of eq 13 or 14; (b) the jackknife procedure; and (c) details of the iteration procedure. Obviously, if $[E_t] \ll K_{i\,app}(V_0/V_i)$, then the log $(1/K_{i\,app})$ will be the same using eq 13 or 14. If $[E_t] \approx K_{i\,app}(V_0/V_i)$, eq 14 will always give the larger (and correct) estimate for log $(1/K_{i\,app})$; in such cases this was the equation finally used to calculate the log $(1/K_{i\,app})$ estimates.

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Inhibitors of Polyamine Biosynthesis. 9. Effects of *S*-Adenosyl-L-methionine Analogues on Mammalian Aminopropyltransferases in Vitro and Polyamine Biosynthesis in Transformed Lymphocytes

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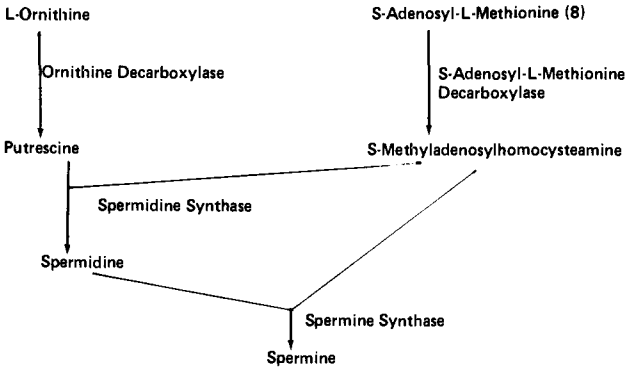
Seven analogues of *S*-adenosyl-L-methionine were studied as inhibitors or substrates for mammalian spermidine and spermine synthases. One of these, *S*-(5′-deoxy-5′-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (**5**), showed a unique spectrum of activities on the polyamine biosynthesis enzymes. It was an inhibitor of *S*-adenosyl-L-methionine decarboxylase from rat liver and spermine synthase from bovine brain and rat ventral prostate. This compound was a substrate for the spermidine synthases from bovine brain and rat ventral prostate but not a substrate for the spermine synthases from these same sources. At concentrations of 0.2 mM and higher, compound **5** blocked the increases in polyamine levels and in [^{3}H]thymidine incorporation induced by concanavalin A in cultured mouse lymphocytes. At approximately a 0.5 mM concentration of **5**, the cellular polyamine levels and the rate of thymidine incorporation were similar to those of the unstimulated lymphocytes. Lower concentrations of **5** (0.02–0.1 mM) produced a dose-dependent increase in thymidine incorporation. A dose-dependent decrease in the cellular polyamine levels was observed in the range of 0.05–0.5 mM of the inhibitor. These results suggest that the effects of **5** on transformed lymphocytes are complex and may not be solely due to the inhibition of polyamine biosynthesis by this compound.

The polyamines appear to play an essential role in cellular metabolism and regulation.[2] The development of specific inhibitors of polyamine biosynthesis has received considerable attention during the past decade. These inhibitors were developed as tools for the study of the function of the polyamines and as potential therapeutic agents. Four enzymes are known to be involved in the synthesis of polyamines in mammalian tissues (Scheme I). Many inhibitors of the first two enzymes in the pathway, L-ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, have been developed.[3-5] However, only a few studies have been carried out on inhibitors of spermidine and spermine synthases.[6-12] Potent and specific inhibitors of these synthases may have some advantages over the currently available inhibitors of the decarboxylases. Specifically, such inhibitors may produce significant depletion of spermine. This has not been al-

Scheme I



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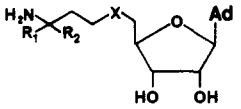
ways possible using inhibitors of L-ornithine decarboxylase.[13] Secondly, the development of compounds which can selectively inhibit one of the synthases, resulting in the depletion of only one of the polyamines, may clarify the relative importance of the individual polyamines in cellular metabolism. Therefore, we began some studies to develop specific inhibitors of spermidine and spermine synthases and delineate the structural features of the substrate, S-methyladenosylhomocysteamine (9), necessary for binding to these enzymes.



