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Induction of spermidine/spermine N^1-acetyltransferase activity in Chinese-hamster ovary cells by N^1N^{11}-bis(ethyl)norspermidine and related compounds

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Treatment of Chinese-hamster ovary (CHO) cells with N^1N^{11}-bis(ethyl)norspermine (BENSM) led to a very large increase in the activity of spermidine/spermine N^1-acetyltransferase (SAT), which rose by about 600-fold within 48 h. Smaller, but still very large increases, were also produced in decreasing order of potency by 3,7,11,15,19-penta-azaheneicosane, N^1N^{12}-bis(ethyl)spermine and by N^1N^{14}-bis(ethyl)homospermine. The rise in acetyltransferase activity was due to an increase in enzyme protein, as indicated by immunoblotting using antibodies directed against rat liver SAT. There was an increase in the content of mRNA for SAT, indicating that BENSM regulates the level of enzyme protein partly by means of a change in transcription or stability of the mRNA. There was also a decreased rate of degradation of the protein in CHO cells treated with the drug. This may be due to the binding of BENSM, which is a competitive inhibitor of the enzyme with a K_i of 120 μM. Exposure to BENSM led to an increased conversion of spermidine into N^1-acetylspermidine and putrescine, a rapid fall in the content of intracellular polyamines and the excretion from the cell of putrescine, N^1-acetylspermidine and spermidine. When polyamine oxidase activity in the treated cells was blocked, increases in N^1-acetylspermidine and N^1-acetylspermine were much greater, and the formation of putrescine was prevented. These results indicate that the induction of SAT facilities the degradation of spermine and spermidine to putrescine and the subsequent excretion of putrescine from the cell. When the degradation of the N^1-acetyl derivatives by polyamine oxidase is blocked, the cells excrete N^1-acetylspermidine instead of putrescine. CHO cells also contained and excreted N^8-acetylspermidine, but its synthesis was not increased in cells treated with BENSM, confirming data obtained *in vitro* that SAT does not produce this derivative.

INTRODUCTION

Spermidine/spermine N^1-acetyltransferase (SAT) is a soluble cytosolic enzyme which may play an important role in the regulation of cellular polyamine concentrations. SAT activity in most mammalian cells is very low under normal conditions, but can be increased by exposure to toxic agents, hormones or polyamines (reviewed by Della Ragione & Pegg, 1984; Pegg, 1986; Seiler, 1987). The increased level of SAT may lead to the conversion of spermine and spermidine into putrescine, since the N^1-acetylated polyamines produced by SAT are the physiological substrates for polyamine oxidase (Seiler, 1987). It has been suggested that the induction of SAT provides a cellular mechanism to decrease excess intracellular polyamine concentrations (Pegg, 1986).

The biochemical mechanism responsible for changes in SAT activity is not well understood, and has been difficult to approach because of the very small amount of SAT present in most mammalian cells even after stimulation (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982; Persson and Pegg, 1984). SAT is induced many-fold in rodent liver in response to hepatotoxins such as CCl_4, but the absolute level of the protein is still low, amounting to only about 1 part in 10^5 of the soluble protein (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982; Persson & Pegg, 1984). Similarly, although vast increases in SAT activity occur in rat liver and in L6 myoblasts in response to exogenous polyamines or methylglyoxal bis(guanylhydrazone) (MGBG), the absolute amount of SAT even at the peak of induction represents less than 0.01% of the protein (Pegg *et al.*, 1985; Pegg & Erwin, 1985; Erwin & Pegg, 1986).

Recently, it has been reported that a number of polyamine analogues act as potent enhancers of SAT activity in rodent and human cells (Erwin & Pegg, 1986; Casero *et al.*, 1989*b*; Libby *et al.*, 1989; Pegg *et al.*, 1989). In particular, the bis(ethyl) derivatives of spermine and of sym-norspermine were especially active in this respect. In the present work we have studied the increase in SAT by N^1N^{11}-bis(ethyl)norspermine (BENSM) and related compounds in Chinese-hamster ovary (CHO) cells. A remarkably high SAT activity was induced by BENSM in these cells, amounting to more than a 500-fold increase in activity, and the induced protein was easily detectable on Western blots. This system provides a particularly useful model to study the changes in SAT activity and the effects of a high SAT content on polyamine metabolism.

MATERIALS AND METHODS

Materials

BESM, BEHSM, BENSM and BEPH were synthesized by published methods (Bergeron *et al.*, 1989). [1-^{14}C]Acetyl-CoA (55 mCi/mmol) was purchased from ICN Radiochemicals, Irvine, CA, U.S.A. [2,3-^{3}H]Putrescine (47.5 Ci/mmol) was obtained from DuPont–New England Nuclear, Boston, MA, U.S.A.

Abbreviations used: SAT, spermidine/spermine N^1-acetyltransferase; BENSM, N^1N^{11}-bis(ethyl)norspermine (3,7,11,15-tetra-azaheptadecane); BESM, N^1N^{12}-bis(ethyl)spermine (3,7,12,16-tetra-azaoctadecane); BEHSM, N^1N^{14}-bis(ethyl)homospermine (3,8,13,18-tetra-azaeicosane); BEPH, 3,7,11,15,19-penta-azaheneicosane, MDL 72521, N^1-methyl-N^2-(2,3-butadienyl)butane-1,4-diamine; CHO, Chinese-hamster ovary.

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MDL 72521 was generously given by Merrell Dow Research Institute, Strasbourg, France. Other biochemical reagents were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Immunoblotting using the horseradish peroxidase method and 4-chloro-1-naphthol was carried out with reagents from Bio-Rad Laboratories, Richmond, CA, U.S.A. Reagents for mRNA purification were obtained from Pharmacia Molecular Biology Division, Piscataway, NJ, U.S.A.

