scribed [A. Pellicer, M. Wigler, R. Axel, S. Silverstein, *Cell* **14**, 133 (1978)] except that 0.1 percent 8-hydroxyquinoline was included during phenol extraction. DNA's were cleaved with Eco RI, and fragments were separated in 0.8 percent agarose gels. DNA was transferred from gels to nitrocellulose filters (*10*). A ^{32}P-labeled p640 probe was hybridized to filters as described [D. Owerbach, W. J. Rutter, J. A. Martial, J. D. Baxter, T. B. Shows, *Science* **209**, 289 (1980)]; the filters were rinsed at 68°C in a buffer of NaCl (0.3*M*) and sodium citrate (0.03*M*) containing 0.1 percent sodium dodecyl sulfate, then dried. The filters were then exposed for up to 1 week at −70°C to Kodak XAR-5 x-ray film with Dupont Cronex Lightning Plus intensifying screens.

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Spermidine Requirement for Cell Proliferation in Eukaryotic Cells: Structural Specificity and Quantitation

Abstract. *Six structural homologs of spermidine and five of its precursor, putrescine, were studied for their ability to prevent cytostasis of cultured L1210 leukemia cells induced by α-difluoromethylornithine (DFMO), a specific inhibitor of putrescine biosynthesis. High-performance liquid chromatography and competition studies with spermidine indicated that the homologs, which vary in the length of the carbon chain separating the amines, penetrated the cells. The structural specificity of the spermidine carrier was defined. Three of the six spermidine homologs supported cell growth during a 48-hour incubation in the presence of DFMO, indicating that a two-carbon extension of spermidine structure was tolerated for biological function. Two of the five putrescine homologs supported growth after being converted by the cells to their respective spermidine homologs. The central nitrogen of spermidine appears to be essential for function since diamines of chain length comparable to that of spermidine did not prevent DFMO cytostasis. No more than 15 percent of the spermidine normally present in L1210 cells was required for cell proliferation in the presence of DFMO.*

Several lines of evidence indicate that polyamines, particularly spermidine, are required for cell proliferation: (i) they are found in greater amounts in growing than in nongrowing tissues (*1*); (ii) prokaryotic and eukaryotic mutants deficient in polyamine biosynthesis are auxotrophic for polyamines (*2*); and (iii) inhibitors specific for polyamine biosynthesis also inhibit cell growth (*3, 4*). Despite this evidence, the precise biological role of polyamines in cell proliferation is uncertain. It has been suggested that polyamines, by virtue of their charged nature under physiological conditions and their conformational flexibility, might serve to stabilize macromolecules such as nucleic acids by anion neutralization (*5, 6*). Any specificity of such interactions would probably reside in the spatial separation of the amines to ensure optimal reactivity at their site of action. We now report the results of a study undertaken to quantitate and define structurally the spermidine requirement of eukaryotic cell proliferation.

Five putrescine and six spermidine homologs of various aliphatic chain lengths were examined for their ability to prevent cytostasis induced in cultured L1210 cells by α-difluoromethylornithine (DFMO). The latter is a highly specific and irreversible inhibitor of ornithine decarboxylase (*4*), which is the initial enzyme in putrescine and spermidine biosynthesis. Treatment of cells with DFMO results in a depletion of intracellular putrescine and spermidine pools, followed by cytostasis. The cytostasis can be readily prevented by including spermidine or its precursor, putrescine, in the DFMO-containing medium. If the medium is supplemented instead with various homologs of spermidine or putrescine, the structural constraints of the biological sites critical for cell proliferation can be mapped, provided that the homologs enter the cells. This test system may not be entirely equivalent to the situation in untreated cells since DFMO results in a marked increase in decarboxylated *S*-adenosylmethionine and *S*-adenosylmethionine decarboxylase (*7*), the enzyme required for the conversion of putrescine to spermidine.

A series of putrescine homologs having the general structure $NH_2(CH_2)_nNH_2$ [abbreviated, DA_n (for diamine), with DA_4 being putrescine itself (Table 1)] and spermidine homologs having the general structure $NH_2(CH_2)_nNH(CH_2)_{n'}NH_2$ [abbreviated, ${}_nTA_{n'}$ (for triamine), with ${}_3TA_4$ being spermidine itself] were accrued (*8*). Their ability to enter L1210 cells was evaluated according to three separate parameters.

1) The spermidine homologs competed effectively with [^{3}H]spermidine for uptake (Table 1). Although most of the spermidine homologs were in the same range of effectiveness, homospermidine (${}_4TA_4$) was clearly the most competitive, with an inhibition constant (K_i) of 3.5. The slightly better competition of ${}_3TA_8$ over other long-chain spermidine homologs could be due to its resemblance, in chain length, to spermine. The putrescine homologs competed poorly with spermidine, and their effectiveness

Table 1. Inhibition of [^{3}H]spermidine uptake into L1210 cells by polyamines or their homologs. The abbreviation for putrescine homologs having the general structure $NH_2(CH_2)_nNH_2$ is DA_n (for diamine) where n is 3 to 8. The abbreviation for spermidine homologs having the general structure $NH_2(CH_2)_nNH(CH_2)_{n'}NH_2$ is ${}_nTA_{n'}$ (for triamine) where n is 3 or 4 and n' is 3 to 8.