	X	R_1	R_2
1	S	COOH	CH_3
2	$\overset{+}{S}CH_3$	COOH	CH_3
3	S=O	COOH	CH_3
4	S	H	CH_3
5	$\overset{+}{S}CH_3$	H	CH_3
6	S=O	H	CH_3
7	NCH_3	H	H
8	$\overset{+}{S}CH_3$	COOH	H
9	$\overset{+}{S}CH_3$	H	H

Recently, we reported the synthesis of a group of simple analogues of S-adenosyl-L-methionine (8) and S-methyladenosylhomocysteamine (compounds 1–7). We also reported the inhibitory activities of these compounds on rat liver S-adenosyl-L-methionine decarboxylase.[14] In this report we describe the effects of these analogues on the spermine synthase from bovine brain and spermine and spermidine synthases from rat ventral prostate. We also report on the effects of one of these compounds on the polyamines and DNA synthesis in proliferating mouse lymphocytes.

Results

Spermidine and spermine synthase activites were measured by determining the amount of radioactive polyamines formed from S-methyladenosyl[^{14}C]homocysteamine and putrescine or spermidine, respectively.

The purified bovine brain spermine synthase has a very high affinity for the substrate S-methyladenosylhomocysteamine. The apparent K_M for this substrate is 0.60 μM and for spermidine is 60 μM.[15] The inhibition by compounds 1–7 of the formation of spermine by the purified enzyme from spermidine and S-methyladenosyl-[^{14}C]homocysteamine was measured in vitro. The target compounds were tested at concentrations of 0.01, 0.1, 0.4, and 1.0 mM. Compounds 1–4, 6, and 7 did not produce significant inhibition of the formation of [^{14}C]spermine under these conditions. However, compound 5 produced a significant inhibition of the enzymatic production of [^{14}C]spermine from spermidine and S-methyladenosyl-[^{14}C]homocysteamine (Table I). Incubation of 5 with [^{14}C]spermidine and bovine brain spermine synthase did not result in the formation of radiolabeled polyamines, indicating that 5 is not a substrate for this enzyme. The inhibition of spermine synthesis by 5′-methyl-5′-deoxythioadenosine at 0.01 and 0.1 mM concentrations are included in Table I for comparison. This compound is a more potent inhibitor of spermine synthase than compound 5.

Table I. Effects of S-(5′-Deoxy-5′-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (5) on Spermine Synthase from Bovine Brain and Spermidine Synthase and Spermine Synthase from Rat Prostate

	% activity[a]		
compd (concn, mM)	brain spermine synthase	prostatic spermidine synthase	prostatic spermine synthase
5 (0.01)	102	ND	ND
5 (0.1)	88	76	66
5 (0.4)	58	ND	ND
5 (1.0)	23	ND	ND
5′-(methylthio)-5′-deoxyadenosine (0.01)	44	ND	ND
5′-(methylthio)-5′-deoxyadenosine (0.1)	12	41	9

[a] ND, not determined.

Table II. Substrate Activity of S-(5′-Deoxy-5′-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (5) for Spermidine and Spermine Synthases

	product formation,[a] pmol/min		
propylamine donor	prostatic spermidine synthase	prostatic spermine synthase	brain spermidine synthase
S-methyladenosyl-homocysteamine	218 (100)	88 (100)	96 (100)
5	56 (26)	0	25 (26)

[a] Numbers in parentheses are the percent substrate activities.

The effects of compounds 1–7 on prostatic spermidine and spermine synthase activities were also determined. Only compound 5 produced significant inhibition of both spermidine and spermine synthase activities. Compound 5 produced slightly greater inhibition of the spermine synthase activity than the spermidine synthase activity (Table I).

The ability of compound 5 to serve as substrate for aminopropyltransferases from rat ventral prostate and bovine brain was further studied. In these studies the enzyme preparation was incubated with [^{14}C]putrescine or [^{14}C]spermidine and S-methyladenosylhomocysteamine or 5. Compound 5 was a substrate for spermidine synthase activity from rat ventral prostate and bovine brain (Table II). In these two systems it had approximately 26% the substrate activity of the natural substrate S-methyladenosylhomocysteamine. Compound 5 was not a substrate for prostatic spermine synthase (Table II).

The effects of 5 on polyamine biosynthesis and thymidine incorporation in transformed lymphocytes were examined. The time course of changes in cellular polyamine levels and [^{3}H]thymidine incorporation produced by treatment of lymphocytes with concanavalin A alone or in combination with 5 is shown in Figure 1. Concanavalin A stimulation produced increases in cellular polyamine levels which paralleled the rise in the rate of [^{3}H]thymidine incorporation. Treatment with 5 (0.5 mM) blocked the increases in polyamine levels and thymidine incorporation normally observed in the transformed lymphocytes.

