at maximal levels requires the addition of an inducer such as IPTG. When DH5α cells were transformed with pINSAT1, SSAT was produced but pINSAT2 gave a 4-fold greater level of SSAT expression (Table 1), and this plasmid was used for further experiments. SSAT activity was expressed in large amounts in cells containing pINSAT2, and expression was increased by IPTG (Table 1). On the basis of a specific activity for pure SSAT of about 45 μmol min^{-1} mg^{-1} (see below), the expression of SSAT when cells were harvested close to the onset of the stationary phase of growth amounted to about 0.3% of the extract protein in the absence of IPTG and was induced to about 2% of the protein when 0.15 mM IPTG was added. The addition of putrescine or spermidine to the cell cultures did not influence the expression of SSAT (Table 1).

The observed SSAT activity in *E. coli* transformed with pINSAT2 was due to the mammalian enzyme since all activity was precipitated by reaction with an antiserum to mammalian SSAT (Table 1). A band of M_r about 21 000 was observed in immunoblots of extracts from the cells separated on PAGE under denaturing conditions and developed with the anti-SSAT antiserum (results not shown). This band, which could also be seen by staining of the gels, was absent from extracts derived from DH5α cells transformed with the pIN-III-A3 vector without a SSAT cDNA insert. There was also another fainter band of protein reacting with the anti-SSAT antiserum that had a M_r of about 23 000. The amount of both immunoreactive bands was increased by the addition of IPTG.

Purification of Recombinant SSAT. The SSAT protein was purified from *E. coli* transformed with pINSAT2 62-fold to a specific activity of approximately 45 μmol of spermidine acetylated min^{-1} (mg of protein)$^{-1}$ with a 50% yield. Analysis of the final material by PAGE under denaturing conditions showed that the final preparation contained two bands. The major band amounting to about 80% of the total corresponded to a M_r of about 21 000 and the larger band (ca. 20%) had a M_r of about 23 000. The protein in both bands reacted with antibodies to SSAT on Western blots, and the sizes corresponded exactly to those present in the crude extracts of *E. coli* transformed with pINSAT2. These proteins could not be separated using additional cycles on the *sym*-norspermidine−Sepharose column or by further chromatography on Affi-Gel Blue (Della Ragione & Pegg, 1982). They were also not separated when the SSAT preparation was run on a Spherogel TSK-G3000-SW size-exclusion column on which only one protein peak corresponding to a M_r of 75 000 was observed. This agrees with the published molecular weight of SSAT which is probably a tetramer (Casero & Pegg, 1993).

These results suggest that the larger band is likely to be derived either from an initiation at the vector sequence 5′ to the initiation codon of the SSAT sequence or to a read-through of the termination codon. The latter possibility was excluded since the same two bands were seen when pINSAT3, which has only five bases of the 3′UTR from the SSAT cDNA, was substituted for pINSAT2, which has 108 bases of the 3′UTR. In order to test the possibility of initiation at an earlier site, the two SSAT bands were transferred to nitrocellulose and subjected to protein sequencing. The smaller band gave a sequence of MKGKEF-PKMAKF− in the first 12 cycles, which corresponds exactly to that expected from the pINSAT2 construct. The upper band gave a sequence of MITDSLELQRVLIMKGKE−, showing that it has an extension of 13 amino acids at the amino terminus. This extension is that predicted from the sequence in the pIN vector if the UAG stop codon at position −15 to −13 is read as CAG which gives a glutamine residue. The plasmid DNA sequence has the correct TAG at this position, and it is not clear why this termination codon is so inefficient. The predicted M_rs of the proteins starting from these two sites are 20 956 and 22 468, which are in reasonable agreement with those observed in the purified protein.

The properties of the recombinant human SSAT were similar to those previously published for the enzyme from H157 cells (Casero et al., 1990). The K_m for spermidine was 60 μM. The K_m for spermine was lower (25 μM), but the maximal rate of reaction was also lower, about 40% of that with spermidine as substrate.