Cell culture

Chinese-hamster ovary CHO (Pro^-) line cells were grown as monolayers in α-MEM (Gibco) with 10% (v/v) fetal-bovine serum, 16 mM-$NaHCO_3$, 12 μg of penicillin/ml and 12 μg of streptomycin/ml at 37 °C in 5% CO_2 in air, at 93% humidity. When exogenous polyamines were added, the medium was supplemented with 1 mM-aminoguanidine to prevent degradation by bovine serum oxidases. The cells were harvested with a buffered 0.25% (w/v) trypsin solution. Cell growth was determined by counting the number of viable cells (determined by dye exclusion) with an electronic cell counter.

Assay of SAT activity, protein and mRNA

The effects of bis(ethyl) polyamine derivatives on SAT activity were determined by adding the derivatives at concentrations of 5–50 μM to the culture medium at either 4 or 24 h after plating as indicated in the legends. The cells were then harvested at various times later, and extracts were prepared by two cycles of freeze–thawing in 50 mM-Tris/HCl (pH 7.5)/2.5 mM-dithiothreitrol/0.1 mM-EDTA. The half-life of SAT was determined after addition of 0.2 mM-cycloheximide to block protein synthesis, and the dishes were harvested at 1 h intervals for up to 4 h.

SAT activity was measured by measuring the conversion of [1-^{14}]acetyl-CoA into [^{14}C]acetylspermidine in an assay medium containing 100 mM-Tris/HCl, pH 7.8, 8 μM-[1-^{14}C]acetyl-CoA and 3 mM-spermidine (Matsui *et al.*, 1981). A unit of SAT activity is defined as the amount of activity needed to produce 1 nmol of product in 10 min at 30 °C. Inhibition of SAT activity by bis(ethyl)polyamine derivatives was investigated by using purified rat liver SAT as described by Della Ragione & Pegg (1983). Immunoprecipitation and immunoblotting of SAT were carried out with a rabbit antiserum as previously described (Persson & Pegg, 1984). Purified rat liver SAT was used as marker protein. Synthesis of SAT protein by translation of mRNA from CHO cells was carried out essentially as described by Kameji & Pegg (1987), by using rabbit reticulocyte lysates which were not treated to remove endogenous polyamines and poly(A)-containing RNA isolated from the CHO cells. The protein corresponding to SAT was precipitated by using anti-SAT antiserum, separated by SDS/PAGE and detected by fluorography (Kameji & Pegg, 1987). The RNA was isolated by the guanidinium thiocyanate procedure (Okayama *et al.*, 1989), followed by passage through columns of poly(dT)–cellulose.

Polyamine analysis

Cell pellets were harvested, extracted and deproteinized with 10% (w/v) trichloroacetic acid, and samples of the supernatant used for determination of polyamine content by ion-pair reversed-phase h.p.l.c. separation and post-column derivative formation with *o*-phthalaldehyde (Seiler & Knödgen, 1985). Media samples were processed by the addition of 50% trichloroacetic acid to give a final concentration of 10%, the protein pellet was removed by centrifugation (15000 ***g*** for 10 min), and the supernatant was filtered through 0.22 μm syringe-type filters, extracted with 3 × 10 vol. of ether, freeze-dried, redissolved, and the polyamine content was determined by h.p.l.c. Polyamine levels were expressed as nmol/mg of protein or as nmol per culture. Protein was determined by the method of Bradford (1976). In some experiments, cells were exposed to 20 μM-MDL 72521 for 24 h before the start of the labelling and throughout the experimental period to inhibit intracellular polyamine oxidase.

The metabolism and excretion of labelled polyamines was determined as follows. CHO-cell cultures were grown for 4 h, and then 0.5 μCi of [2,3-^{3}H]putrescine (47.5 Ci/mmol)/ml was added in the presence of 1 mM-aminoguanidine and the cells were grown for a further 20 h to label the internal polyamine pools. The cells were then washed three times in phosphate-buffered saline (116 mM-NaCl/6.3 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.2) to remove the exogenous [2,3-^{3}H]putrescine, and fresh medium containing no drugs or 25 μM-BESM or -BENSM was added. Samples of cells and medium were harvested at various times later and prepared for h.p.l.c. as described above. The eluate from the h.p.l.c. was monitored for radioactivity with a Radiomatic FLO-ONE/Beta A-100 radioactivity detector (Radiomatic Instruments, Tampa, FL, U.S.A.). Control experiments in which each of the polyamines and acetyl derivatives were added to the culture medium and incubated for 48 h in the absence of cells indicated that the recovery of these compounds from the medium was better than 80% and that there was no significant degradation of the polyamines.

RESULTS

Exposure of CHO cells to the bis(ethyl) polyamines produced a rapid and large increase in the capacity to acetylate spermidine (Table 1). This increase was readily apparent at 24 h after adding the drugs, but a maximal increase required exposure for 72–96 h. All four of the bis(ethyl) derivatives tested produced a large increase in the activity, but BENSM and BEPH were most active, followed by BESM and BEHSM.

In order to determine to what extent the rise in the capacity to acetylate spermidine was due to an increase in SAT activity, samples of extracts from control and BENSM-treated CHO cells were treated with control antiserum and with antiserum to SAT (Table 2). The loss in acetylase capacity produced by the antiserum to SAT was more than 95% in the samples from the BENSM-treated cells, indicating that the great majority, if not all, of the increased activity is due to SAT. In contrast, the sensitivity of the acetylase activity in the control cells to anti-SAT antiserum varied between 15% and 66%. The SAT activity in each of the samples was calculated by subtracting the activity found after treatment with the antiserum to SAT from that found when a control antiserum was used. Treatment with BENSM produced a very large induction of SAT, rising from a 51-fold increase at 24 h at 632-fold at 48 h and 834-fold at 72 h (Table 2). The SAT activity in the control untreated CHO cells showed a peak at 24 h and then declined.