The effects of varied concentrations of 5 on polyamine accumulation and [^{3}H]thymidine incorporation in concanavalin A stimulated lymphocytes were also studied (Table

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Table III. Effects of Varied Concentrations of S-(5'-Deoxy-5'-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (5) on Polyamine Accumulation and [^{3}H]Thymidine Incorporation in Concanavalin A Stimulated Lymphocytes

conditions of incubation[a]	polyamine levels, pmol/g of DNA[b]			[^{3}H]thymidine incorporation (cpm/10^6 viable cells)/h[b]
	putrescine	spermidine	spermine	
unstimulated	3.8	15.4	23.6	2 160
stimulated	16.0	44.8	53.3	14 900
+0.02 mM 5	17.8 (114)	67.3 (176)	53.3 (100)	15 300 (102)
+0.05 mM 5	11.0 (50)	39.8 (83)	36.4 (43)	18 000 (124)
+0.10 mM 5	8.9 (41)	38.7 (79)	37.3 (46)	20 200 (142)
+0.20 mM 5	6.8 (25)	36.4 (71)	36.6 (43)	9 710 (59)
+0.35 mM 5	4.2 (3)	22.5 (24)	32.3 (29)	4 270 (17)
+0.50 mM 5	3.5 (0)	14.6 (0)	21.8 (0)	2 360 (2)

[a] Cultures were harvested 24 h after addition of concanavalin A and 5. [b] Numbers in parentheses are percent of control (stimulated with concanavalin A); measured values are means of at least two separate determinations; the coefficient of variation of these measurements is not more than ±5%.

Table IV. Effects of the Addition of Polyamines on the Intracellular Concentration of Polyamines and [^{3}H]Thymidine Incorporation in Concanavalin A Stimulated Lymphocytes in the Presence or Absence of S-(5'-Deoxy-5'-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (5)

conditions of incubation[a]	polyamine levels, pmol/g of DNA[b]			[^{3}H]thymidine incorporation, (cpm/10^6 viable cells)/h[b]
	putrescine	spermidine	spermine	
		At 24 h		
unstimulated	2.2	12.9	22.0	643
stimulated	8.6	28.5	38.6	7 310
stimulated + 5 (0.5 mM)	4.3 (33)	17.5 (29)	29.2 (43)	2 760 (32)
		At 48 h		
unstimulated	2.1	12.6	22.4	764
stimulated	13.3	90.5	59.8	49 400
stimulated & putrescine (50 μM)	29.5 (245)	101.3 (114)	53.6 (84)	30 700 (62)
stimulated & spermidine (50 μM)	7.8 (51)	157.3 (186)	52.5 (80)	39 200 (79)
stimulated & spermine (50 μM)	5.3 (29)	38.2 (33)	98.2 (203)	37 100 (75)
stimulated + 5 (0.5 mM)	5.3 (29)	19.3 (9)	18.3 (0)[c]	1 660 (2)
stimulated + 5 (0.5 mM) & putrescine (50 μM)	7.4 (47)	21.7 (12)	22.7 (1)[c]	1 770 (2)
stimulated + 5 (0.5 mM) & spermidine (50 μM)	10.3 (73)	26.2 (17)	19.5 (0)[c]	1 260 (1)
stimulated + 5 (0.5 mM) & spermine (50 μM)	4.4 (21)	20.0 (9)	21.6 (0)[c]	2 930 (4)

[a] Cultures were treated with concanavalin A and 5, where used, at time 0. The polyamines were added at 24 h and cells were harvested at 48 h. [b] Numbers in parentheses are percent of control, [(treatment − unstimulated)/(stimulated − unstimulated)] × 100; measured values are means of at least two separate determinations; the coefficient of variation of these measurements is not more than ±5%. [c] Spermine concentrations in these cultures were lower than the unstimulated cells.

III). At concentrations of 0.2 mM and higher, 5 produced a dose-dependent decrease in both polyamine levels and thymidine incorporation. At approximately a 0.5 mM concentration of 5, the cellular polyamine levels and the rate of thymidine incorporation were similar to those of the unstimulated lymphocytes. Lower concentrations of 5 (0.02–0.01 mM) produced a dose-dependent increase in thymidine incorporation in spite of dose-dependent decreases in the cellular polyamine levels.