Ability of SSAT To Attack Other Substrates. The pure recombinant human SSAT was used to test whether certain drugs which contain aminopropyl groups were substrates for this enzyme. The standard assay for SSAT is based on the procedure introduced by Libby (1978) in which enzyme and the putative amine substrate are incubated with [^{14}C]acetyl-CoA. The reaction mixture is then placed on a cellulose phosphate disk, and radioactivity binding to the disk is assayed. This method gives excellent and quantitative results with spermidine or spermine as substrates since the acetyl derivatives of these polyamines are strong cations and bind tightly to the disk while acetyl-CoA is not retained. How-

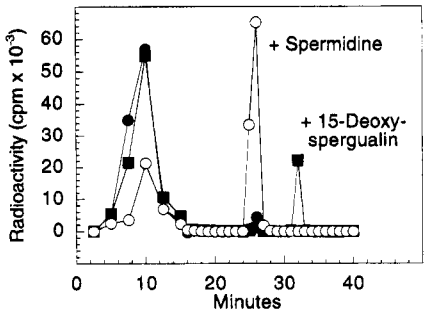

FIGURE 1: Acetylation of deoxyspergualin by recombinant SSAT. Purified SSAT was incubated for 30 min in an solution containing 50 mM Tris-HCl, pH 7.5, 1.5 mM dithiothreitol, 0.05 mM EDTA, 40 nCi of [^{14}C]acetyl-CoA, and either no amine acceptor (filled circles), 1 mM spermidine (open circles), or 1 mM deoxyspergualin (boxes). The products were then separated by HPLC, and radioactivity was determined as shown.

ever, this method is not satisfactory for testing the ability of other compounds to serve as potential substrates if their acetylated derivatives are unlikely to bind tightly to cellulose phosphate. Therefore, these compounds were incubated with SSAT and [^{14}C]acetyl-CoA and the resulting products separated by HPLC using a system in which acetyl-CoA and acetate were eluted within the first 12 min.

15-Deoxyspergualin was clearly a substrate for recombinant human SSAT. When substituted for spermidine in the standard assay, there was a concentration-dependent incorporation of label into material that bound to cellulose phosphate disks (results not shown). When the products of the reaction were run on HPLC, there was a radioactive product peak at 32.5 min that was well separated from the N^1-acetylspermidine peak which eluted at 27 min (Figure 1). The approximate K_m for deoxyspergualin was 12 μM, and the rate of reaction at saturating substrate concentration was about 18% of that of spermidine. BDAP was also acetylated but was only a weak substrate with a K_m almost an order of magnitude higher than that for spermidine and a maximal rate of reaction of only 1.3% that of spermidine. The radioprotective drug, WR-1065, was also acetylated by SSAT at a rate slightly less than 10% of that of spermidine and with K_m 10 times greater. The related compounds WR-2721 and WR-44923 were also acetylated by SSAT at rates similar to that of WR-1065, but WR-2822 was not acetylated. WR-1065 reacted quite readily with acetyl-CoA in the absence of enzyme, but all results were corrected for this nonenzymatic reaction.

Effect of SSAT Expression on Cell Growth and Polyamine Content. The *E. coli* DH5α cells containing pINSAT2 grew more slowly than cells containing a control pIN-III-A3 plasmid without a cDNA insert (Figure 2). There was a 2−2.5-fold increase in doubling time with the slightly greater increase when IPTG was added. The addition of polyamines (putrescine plus spermidine) counteracted this reduction in growth rate (Figure 2A) without affecting the level of expression of SSAT (Table 1). Polyamine addition completely restored the normal growth rate to cultures without IPTG but only partially restored the rate in the presence of IPTG (Figure 2A).

When putrescine and spermidine were added separately to cultures of *E. coli* cells containing pINSAT2, either polyamine was able to increase the growth rate of cultures which lacked IPTG but only putrescine increased growth in