The increase in SAT activity brought about by the bis(ethyl) spermine derivatives appears to be due to an increased accumulation of the enzyme protein. Western blots of extracts of cells treated with these compounds developed with antisera to SAT showed a strong band corresponding to a protein of approx. 18000 M_r which was not detected in the control cell extracts. This band was coincident with a marker of purified rat liver SAT (Fig. 1). Since the SAT band could not be detected in the control cells, the extent of increase in the protein could not be ascertained, but at least a 50-fold increase in protein was produced within 48 h of treatment.

Experiments in which cycloheximide was added to CHO cells at the same time as BENSM showed that the rise in acetylase activity which was produced 6–9 h later was completely dependent on continuing protein synthesis (Table 3). Treatment with actinomycin D to block RNA synthesis decreased the

Table 1. Stimulation of SAT activity in CHO cells by bis(ethyl) polyamine derivatives

The cells were grown for 24 h before addition of the compound shown, except for the 96 h time points, where the compounds were added 4 h after plating. Extracts were prepared at the times indicated and the acetylase activity was measured. Results are shown as means ± S.D. for at least four measurements; N.D., not determined.

Treatment	Acetylase activity (units/mg) measured at time indicated			
	24 h	48 h	72 h	96 h
Control	1.1±0.1	0.5±0.1	0.4±0.1	0.2±0.1
10 μM-BENSM	51.6±1.6	88.0±1.8	84.9±11.5	N.D.
25 μM-BENSM	56.2±5.6	88.9±4.9	108.9±10.0	182.6±11.7
10 μM-BESM	14.5±1.6	35.1±3.2	52.9±2.3	N.D.
25 μM-BESM	15.7±1.0	40.6±2.1	85.5±7.1	121.1±26.8
10 μM-BEHSM	6.4±0.7	16.3±0.5	26.8±1.3	N.D.
25 μM-BEHSM	6.6±0.6	16.7±1.5	31.2±3.2	58.8±11.1
10 μM-BEPH	N.D.	76.2±4.3	83.4±9.3	N.D.
25 μM-BEPH	N.D.	81.8±5.6	111.5±15.6	N.D.

Table 2. Immunoprecipitation of acetylase activity in CHO-cell extracts by antiserum to SAT

The extracts were prepared and 100 μl samples were incubated at 4 °C for 15 h in the presence of 3 ml of the serum indicated, 1% bovine serum albumin, 0.02% Brij 35 and 1 mM-spermidine in a total volume of 110 μl. Protein A adsorbent (50 μl) was then added and the mixture incubated with shaking for 90 min at 4 °C. The precipitate was removed by centrifugation at 15000 *g* for 3 min, and the acetylase activity present in the supernatant was measured. The SAT activity was calculated by subtracting the activity found after treatment with anti-SAT serum from that found in extracts treated in the same way with normal rabbit serum. The results were not corrected for the small extent of loss of activity in response to overnight incubation with the normal rabbit serum, which is indicated by the difference between the first two columns of values.

Treatment	Acetylase activity (units/mg)			
	No serum treatment	Control-serum treated	Anti-SAT-serum treated	SAT activity
Control, 0 h	0.31	0.34	0.29	0.05
Control, 24 h	1.27	0.97	0.33	0.64
Control, 48 h	0.36	0.33	0.24	0.09
Control, 72 h	0.32	0.31	0.23	0.09
BENSM, 24 h	38.1	34.2	1.3	32.9
BENSM, 48 h	69.1	58.4	1.5	56.9
BENSM, 72 h	80.8	76.2	1.1	75.1

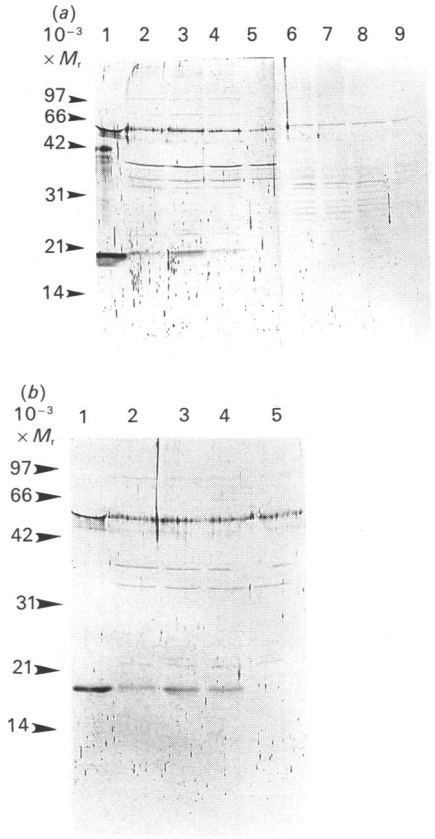

Fig. 1. Immunoblotting, using rabbit anti-SAT, of extracts from CHO cells treated with bis(ethyl)spermine derivatives

Extracts were prepared from cells exposed to the compounds for 48 h, separated by SDS/PAGE, and the proteins transferred to nitrocellulose membranes and subjected to immunoblotting with normal rabbit serum or with rabbit anti-SAT serum. In panel (*a*), lanes 1–5 were developed with rabbit anti-SAT serum and lanes 6–9 with control serum. Lane 1 shows results for purified rat SAT (190 units), lanes 2 and 6 for BEHSM-treated cells (84 units), lanes 3 and 6 for BENSM-treated cells (138 units), lanes 4 and 8 for BESM-treated cells (115 units) and lanes 5 and 9 for control cells (0.6 unit). In panel (*b*) all lanes were developed with a different rabbit anti-SAT serum. Lane 1 shows results for purified rat SAT (190 units), lane 2 for BEHSM-treated cells (84 units), lane 3 for BENSM-treated cells (138 units), lane 4 for BESM-treated cells (115 units) and lane 5 for control cells (0.6 unit). In both panels, all lanes except lane 1 were loaded with 135 μg of cell protein. The bands corresponding to an M_r of about 60000 were seen with all sera tested which came from rabbits immunized with Freund's adjuvant, including those raised to several other proteins (results not shown), and may represent a reaction with a bacterial protein present in one of the reagent solutions.

increase by 57% at 6 h and by 66% at 9 h, suggesting that increased accumulation of the mRNA for SAT contributes to the induction, but is not responsible for the entire increase. These experiments with inhibitors of protein and RNA synthesis could only be carried out over a short time period because of the general toxic effects of the inhibitors.