The effects on [^{3}H]thymidine incorporation and intracellular polyamine concentrations produced by the addition of polyamines to concanavalin A stimulated lymphocytes treated with 5 or no inhibitors are shown in Table IV. In these experiments, concanavalin A and 5, where used, were added at 0 time and the polyamines, at 50 μM concentrations, were added 24 h later. The [^{3}H]thymidine incorporation and the intracellular polyamine concentrations in the stimulated lymphocytes were measured at 48 h. Exogenously added polyamines were not toxic to lymphocytes and altered the cell viability and [^{3}H]thymidine incorporation only slightly (Table IV). At 48 h, i.e., 24 h after the addition of 50 μM putrescine, spermidine, or spermine to lymphocytes stimulated with concanavalin A, the intracellular concentration of the particular polyamine added was higher than that produced by concanavalin A alone. Interestingly, the addition of one polyamine also led to changes in the concentrations of the other polyamines. With the exception of putrescine addition which resulted in an increase in both putrescine and spermidine concentrations, the addition of one polyamine always reduced the increase in the other polyamines caused by concanavalin A stimulation.

The addition of polyamines did not produce a reversal of the inhibitory effects of 5 on [^{3}H]thymidine incorporation in stimulated lymphocytes. Furthermore, the addition of polyamines did not restore the intracellular concentrations of polyamines to those observed in stimulated lymphocytes untreated with 5.

Discussion

The results presented here provide some general and useful information on the requirements for binding of

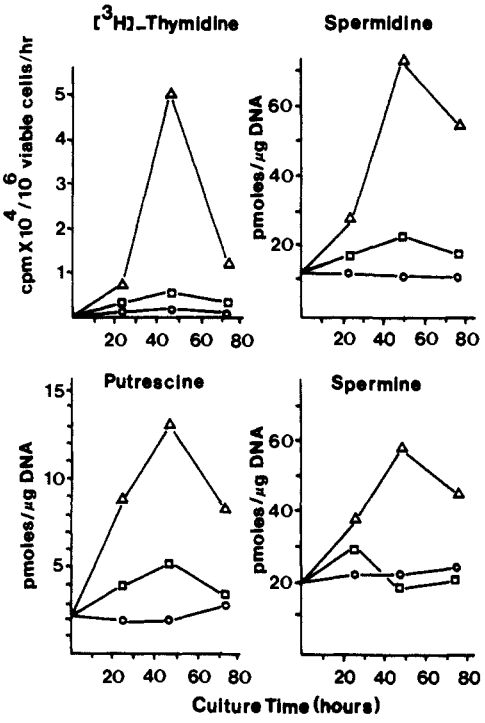

Figure 1. Time course of the effects of compound **5** on [^{3}H]-thymidine incorporation and intracellular levels of polyamines in concanavalin A stimulated lymphocytes. Mouse spleen lymphocytes (5×10^6 cells/mL) were cultured without concanavalin A (○), with 1 mg/mL concanavalin A (△), or with 1 mg/mL concanavalin A and 0.5 mM **5** (□) in RPMI 1640 medium supplemented with 1% heat-inactivated human serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin.

ligands to the aminopropyltransferases from rat prostate and bovine brain. It appears that the presence of the α-carboxyl group, such as in compound **2** and *S*-adenosyl-L-methionine (**8**), interferes with the binding of the ligand to the enzyme. This should be expected to ensure that **8**, which is present intracellularly in much higher concentrations than *S*-methyladenosylhomocysteamine (**9**), will not compete for binding to the enzymes. The presence of a sulfonium group with a fully developed positive charge appears to be essential for binding of substrate analogues to the enzyme. This was demonstrated by the lack of inhibitory activity of compounds **4** and **6**. Since the thioethers 5′-methyl-5′-deoxythioadenosine, 5′-ethyl-5′-deoxythioadenosine, and 5′-methyl-5′-deoxytubercidin are potent inhibitors of spermine and spermidine synthases,[7,8,11] the inactivity of the thioether **4** was unexpected. This observation suggests that the requirements for binding of the substrate and its analogues are different than those for the product and its analogues. However, it is not known yet whether the substrate and product bind to the same site or to two different sites. Finally, it appears that replacement of the α-hydrogen in the substrate with a methyl group, such as in **5**, does not preclude binding of the ligand to the enzymes.