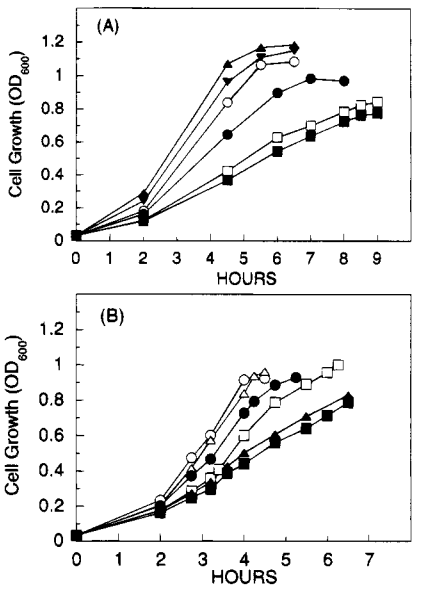

FIGURE 2: Effect of pINSAT2 on growth of *E. coli*. In the experiment shown in panel A, DH5α cells transformed with pIN-III (▲ or ▼) or pINSAT2 (●, ○, ■, or □) were grown in the presence (●, ■, ▲, or ▼) or absence (○ or □) of 0.15 mM IPTG and in the presence (●, ○, or ▲) or absence (■, □, or ▼) of polyamines (25 mM putrescine and 2.5 mM spermidine) as indicated. In the experiment shown in panel B, DH5α cells transformed with pINSAT2 were grown in the presence (●, ■, or ▲) or absence (○, □, or △) of 0.15 mM IPTG and in the presence of 25 mM putrescine (● or ○) or 2.5 mM spermidine (▲ or △) or no polyamine (■ or □) a shown. Results are the mean of three or four separate estimations that agreed within ±10%.

the cultures with IPTG (Figure 2B). Although 25 mM putrescine was used in these experiments, as little as 20 μM gave a significant increase in the growth rate of cells containing pINSAT2 in the presence of IPTG, and 0.2 mM gave a maximal effect (results not shown). In contrast, spermidine had no effect on growth of these cultures at levels from 20 μM to 2.5 mM.

The expression of SSAT in DH5α cells led to the complete loss of spermidine and an increase in N^1-acetylspermidine (Table 2). The loss of spermidine occurred even in cultures where SSAT production was limited by the lack of IPTG. However, when exogenous spermidine was added to cultures lacking IPTG, some free spermidine was present in the cells, but in the cell cultures induced with IPTG, which had much higher levels of SSAT, there was no free spermidine. This suggests that the growth of pINSAT2-transformed cells induced with IPTG can not be restored by spermidine due to the immediate conversion of all the spermidine taken up to its acetylated derivative, while in noninduced cultures, the small pool of free spermidine can stimulate growth. In order to test this hypothesis, the effect of the addition of 1-methylspermidine, which is not a substrate for SSAT (Lakanen et al., 1992), was examined. The presence of 0.1−1 mM 1-methylspermidine did stimulate the growth of cells containing pINSAT2 even in the presence of IPTG (results not shown), and there was a substantial pool of 1-methylspermidine in these cells (Table 2).

The addition of exogenous putrescine to the cells expressing SSAT raised intracellular putrescine (Table 2). No acetylated putrescine was found. This is consistent with the known specificity of SSAT which does not attack putrescine (Matsui et al., 1981; Della Ragione et al., 1983). The partial

Table 2: Effect of SSAT Expression and Polyamine Addition on Polyamine Levels in *E. coli*[a]

plasmid and addition	polyamines (nmol/mg of protein)				
	putrescine	spermidine	N^1-acetylspermidine	N^8-acetylspermidine	1-methylspermidine
pIN-III + IPTG	93 ± 2	26 ± 3	4.2 ± 0.4	1.6 ± 0.2	
pIN-III + IPTG + putrescine	131 ± 16	24 ± 2	2.9 ± 0.5	1.4 ± 0.1	
pIN-III + IPTG + spermidine	71 ± 4	30 ± 2	9.9 ± 2.3	3.8 ± 0.4	
pIN-III + IPTG + 1-methylspermidine	63 ± 6	15 ± 2	2.8 ± 0.8	1.1 ± 0.1	50 ± 10
pINSAT2 + IPTG	112 ± 17	<0.3	31 ± 6	<0.3	
pINSAT2 + IPTG + putrescine	211 ± 10	<0.3	30 ± 7	<0.3	
pINSAT2 + IPTG + spermidine	85 ± 5	<0.3	64 ± 15	2.2 ± 0.5	
pINSAT2 + IPTG + 1-methylspermidine	77 ± 7	<0.3	20 ± 3.5	<0.3	26 ± 2
pINSAT2	120 ± 16	1.4 ± 0.3	25 ± 3	<0.3	
pINSAT2 + putrescine	194 ± 38	1.7 ± 0.5	24 ± 5	<0.3	
pINSAT2 + spermidine	79 ± 4	3.4 ± 0.4	51 ± 8	<0.3	

[a] DH5α cells transformed with pIN-III or pINSAT2 were grown in the presence or absence of 0.15 mM IPTG, 2.5 mM spermidine, 1 mM 1-methylspermidine, or 2.5 mM putrescine as shown for 4 h. Cells were harvested, and intracellular polyamine content was then measured. Results are shown as the mean ± SD for three cultures.