The content of mRNA for SAT was investigated directly by the isolation of poly(A)-containing RNA from the treated cells, followed by its translation in a reticulocyte lysate in the presence of [^{35}S]methionine. The radioactive SAT was then isolated by immunoprecipitation, followed by SDS/PAGE. As shown in Fig. 2, a protein band of M_r about 18000 was found after translation of mRNA from cells treated with BENSM, and this band was more prominent in extracts from such cells than from controls. Accurate quantification of the extent of increase was not possible, because the synthesis of SAT in response to mRNA from control cells was very small, but scanning with a densitometer indicated that at least a 10-fold increase had occurred. Even allowing for the imprecision in this measurement, it does not appear that all of the increase in SAT can be accounted for by an increase in mRNA, particularly since the SAT activity increased substantially between 48 and 96 h, whereas the mRNA content did not (results not shown).

Another possible mechanism by which the SAT content might be increased is via the stabilization of the protein, since SAT normally turns over very rapidly (Matsui & Pegg, 1980; Pegg, 1986). The decline in acetylase activity was therefore measured after protein synthesis was blocked by exposure to cycloheximide

Table 3. Effects of inhibitors of protein and RNA synthesis on the induction of acetylase activity by BENSM in CHO cells

CHO cells were grown for 24 h, and then 25 μM-BENSM was added to half of the cultures. At the same time some dishes were treated with actinomycin D (0.5 mg/ml) or cycloheximide (0.2 mM) and the cells harvested at 6 or 9 h as shown, and the acetylase activity was measured. Results are shown as means ± S.D. for at least four estimations.

Treatment	Acetylase activity (units/mg) after time shown	
	6 h	9 h
Control	0.22 ± 0.04	0.50 ± 0.10
Control + actinomycin D	0.15 ± 0.02	0.16 ± 0.05
Control + cycloheximide	0.19 ± 0.05	0.19 ± 0.04
BENSM	6.8 ± 1.0	12.1 ± 1.8
BENSM + actinomycin D	2.9 ± 0.6	5.2 ± 0.9
BENSM + cycloheximide	0.18 ± 0.02	0.18 ± 0.03

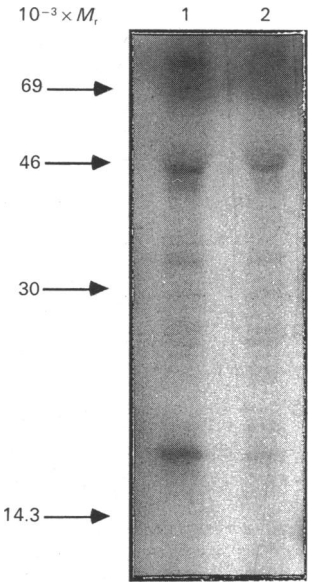

Fig. 2. Translation of mRNA for SAT in CHO cell extracts

Poly(A)-containing RNA was isolated from CHO cells treated with BENSM for 48 h (lane 1) or from control CHO cells (lane 2), and translated in a reticulocyte-lysate system containing [^{35}S]methionine. The translation products were then precipitated by reaction with rabbit anti-SAT antiserum and these proteins were separated by SDS/PAGE and localized by fluorography.

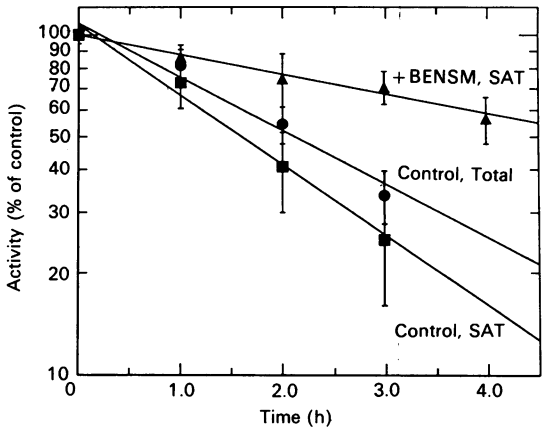

Fig. 3. Half-life of SAT in CHO cells treated with 25 μM-BENSM

CHO cells were plated at a density of 8×10^5 cells/dish, and 25 μM-BENSM was added to half of the dishes as indicated. The cells were grown for 48 h. At this time, 0.2 mM-cycloheximide was added to block protein synthesis, and the dishes were harvested at hourly intervals as indicated. The total acetylase activity in control (Control, Total, ●) and BENSM-treated cells and the activity immunoprecipitable by antiserum to SAT in control (Control, SAT, ■) and BENSM-treated (+BENSM, SAT, ▲) cells were both measured and expressed as a percentage of the activity present at the time of addition of cycloheximide. There was no significant difference between the total acetylase activity and the immunoprecipitable activity in the extracts from the BENSM-treated cells, and only the latter is plotted. The absolute values for these activities at zero time were 1.4 ± 0.3 units for the control total activity, 0.85 ± 0.04 for the control cells SAT, 77.1 ± 9.2 for the BENSM-treated cell total activity and 74.4 ± 6.8 for the BENSM SAT.

Table 4. Inhibition of rat liver SAT by bis(ethyl)spermidine derivatives

Rat liver SAT was used as a source of enzyme, and the assays were carried out with the spermidine concentration shown.