It should be pointed out that some of these findings are not in agreement with the published results of other workers.[6,10,12] Hibasami et al.[10,11] reported that *S*-adenosyl-L-methionine and some of its carboxyl-containing analogues were potent inhibitors of spermidine synthase and spermine synthase from rat prostate. Furthermore, these authors have shown that some analogues of *S*-adenosylhomocysteamine were also inhibitors of these synthases. More recently, Tang et al. reported that *S*-adenosyl-3-thio-1,8-diaminooctane was a more potent inhibitor of spermidine synthase than its corresponding sulfonium salt.[12] Interestingly, spermidine synthases were found to be strongly inhibited by high concentrations of the substrate.[6,11,16] In view of the complex kinetics of the spermidine synthases, it is not possible to extrapolate from the results of the studies on the substrate activity of **5**, in which only one concentration of **5** or the substrate **9** was used.

An interesting profile of inhibitory activities of compounds **1**–**7** on the polyamine biosynthesis enzymes emerges from these studies. Previously, compounds **2**, **5**, and **7** were found to be inhibitors of AdoMet decarboxylase from rat liver in vitro.[14] Although **5** and **7** are analogues of *S*-methyladenosylhomocysteamine, the product of the decarboxylation reaction, the mode of binding of these two inhibitors to the enzyme appears to be different.[14] Compounds **5** and **7** are both analogues of the substrate for the aminopropyltransferases enzymes, but only **5** was able to bind to these enzymes. However, the interaction between **5** and the two transferases were different. Compound **5** was a substrate for the spermidine synthases but not for spermine synthases.

Compound **5** has a unique spectrum of activities on the polyamine biosynthesis enzymes. It is an inhibitor of both AdoMet decarboxylase and spermine synthase and a substrate for spermidine synthase. Compound **2**, on the other hand, produced inhibition of only AdoMet decarboxylase. The study of the effects of **2** and **5** on polyamines biosynthesis and thymidine incorporation in concanavalin A transformed lymphocytes provided valuable information. Compound **2** produced a selective inhibition of spermine synthesis and thymidine incorporation in these cells.[17] This inhibition was accompanied by dose-dependent increases in the cellular levels of putrescine and spermidine at 24 h after the addition of the inhibitor. Interestingly, **5** produced an inhibition of the biosynthesis of all three polyamines at 24 h.

Compound **5** is a considerably more potent inhibitor than **2** of both polyamine biosynthesis and thymidine incorporation in transformed lymphocytes. At the highest concentration tested (2.0 mM), compound **2** produced only a 78% inhibition of thymidine incorporation.[15] At approximately 0.5 mM **5**, the increase in thymidine incorporation produced by treatment with concanavalin A was completely inhibited.

Compounds **2** and **5** are sulfonium salts and are not expected to enter cells readily by a simple (passive) diffusion mechanism. It is possible that only a small fraction of these compounds entered the cells to produce the observed effects. This would indicate that the intrinsic activities of these compounds is much greater than the observed activity. On the other hand, these compounds may be transported into cells by a facilitated diffusion mechanism. *S*-Adenosyl-L-methionine and some other nucleosides have been shown to enter mammalian cells by a facilitated diffusion mechanism.[18-20]

To determine if the antiproliferative effects produced by **5** were due to polyamine depletion, we attempted to reverse these effects by the addition of polyamines to cell cultures treated with **5**. The addition of polyamines did

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not produce a reversal of the inhibitory effects of 5 or [^{3}H]thymidine incorporation nor restore the intracellular concentrations of polyamines to those observed in untreated lymphocytes. Thus, the inability of exogenous polyamines to reverse the antiproliferative effects of 5 on stimulated lymphocytes is simply a reflection of the inability of this treatment to restore intracellular levels of polyamines. Although these results do not confirm that the antiproliferative effects of 5 are due to the depletion of polyamines, they are not inconsistent with such a conclusion.

The stimulatory effects produced by lower concentrations of 5 (0.02–0.1 mM) on thymidine incorporation were unexpected (Table IV). At this range of concentrations the compound produced a dose-dependent increase in thymidine incorporation. However, with the exception of the lowest concentration tested, a dose-dependent decrease in the cellular polyamine levels was also observed in the range of concentrations of inhibitor tested. The mechanism of enhancement of thymidine incorporation by low concentrations of 5 is not known and is currently under investigation.