Table 3: Effect of BESM and pINSAT2 on Polyamine Content and SSAT Activity[a]

plasmid	BESM	polyamines (nmol/mg of protein)				SSAT activity [nmol min^{-1} (mg of protein)$^{-1}$]
		putrescine	spermidine	N^1-acetylspermidine	BESM	
pIN-III	0	85 ± 17	31 ± 3	2.5 ± 0.5		14 ± 3
pIN-III	0.1	118 ± 8	21 ± 2	3.3 ± 0.3[b]	7 ± 1	8 ± 2
pIN-III	1.0	110 ± 12	24 ± 2	4.8 ± 1.2[b]	72 ± 2	6 ± 1
pINSAT2	0	102 ± 13	<0.3	38 ± 7		773 ± 7
pINSAT2	0.1	139 ± 24	<0.3	32 ± 4	35 ± 2	1355 ± 38
pINSAT2	1.0	129 ± 11	<0.3	28 ± 5	167 ± 19	1068 ± 90

[a] DH5α cells transformed wtih pIN-II or PINSAT2 as indicated were grown in the presence of 0.15 mM IPTG and the BESM concentration shown. Cells were harvested at an approximate OD_{595} of 0.6 (except for the group with pINSAT2 and 1 mM BESM, which only reached 0.45). SSAT levels and intracellular polyamine content were then measured. Results are shown as the mean ± SD for three cultures. [b] N^8-Acetylspermidine was also present in these samples at levels of 3.1 and 4.9 nmol/mg of protein, respectively. No other samples had detectable levels of N^8-acetylspermidine.

reversal of growth inhibition due to SSAT induction brought about by putrescine, therefore, seems to be due to the increase in putrescine levels, which at very high concentrations can substitute for spermidine.

Substantial amounts of N^1-acetylspermidine were present in the media of cultures of cells expressing SSAT (Figure 3A). This production was increased even more when spermidine was added to the medium, with levels reaching almost 0.5 mM after 6 h. Control *E. coli* cultures lacking the human SSAT did not excrete N^1-acetylspermidine unless spermidine was added, and even then, the amount was less than 20% of that found in the cells producing SSAT (Figure 3A). N^8-Acetylspermidine was excreted only in the cultures supplemented with spermidine, and its production was reduced by the expression of SSAT presumably because most of the spermidine is converted to N^1-acetylspermidine by SSAT (Figure 3B). Thus, it appears that much of the acetylated spermidine produced within the cell is excreted into the medium.

Effects of SSAT Expression on Sensitivity of Cell Growth to BESM. BESM is a potent inducer of SSAT in human tumor cells and is very strongly inhibitory to their growth (Casero & Pegg, 1993; Casero et al., 1989; Shappell et al., 1992). Addition of BESM had only a small effect on the content of SSAT in *E. coli* DH5α cells containing pINSAT2, increasing the level by less than 2-fold (Table 3), but BESM was very strongly inhibitory to the growth of these cells and had much less effect on the growth of cells containing the control pIN-III-A3 plasmid (Figure 4). Putrescine levels were slightly increased in the cells expressing SSAT, but

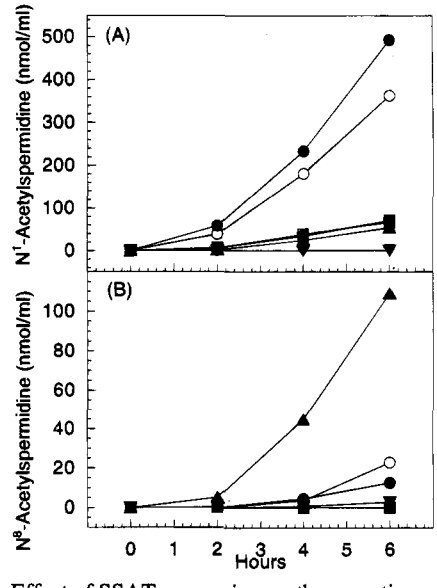

FIGURE 3: Effect of SSAT expression on the excretion of acetylated spermidine by *E. coli*. DH5α cells transformed with pIN-III (▲, ▼, or △) or PINSAT2 (●, ○, ■, or □) were grown in the presence (▲, ▼, ●, or ■) or absence (△, ○, or □) of 0.15 mM IPTG and in the absence (△, ▼, □, or ■) or presence (△, ▲, ○, or ●) of 2.5 mM spermidine, for the times shown. The content of polyamines in the medium was then measured. Results are shown for N^1-acetylspermidine (panel A) and N^8-acetylspermidine (panel B) as the mean for three cultures which agreed within ±10%.