Addition	Acetylase activity (% of control) with spermidine substrate concn. shown:		
	0.1 mM	1 mM	3 mM
1 mM-BENSM	3	10	19
0.125 mM-BENSM	10	34	55
1 mM-BESM	6	22	44
0.125 mM-BESM	19	50	86
1 mM-BEHSM	23	49	75
0.125 mM-BEHSM	74	90	97

(Fig. 3). When total acetylase activity was measured in the control cells, it declined with a half-life of about 2.1 h. However, when immunoprecipitation with anti-SAT serum was used to ensure that only SAT was measured, the half-life in the control cells was estimated to be 1.5 h. The acetylase activity in the BENSM cells was much more stable, and declined with a half-life of about 5 h.

When extracts from untreated CHO cells were used as a source of enzyme for the acetylation of spermidine, most of the product formed (82%) was N^8-acetylspermidine and only 18% was N^1-acetylspermidine. However, more than 90% of the product was N^1-acetylspermidine when extracts from cells treated with BENSM for 48 h were used. These results are consistent with the hypothesis that the increased acetyltransferase activity is indeed SAT, since this enzyme is known to form exclusively the N^1-acetylspermidine isomer (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982). They also indicate that untreated CHO cells are able to form both N^8- and N^1- acetylspermidine and that the capacity to form N^1-acetylspermidine may be increased after exposure to the bis(ethyl) spermine derivatives. However, these compounds may also act as inhibitors of SAT, since they resemble the substrate for the enzyme. This possibility was tested by studying their effect on the acetylation of spermidine by purified rat liver SAT (Table 4). All of the compounds were inhibitory, but BENSM was more active than BESM, which in turn was more active than BEHSM. The inhibition by BENSM and by BESM was investigated in more detail, and was found to be competitive with respect to spermidine (results not shown). Analysis by Dixon plots indicated that the K_i for BENSM was 0.12 mM and that for BESM was 0.37 mM.

In order to examine the physiological consequences of the induction of SAT, intracellular and extracellular polyamine

Table 5. Polyamine contents of CHO cells and media after exposure to BESM and BENSM

Results, which are rounded up to two significant figures, are the means of at least three estimations which agreed within ± 10 %.

Addition and time	Polyamine content (nmol/culture)						
	Putrescine	Spermidine	N^1-Acetyl-spermidine	N^8-Acetyl-spermidine	Spermine	N^1-Acetyl-spermine	Total
None, 0 h (Intracellular)	0.80	2.8	< 0.1	< 0.1	1.6	< 0.1	5.2
None, 24 h (Intracellular)	2.0	7.8	< 0.1	< 0.1	5.5	< 0.1	15.3
None, 24 h (Media)	< 0.1	0.50	0.21	0.36	0.41	< 0.1	1.5
None, 48 h (Intracellular)	2.3	14.4	< 0.1	0.17	10.9	< 0.1	27.8
None, 48 h (Media)	< 0.1	0.87	0.38	0.93	0.62	< 0.1	2.8
BESM, 24 h (Intracellular)	< 0.1	0.80	< 0.1	< 0.1	1.8	< 0.1	2.6
BESM, 24 h (Media)	0.42	0.71	0.67	0.16	0.24	< 0.1	2.2
BESM, 48 h (Intracellular)	< 0.1	0.29	< 0.1	< 0.1	0.9	< 0.1	1.2
BESM, 48 h (Media)	1.2	0.74	0.78	0.29	0.36	< 0.1	3.4
BENSM, 24 h (Intracellular)	0.28	0.95	0.11	< 0.1	1.4	< 0.1	2.7
BENSM, 24 h (Media)	0.81	0.46	0.35	0.24	0.18	< 0.1	2.0
BENSM, 48 h (Intracellular)	0.49	0.26	< 0.1	< 0.1	0.83	< 0.1	1.8
BENSM, 48 h (Media)	1.5	0.42	0.62	0.46	0.18	< 0.1	3.2
MDL 72521, 0 h (Intracellular)	0.39	3.2	0.11	< 0.1	1.9	< 0.1	5.7
MDL 72521, 24 h (Intracellular)	0.95	7.7	0.19	< 0.1	4.8	< 0.1	13.6
MDL 72521, 24 h (Media)	< 0.1	0.29	0.71	0.37	0.39	< 0.1	1.7
MDL 72521, 48 h (Intracellular)	1.9	15.3	0.44	< 0.1	11.7	< 0.1	29.3
MDL 782521, 48 h (Media)	< 0.1	0.91	1.1	0.95	0.68	< 0.1	3.6
MDL 72521 + BENSM, 24 h (Intracellular)	< 0.1	0.83	0.25	< 0.1	1.5	0.35	2.9
24 h (Media)	< 0.1	0.43	1.4	0.18	0.19	0.59	2.8
MDL 72521 + BENSM, 48 h (Intracellular)	< 0.1	0.37	0.17	< 0.1	0.72	0.20	1.5
48 h (Media)	< 0.1	0.38	2.8	0.37	0.22	0.61	4.4

levels were measured in CHO cells treated with 25 μM-BESM or -BENSM (Table 5). Treatment with BESM or BENSM led to a dramatic decrease in the content of all of the major intracellular polyamines; within 48 h of treatment with either compounds, putrescine decreased from 4.7 to 0.8 nmol/mg of (83 % decrease), spermidine from 17.9 to 0.9 nmol/mg (95 %) and spermine from 10.6 to 1.2 nmol/mg (89 %). The bis(ethyl)spermine derivatives substantially inhibited the growth of the CHO cells, with 63 %, 56 % and 80 % decreases in cell number after 96 h in response to BENSM, BESM and BEHSM respectively (results not shown), so the extent of depression of polyamine accumulation in the culture is even greater than indicated by the values expressed per mg of protein. When the results are expressed as the total polyamine present in the culture, both intracellular and in the medium (Table 5), it is clear that the addition of BESM or BENSM completely prevented the synthesis *de novo* of polyamines during the 48 h period; there was no significant increase in total polyamine content in the presence of either of these two drugs, whereas the total polyamine content in the growing control culture increased 6-fold. This is consistent with previous reports that bis(ethyl) spermine derivatives prevent the synthesis *de novo* of polyamines by decreasing the activity of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (Porter *et al.*, 1987*a*,*b*; Pegg *et al.*, 1988; Porter & Bergeron, 1988).