The results suggest that the effects of 5 on transformed lymphocytes are complex. The inhibition of thymidine incorporation produced by concentrations of 5 greater than 0.2 mM may not be due solely to the inhibition of the biosynthesis of polyamines.

Experimental Section

Chemicals. Compounds 1–7 were synthesized as described in ref 14. 5′-Deoxy-5′-(methylthio)adenosine was obtained from Sigma Chemical Co.

DL-[2-^{14}C]Methionine (sp act. 4.08 Ci/mol) was obtained from New England Nuclear Corp. Labeled *S*-adenosylmethionine was prepared from DL-[2-^{14}C]methionine essentially as described by Pegg and Williams-Ashman.[21] Radioactive *S*-methyladenosylhomocysteamine was prepared from *S*-adenosylmethionine labeled at the C-2 position of the methionine moiety using *S*-adenosylmethionine decarboxylase from *E. coli* purified through step 3 by the method of Wickner et al.[22] The product was first purified on a Dowex 50 (H^+) column, followed by preparative paper electrophoresis as previously described.[23] Preparation of spermine–Sepharose has been described previously in detail.[24]

Assay of Spermine Synthase Activity. The assay of spermine synthase activity was principally performed as previously described.[15] The standard reaction mixture contained, in a total volume of 0.1 mL, 20 μM ^{14}C-labeled *S*-methyladenosylhomocysteamine, 1 mM spermidine, 5 mM dithiothreitol, 0.05 mg of enzyme protein, inhibitors at concentrations of 0.01–1.0 mM, and 0.1 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 37 °C for 20 min, and the reaction was stopped by the addition of 10 μL of 1 M potassium hydroxide.

Spermine synthase was purified from bovine brain using two spermine–Sepharose affinity columns.[15]

Assay of Spermidine Synthase Activity. The assay of spermidine synthase activity was determined as previously described by Raina et al.[25] The standard reaction mixture contained, in a total volume of 0.1 mL, 20 μM ^{14}C-labeled *S*-methyladenosylhomocysteamine, 1 mM putrescine, enzyme protein, and inhibitors at 0.1 mM concentrations. The mixture was incubated at 37 °C for 15 min.

Substrate Activity of 5 for Spermidine and Spermine Synthases. The reaction mixture contained, in a total volume of 0.2 mL, 0.13 mM *S*-methyladenosylhomocysteamine or 5, 0.6 mM [^{14}C]putrescine for measurement of spermidine synthase activity or [^{14}C]spermidine for measurement of spermine synthase, and 1.57 mg of soluble supernatant protein of rat ventral prostate or 0.09 mg of enzyme protein purified 30-fold from the soluble fraction of bovine brain homogenate. The mixture was incubated at 37 °C for 20 min. The reaction was stopped by the addition of 20 μL of 10% perchloric acid containing unlabeled polyamines. The formation of labeled polyamines was measured in the acid supernatant by paper electrophoresis.[23,26]

Lymphocyte Culture. Lymphocytes from mouse spleens were isolated and cultured as described by Wang et al.[27] DNA, RNA, or protein synthesis in lymphocytes was determined by the incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine into trichloroacetic acid precipitable material during a 1-h pulse period.[24]

Determination of DNA Content. Approximately 2–3 × 10^7 lymphocytes (4 mL of a culture suspension containing 4–6 × 10^6 cells/mL) were collected by centrifugations, extracted with 1.0 mL of 5% trichloroacetic acid, and stored at −20 °C until assayed. The DNA content of trichloroacetic acid precipitable material was determined using the method of Schneider[28] with calf thymus DNA as standard.

Determination of Intracellular Polyamines. Approximately 1–3 × 10^7 cells (3–5 mL of a culture suspension containing 4–6 × 10^6 cells/mL) were collected by centrifugations and extracted with 0.5 mL of 0.3 N perchloric acid, and the extract was stored at −20 °C until analysis. A simple modification of the procedure of Newton et al.[29] was used for the determination of the polyamines in the acid extract. This modification involved the use of *n*-hexane in place of cyclohexane in the high-performance LC solvent. Duplicate samples were analyzed twice for polyamine concentration. The polyamine concentrations are expressed as picomole per microgram of DNA.

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