spermidine content was completely depleted. These results provide strong evidence that the conversion of spermidine to its N^1-acetyl derivative renders cells more sensitive to

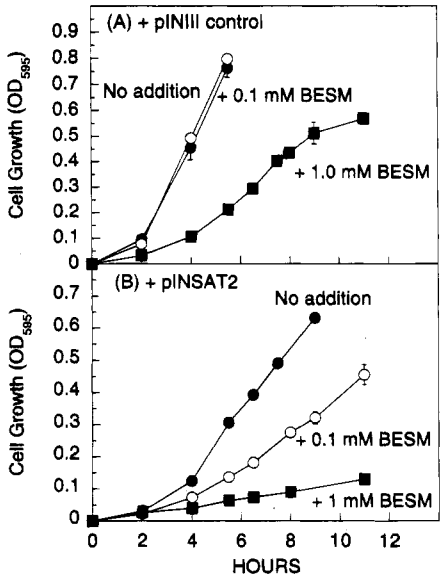

FIGURE 4: Effect of SSAT expression on inhibition of cell growth by BESM. DH5α cells transformed with pIN-III (panel A) or PINSAT2 (panel B) were grown in the presence of 0.15 mM IPTG and in the presence of 0.1 mM (○), 1 mM (■), or without BESM (●) for the times indicated. Results are shown ±SD for three to four estimations.

BESM. Part of this effect may be due to the increased uptake/retention of BESM by the cells when SSAT is present (Table 3).

DISCUSSION

The role of SSAT in cellular physiology has been difficult to study because all of the known inducers are either polyamines or analogs or substances/conditions that perturb cellular polyamine pools (Casero & Pegg, 1993). It has therefore been impossible to design experiments that test the extent to which changes solely in SSAT levels alter polyamine levels and cell growth. Attempts to express SSAT in mammalian cells by inserting the cDNA into appropriate expression vectors have failed because the mRNA produced from these vectors is not translated unless polyamines or analogs are used to alter intracellular polyamine levels or distribution (Parry et al., 1995). This regulation mechanism may involve specific eukaryotic proteins interacting with the SSAT protein or mRNA since the present results show that it does not occur in *E. coli*. There was only a small (at most 2-fold) increase in SSAT levels in cells treated with BESM, which gives a huge induction of SSAT in mammalian cells (Casero & Pegg, 1993; Casero et al., 1989; Pegg et al., 1990; Libby et al., 1989; Shappell et al., 1992). This small increase may be due to a stabilization of the SSAT protein (Pegg et al., 1990). Human SSAT was expressed well in *E. coli* from the pIN-III(lpp^{P-5}) vector. The protein was present at a level of about 2% of the soluble protein, which is comparable to the level of expression of other enzymes such as ornithine decarboxylase (Poulin et al., 1992) and *S*-adenosylmethionine decarboxylase (Shantz et al., 1992) from this vector.

However, in contrast to the expression of these decarboxylases which had no effect on growth of the host *E. coli*, expression of SSAT led to a significant reduction in the cell growth rate. This reduction is likely to be due to the action of SSAT on intracellular spermidine since addition of polyamines overcame the inhibition. When SSAT induction was limited by the absence of the inducer IPTG, either spermidine or putrescine could improve growth, but when SSAT levels were maximal, only putrescine was effective. This finding can be explained by the observations that all of the spermidine taken up by the cells was acetylated by the very high levels of SSAT. When 1-methylspermidine, which is not a substrate for SSAT (Lakanen et al., 1992), was used instead of spermidine, growth was increased.