There was a major redistribution of the polyamines in the drug-treated cultures. In the control cultures, only small amounts (about 10 %) of the total polyamine were present in the medium, whereas in the cells treated with BESM or BENSM about 45 % of the total polyamine was present in the medium after 24 h and about 70 % after 48 h. The excretion of putrescine was stimulated to the greatest extent, but there was also an increased fraction of the total spermidine present in the medium (Table 5).

N^8-Acetylspermidine and N^1-acetylspermidine were present in the cells and were excreted into the medium, but at most times the intracellular levels were too low for accurate measurement. BESM and BENSM decreased the excretion of N^8-acetylspermidine, but this is not unexpected, since the amount of its precursor, spermidine, was greatly diminished. However, formation and subsequent excretion of N^1-acetylspermidine were clearly increased by exposure to these agents, particularly when the decreased spermidine content is taken into account. The possible role of polyamine oxidase in decreasing the levels of acetyl polyamines was examined by exposing the CHO cells to MDL 72521, a potent inhibitor of polyamine oxidase (Bolkenius *et al.*, 1985; Seiler, 1987). Treatment with MDL 72521 completely prevented the excretion of putrescine in cells treated with BENSM and led to a large increase in the excretion of N^1-acetylspermidine from these cells (Table 5). Also, N^1-acetylspermine, which was not detected under any of the other culture conditions, was found in both the cells and the medium of cultures treated with the combination of MDL 72521 and BENSM.

Since the interconversion of the polyamines can only be inferred indirectly from measurements of polyamine content, the polyamine pools were labelled by exposure to [^{3}H]putrescine for 24 h before treatment with BENSM or BESM. The labelled polyamines present in the cells and in the medium were then isolated and quantified (Figs. 4 and 5). The results were expressed as the percentage of the total radioactivity present represented by each of the polyamines. There was no significant decline in this total over the 48 h period, indicating that there was little

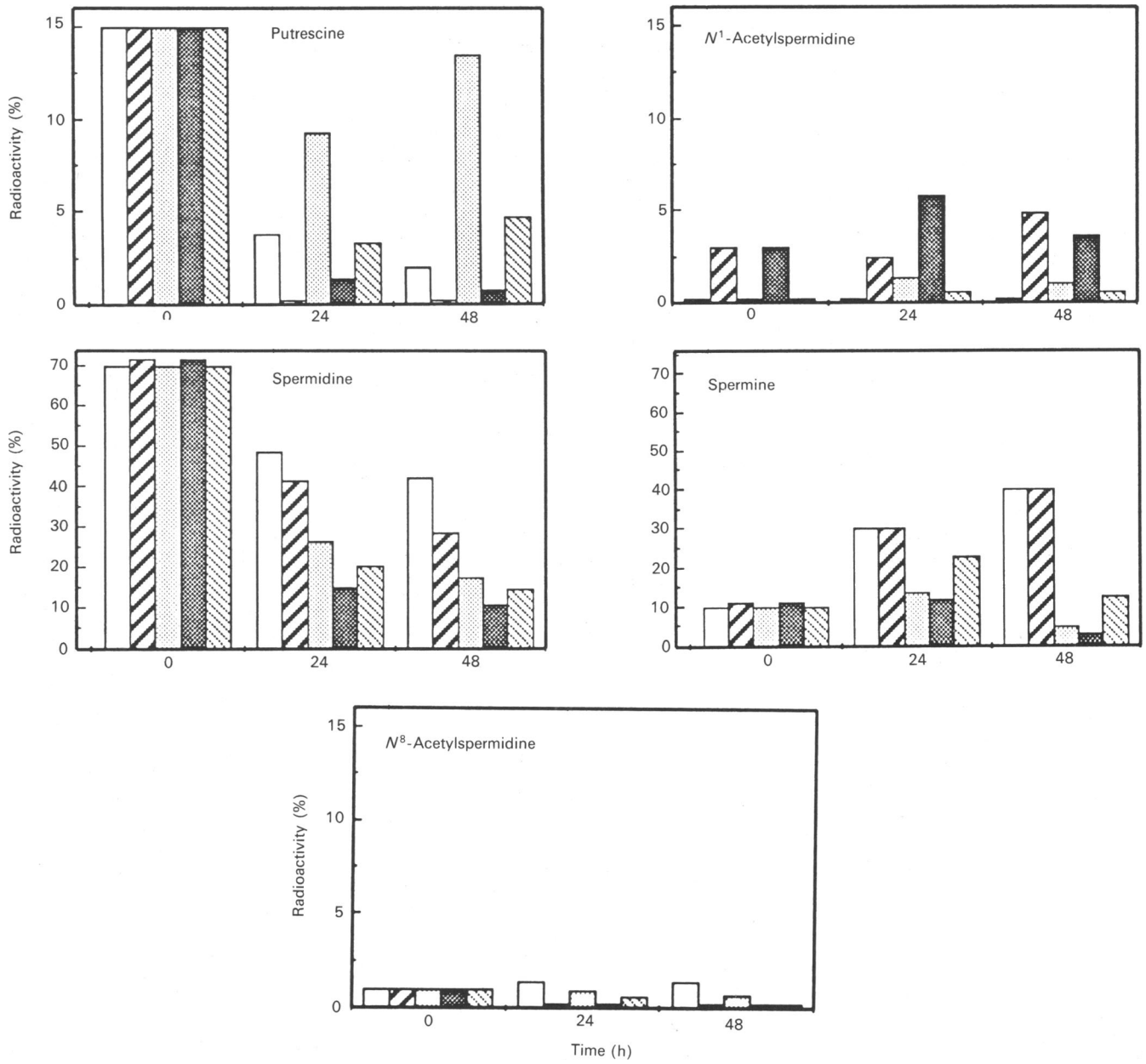

Fig. 4. Effect of BENSM and BESM on labelled intracellular polyamine pools in CHO cells

CHO-cell cultures were labelled by exposure to 0.5 μCi of [2,3-^{3}H]putrescine/ml as described in the Materials and methods section, and then exposed to no addition (□; control), 20 μM-MDL 72521 (▨), 25 μM-BENSM (▩), 25 μM-BESM (▧) or 20 μM-MDL 72521 + 25 μM-BENSM (■) for 24 and 48 h, and the content of labelled polyamines was determined. The results are expressed as percentages of the total label in the culture (both intracellular and in the media) that was present in the intracellular polyamine indicated. Results shown are means of three separate determinations which agreed within ±10%.

degradation of the polyamines to other metabolites. At zero time (20 h after exposure to labelled putrescine) about 16% of the radioactivity in the cells was present in putrescine, 71% in spermidine and 11% in spermine (Fig. 4). In the control cells, the label present in putrescine and spermidine decreased and that in spermine increased, which is as expected from the order of these compounds in the polyamine-biosynthetic pathway (Tabor & Tabor, 1984; Pegg, 1986). In the cells treated with BESM or BENSM, there was a substantial decline in the intracellular labelling of spermine, a smaller decline in labelled spermidine and an increase in the content of labelled N^1-acetylspermidine and putrescine (Fig. 4). There was an even larger increase in the excretion of labelled putrescine and labelled N^1-acetylspermidine into the medium, and there was also a rise in the excretion of labelled spermidine (Fig. 5). The formation and excretion of labelled putrescine from the BENSM-treated cells was blocked by MDL 72521, and in these cells there was a large increase in the excretion of radioactive N^1-acetylspermidine (Fig. 5). Labelled N^1-acetylspermine was found only in the cells and media from cultures treated with BENSM and MDL 72521 (results not shown).

DISCUSSION

The increased acetyltransferase activity produced in CHO cells by exposure to bis(ethyl)spermine derivatives clearly does result

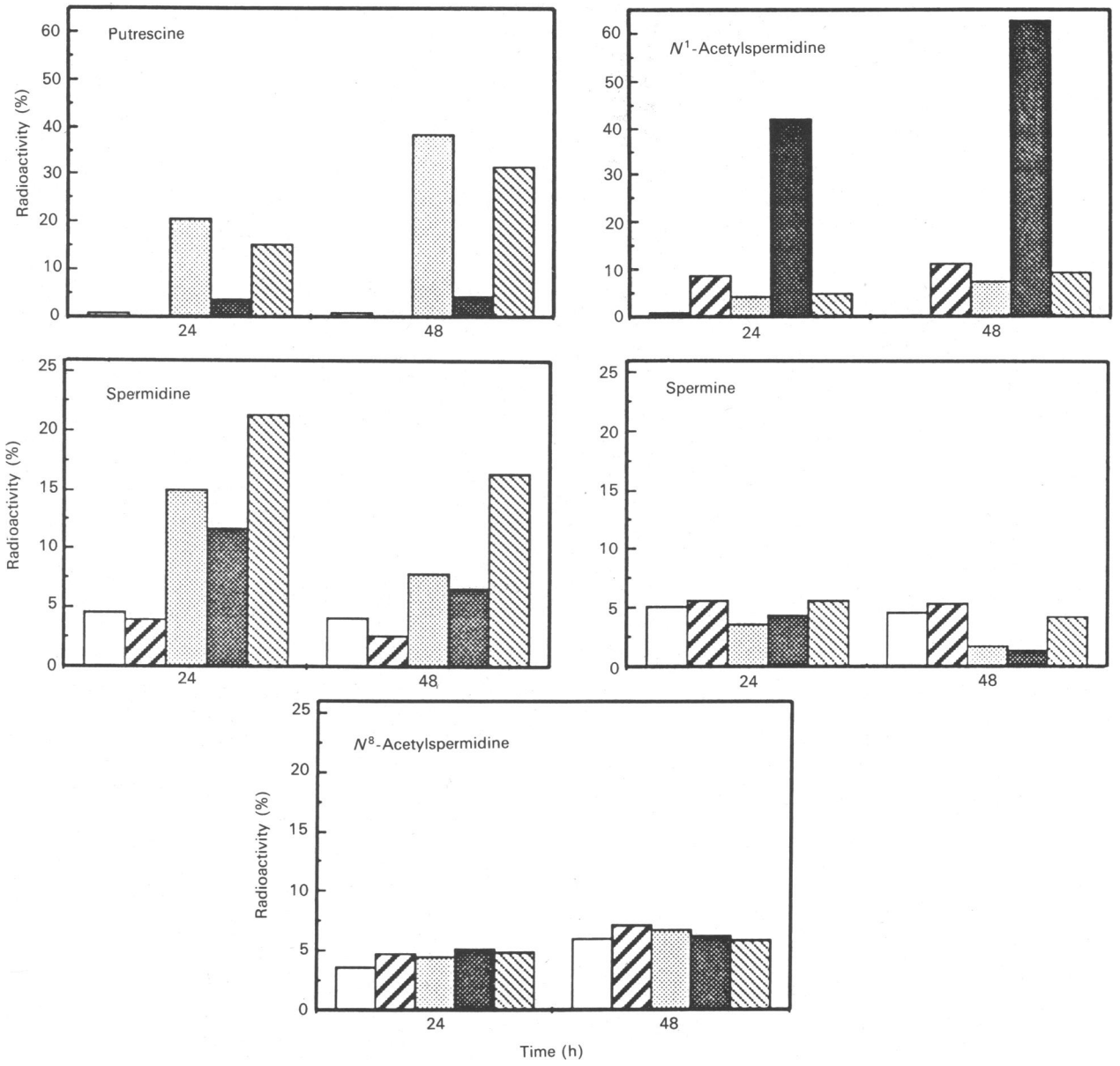

Fig. 5. Effect of BENSM and BESM on excretion of labelled polyamines from CHO cells