Our results, therefore, indicate that N^1-acetylspermidine cannot support a maximal growth rate of *E. coli* but that either spermidine or putrescine (albeit at much higher levels) is sufficient. These conclusions are in agreement with results obtained with *E. coli* mutants defective in polyamine synthesis. Such mutants when maintained in culture conditions so that they contain no detectable polyamines are able to grow at a reduced rate of about 30% that seen in the presence of polyamines (Tabor & Tabor, 1985). Mutants unable to synthesize spermidine from putrescine grow at a rate of 75−100% of the wild type (Tabor et al., 1978; Xie et al., 1993). Although *E. coli* can grow in the absence of polyamines, the cells are more sensitive to a number of conditions including oxidative stress (Minton et al., 1990), indicating that this process requires polyamines. The expression of SSAT may be a useful way to study such polyamine functions in *E. coli* since it is difficult to remove all polyamines from the culture medium and SSAT induction could be used to rapidly remove intracellular spermidine and/or prevent its accumulation. The pIN-III vector/DH5α host cell system as used in our experiments is not ideal for this purpose owing to the leakiness of the repression of expression, but the use of other bacterial strains expressing the *lac* repressor to high levels and greater care to ensure the absence of all *lac* inducers from the medium would allow for a more completely inducible expression system.

The content of acetylated polyamines in *E. coli* is normally low but can be increased rapidly by exposure to stress from cold, heat, alkali, or toxic chemicals (Tabor, 1968; Tabor & Dobbs, 1970; Carper et al., 1991). An enzyme acetylating polyamines has been found in *E. coli* extracts (Carper et al., 1991; Matsui et al., 1982; Fukuchi et al., 1994). It appears that acetylation occurs under conditions unfavorable to growth and provides a means of reducing the active polyamine concentration. Both N^1-acetylspermidine and N^8-acetylspermidine are formed by the *E. coli* acetylase (Tabor & Dobbs, 1970; Tabor & Tabor, 1985; Carper et al., 1991; Fukuchi et al., 1994; Table 2 and Figure 3) whereas the SSAT is absolutely specific for the formation of N^1-acetylspermidine (Della Ragione & Pegg, 1984; Casero & Pegg, 1993). The formation of acetylated derivatives occurs when *E. coli* is grown in the presence of high concentrations of exogenous spermidine (Tabor & Dobbs, 1970; Carper et al., 1991; Kakegawa et al., 1991; Figure 3). Acetylated polyamines did not stimulate growth of polyamine-requiring mutants or replace polyamines in stimulation of protein synthesis and binding to RNA (Kakegawa et al., 1991). In mammalian cells, N^1-acetylspermidine and N^1-acetylspermine are both excreted and degraded by polyamine oxidase (Casero & Pegg, 1993; Kakegawa et al., 1991), but this enzyme has not been observed in *E. coli*. There was significant excretion of acetylated polyamines in *E. coli* expressing SSAT particularly when supplemented with exogenous spermidine (Figure 4). It appears that excess polyamines are converted to the physiologically inert acetylated form and either excreted or retained as the inert form

until deacetylated. The accumulation of acetylated polyamines may aid in the responses to stress by reducing the growth rate or in other ways. The DH5α *E. coli* expressing SSAT were not more resistant to stress by cold shock or by exposure to alkali (L. Wiest and A. E. Pegg, unpublished observations), but this experiment is not conclusive since the DH5α strain contains the endogenous acetylase and acetylated polyamines accumulate in stressed cells even in the absence of SSAT expression. An experiment in which the SSAT expression plasmid was used in an *E. coli* strain described by Carper et al. (1991) that does not produce acetylated polyamines would be more informative.

It has been suggested that the very high level of induction of SSAT in mammalian cells by BESM and related compounds is related to the inhibition of growth by these polyamine analogs. This is supported by a correlation between the extent of SSAT induction and growth inhibition in a series of tumor cell lines (Casero & Pegg, 1993; Casero et al., 1989; Saab et al., 1993; Shappell et al., 1993; Libby et al., 1989; Della Ragione & Pegg, 1982; Shinki & Suda, 1989). However, other experiments suggest that the analogs are themselves antiproliferative, possibly by virtue of their abilities to interact with DNA (Basu et al., 1989, 1993; Albanese et al., 1993; Fukuchi et al., 1992). Our results suggest that both suggestions may be correct. The inhibition of growth of *E. coli* by BESM was greatly increased by the expression of SSAT. This finding provides the first demonstration that high levels of SSAT render cells sensitive to BESM. It is likely that the conversion of spermidine to its N^1-acetyl derivative permits the increased uptake/retention of BESM and allows its binding to intracellular sites needed for its antiproliferative action. Thus, the ability to induce SSAT may play a significant factor in the cellular response to bis(ethyl) polyamines by acetylating intracellular polyamines and altering the cellular environment so that the analogs are more inhibitory to growth.

SSAT was readily purified from cultures of *E. coli* DH5α containing pINSAT2. Using affinity chromatography on *sym*-norspermidine−Sepharose as the major purification step, apparently pure material was obtained with a 62-fold purification in 54% yield. This provides an excellent system for the isolation of large amounts of SSAT for the production of antibodies and investigation of structure/function relationships. The final specific activity of the recombinant SSAT was similar to that reported for the human protein isolated from H157 cells (Casero et al., 1990), and the K_m values for spermidine and spermine agree with those reported earlier. This suggests that the small extension at the amino terminus produced by the cloning method does not affect the activity. It also is consistent with the lack of any essential posttranslational modifications of the SSAT needed for activity. This agrees with studies in mammalian cells showing that changes in SSAT activity are brought about by alterations in the amount of immunoreactive enzyme protein rather than by changes in the catalytic effectiveness of the protein (Casero & Pegg, 1993; Persson & Pegg, 1984; Pegg & Erwin, 1985; Erwin & Pegg, 1986).

Results with the recombinant SSAT confirm and extend previous work showing the specificity of this enzyme toward acetylation of the primary amino group of structures R−NH-$(CH_2)_3NH_2$ (Della Ragione & Pegg, 1984; Della Ragione et al., 1983). Our finding that 15-deoxyspergualin and BDAP are substrates shows that, for the human SSAT, R− can be either quite large or a small hydrophobic group. The acetylation of 15-deoxyspergualin by SSAT is consistent with the results on the disposal of this drug in human cancer patients. The major metabolite obtained after treatment with deoxyspergualin was des(aminopropyl)deoxyspergualin (Muindi et al., 1991). The removal of the aminopropyl group would be consistent with the acetylation by SSAT followed by the oxidative cleavage by polyamine oxidase removing the acetylated aminopropyl group. In preliminary experiments with the rat SSAT, we were unable to find any evidence for acetylation of deoxyspergualin (L. Wiest and A. E. Pegg, unpublished observations). This suggests that there may be a significant species-dependent difference in the substrate specificity of SSATs from mammalian sources. This is quite surprising because the rat and the human enzymes are quite similar in all other properties that have been compard (Della Ragione & Pegg, 1982; Casero et al., 1990), and the human, mouse, and hamster SSAT are very similar with only eight amino acid differences (Casero & Pegg, 1993).

The acetylation of WR-1065 and related compounds also conforms to the substrate specificity. WR-1065, WR-2721, and WR-44923, which all contain an aminopropyl group, were acetylated by SSAT, but WR-2822, which has an aminoethyl group, in this position was not. WR-1065 has been shown to be a powerful protective agent against the toxicity of both radiation and certain chemotherapeutic agents (Snyder & Schroeder, 1994; Green et al., 1994). It is responsible for the protective activity of WR-2721 which is rapidly converted to it by the action of phosphatases (Smoluk et al., 1988). Our results show that both compounds are likely to be acetylated by SSAT, but since WR-2721 has a very short half-life, all of the acetylation is likely to occur via WR-1065. The acetylated derivative is then likely to be degraded by the SAT/polyamine oxidase system. WR-1065 was found to be a substrate for polyamine oxidase under unphysiological conditions *in vitro* (Gaugas, 1982), but this enzyme requires acetylation of the substrate before acting on most substrates under physiological conditions (Casero & Pegg, 1993; Seiler, 1987; Morgan, 1986). Since SSAT activity is normally very low, the extent of metabolism of 15-deoxyspergualin and WR-1065 may be quite limited. However, the wide variety of drugs and other conditions that lead to SSAT induction (Casero & Pegg, 1993) suggest that metabolism might be altered substantially by the combination with a treatment inducing SSAT. It is also possible that 15-deoxyspergualin and WR-1065 might themselves act as inducers of SSAT as well as substrates.

ACKNOWLEDGMENT

We thank Dr. J. K. Coward for the gift of 1-methylspermidine and Dr. R. Bergeron for the N^1,N^{12}-bis(ethyl)-spermine. We are most grateful to Dr. R. A. Casero for many helpful discussions and for the measurement of BESM levels.

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BI942523N