Experimental details and symbols are given in the legend to Fig. 4. The results show the percentage of the total label in the culture (both intracellular and in the media) that was present in the polyamine indicated in the medium. Results shown are means of three separate determinations which agreed within $\pm 10\%$.

from an increase in the amount of SAT protein. The properties of the induced enzyme are similar to those described for rat liver SAT (Della Ragione & Pegg, 1982, 1983), there is an increase in immunoreactive protein detected on Western blots with antibodies to rat SAT, and the subunit M_r as determined from SDS/PAGE is similar to that of rat SAT (Persson & Pegg, 1984). The increased SAT content is mediated by an increase in its mRNA and by a decreased rate of turnover of the enzyme protein. The extent of induction of SAT is vast, and the very low levels of SAT protein and mRNA in control cells make it very difficult to determine whether the observed increases in mRNA and in protein stability can account for all of the change. Other factors such as an increase in the rate of synthesis of the protein at the level of translation are therefore not ruled out. Further mechanistic studies require the production of cDNA probes for SAT. These have been difficult to obtain, because of the low abundance of this protein, but the large increase in SAT mRNA produced by BENSM should enable the molecular cloning of SAT. In this respect, the increase in acetyltransferase activity produced by BESM in H157 human large-cell lung carcinoma cells (Casero *et al.*, 1989*a*), which is also due to SAT induction (Casero *et al.*, 1990), may be even more useful, since the amount of SAT in these cells is even greater than that in stimulated CHO cells.

Previous studies have shown that SAT activity is increased by a number of factors, including hepatotoxins, hormones, lectins, growth factors and heat shock (Matsui & Pegg, 1980; Matsui *et al.*, 1981; Danzin *et al.*, 1982; Della Ragione & Pegg, 1984; Persson & Pegg, 1984; Shinki *et al.*, 1985; Steffanelli *et al.*, 1986; Ekstrom *et al.*, 1989; Harari *et al.*, 1989*a*,*b*; Matsui-Yuasa *et al.*, 1989). One possible unifying factor which might mediate stimulation by these agents is an increase in the free intracellular polyamine concentration, owing to the overproduction of polyamines or the release of intracellular polyamines from some

bound form (Erwin & Pegg, 1986; Pegg, 1986). It is possible that the extremely powerful induction of SAT by the bis-(ethyl)spermine analogues is due to a combination of their ability to release intracellular polyamines from bound sites and their resemblance to the natural polyamines. Furthermore, their inability to act as substrates prevents the induced SAT activity from leading to a decrease in their concentration.

The bis(ethyl)spermine derivatives are strongly inhibitory to cell growth (Bergeron *et al.*, 1988, 1989; Pegg, 1988; Porter & Bergeron, 1988; Casero *et al.*, 1989*a*; Pegg *et al.*, 1989). The extent to which the induction of SAT contributes to this growth inhibition is unclear. At first sight, there does not appear to be a good correlation between the extent of growth inhibition and the potency of the various compounds in increasing SAT activity. However, the weaker inducer of activity, BEHSM, is also a much weaker inhibitor of this enzyme, so the rise in the cellular capacity to acetylate polyamines may be proportionally greater than with BENSM, which is a stronger inhibitor.

The studies of the polyamine content and interconversion of CHO-cell cultures treated with BENSM or BESM show clearly the importance of SAT and polyamine oxidase in polyamine metabolism and interconversion. Induction of SAT and the degradation of the acetylated products produced by polyamine oxidase leads to the conversion of spermine into spermidine and of spermidine into putrescine. When polyamine oxidase activity was blocked by MDL 72521, the N^1-acetylspermidine and N^1-acetylspermine formed by SAT accumulated and putrescine production was prevented. A second consequence of the exposure of cells to the bis(ethyl)spermine derivatives is the release of intracellular polyamines from the cells. Putrescine, N^1-acetylspermidine and spermidine are all excreted in response to BESM or BENSM in cells possessing polyamine oxidase activity. When polyamine oxidase was blocked, the excreted polyamines were in the form of N^1-acetylspermidine and spermidine (Fig. 4). These results agree with recent studies in HT 29 cells in that treatment with BESM and related compounds caused the release of polyamines from the cell (Pegg *et al.*, 1989). However, in the HT 29 cell system, the major increase in excreted polyamines was in the form of spermidine, with smaller amounts of N^1-acetylspermidine. Putrescine release was not enhanced over the background level. These differences can be explained by the greater induction of SAT in CHO cells and by the fact that HT 29 cells have very low levels of polyamine oxidase (A. E. Pegg, unpublished work).

The presence of N^8-acetylspermidine in CHO cells, which was also reported recently by Hyvönen (1989), is unusual for mammalian cells. Although it is well known that N^8-acetylspermidine can be formed by a cellular enzyme which may be the same as the nuclear histone acetyltransferase (Erwin *et al.*, 1984; Pegg, 1986), this derivative is readily de-acetylated back to spermidine, and the N^8-acetylspermidine only accumulates in cells treated with inhibitors of the deacetylase (Mamont *et al.*, 1984; Seiler, 1987; Dredar *et al.*, 1989; Marchant *et al.*, 1989). This derivative is clearly also excreted into the medium [Table 5 and Hyvönen (1989)], but its production and excretion are not enhanced by the bis(ethyl)spermine derivatives. This provides supporting evidence that the acetyltransferase that is induced is SAT, since SAT does not form N^8-acetylspermidine (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982; Persson & Pegg, 1984).

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