Homolog	K_i* (μ*M*)	[^{3}H]Spermidine uptake inhibition†	
		Picomoles per 10^7 cells-min	Percent of control
None		56.1	100
DA_3	> 500	54.0	96
DA_4 (putrescine)	171.3	44.6	80
DA_5	459.0	54.0	96
DA_6	63.2	40.1	71
DA_7	18.2	23.0	41
DA_8	22.1	25.2	45
${}_3TA_3$	8.4	16.1	29
${}_4TA_4$	3.5	7.3	13
${}_3TA_5$	12.3	19.8	35
${}_3TA_6$	13.1	19.6	35
${}_3TA_7$	13.0	20.0	36
${}_3TA_8$	7.8	13.5	24
Spermine	9.1	17.1	30

*Prewarmed L1210 cells (5×10^6) were incubated for 20 minutes in 1 ml of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10 μ*M* [^{3}H]spermidine and 100 μ*M* homolog. Uptake data were fitted by computer for competitive inhibition; the Michaelis constant for spermidine uptake was 2.0 μ*M*, and the maximum velocity of the reaction was 117 pmole/min per milligram of protein. †Cells were incubated for 20 minutes at 37°C with 10 μ*M* [^{3}H]spermidine plus 100 μ*M* putrescine or spermidine homolog.

seemed related to the similarity of their chain length to that of spermidine, so that DA_7 was most competitive and DA_3 the least competitive. It is possible that putrescine and its homologs enter the cell by a mechanism different from the spermidine carrier.

2) The spermidine homologs prevented cytotoxicity induced by the anticancer agent methylglyoxal bis(guanylhydrazone) (MGBG) to an extent quantitatively similar to that of their inhibition of [^{3}H]spermidine uptake (data not shown) (*9*). The rationale for this assay is based on evidence that MGBG utilizes the spermidine transport carrier mechanism and thus competes with spermidine for uptake (*10*).

3) The spermidine homologs, $_3TA_3$ through $_3TA_6$ and the putrescine homologs, DA_3 through DA_7, were quantitated intracellularly by high-performance liquid chromatography (HPLC) of the acid-soluble portion of cells incubated with DFMO and the homologs for 48 hours (Table 2). Although $_3TA_7$, $_3TA_8$, and DA_8 were also detected in significant amounts, their prolonged retention times made absolute quantitation unreliable. The diamines DA_5, DA_6, and DA_7 were converted biologically to aminopropyl derivatives, which on chromatography gave results identical with those of $_3TA_5$, $_3TA_6$, and $_3TA_7$, respectively. Neither the short diamine DA_3 nor the long diamine DA_8 were similarly metabolized by the cells. Taken together the results indicate that all of the putrescine and spermidine homologs enter L1210 cells and that the spermidine homologs probably utilize the spermidine transport mechanism to do so.

Spermidine itself ($_3TA_4$) completely prevented DFMO-induced cytostasis at concentrations of 1 μM, but not 0.1 μM (Table 3). Although exogenous spermidine fully supported growth, the intracellular pools of spermidine (420 pmole per 10^6 cells) were only about 15 percent of those (2785 pmole per 10^6 cells) of control cells (*11*). Apparently the basal level of intracellular spermidine represents a considerable excess, or most of it is effectively compartmentalized within the cell.

Although all of the homologs entered L1210 cells, only certain of them were able to substitute for spermidine in supporting growth during the 48-hour incubation in the presence of DFMO (Table 3). When the trimethylene portion of spermidine was kept constant, a two-carbon extension of the tetramethylene portion of the molecule was tolerated for function. Whereas $_3TA_5$ and $_3TA_6$ (at 10 μM) supported cell growth in the presence of DFMO, $_3TA_6$ (at 1 μM), $_3TA_7$, and $_3TA_8$ did not. By contrast, shortening of this same portion of the spermidine molecule by one carbon, resulted in a loss of biological activity as seen with norspermidine ($_3TA_3$) (Table 3), although this particular homolog was difficult to evaluate definitively because of inherent toxicity (*12*). Extension of the trimethylene portion of spermidine by one carbon was well tolerated since the symmetrical homolog, homospermidine ($_4TA_4$), substituted very effectively for spermidine. Spermine seemed unable to fulfill the biological function of spermidine since intracellular spermine pools were consistently elevated with DFMO treatment. Although DFMO-treated cells grew in the presence of exogenous spermine, this has been attributed to its limited conversion back to spermidine via polyamine oxidase (*4*). Indeed, about 15 percent of the normal spermidine pool was detected in cells incubated in DFMO and spermine (Table 2), as opposed to none in cells treated with DFMO alone. Polyamine oxidase appears to be inaccessible to cellular spermine pools, even

Table 2. HPLC analysis of polyamine pools in cultured L1210 cells treated with DFMO and polyamines or their homologs (1 μM). Cells were treated exactly as described in Table 3, then washed and extracted with 0.6*M* perchloric acid. HPLC analysis of the extract was as described in (*16*). DA_4, putrescine; $_3TA_4$, spermidine.

Treatment		Polyamines (picomoles per 10^6 cells)				
DFMO	Homolog	Putrescine	Spermidine	Spermine	Putrescine homolog	Spermidine homolog
None	None	441	2785	720		
1 m*M*	None			900		
1 m*M*	DA_3			1173	990	
1 m*M*	DA_4	33	928	1382		
1 m*M*	DA_5			824	30	390*
1 m*M*	DA_6			791	231	411*
1 m*M*	DA_7			963	168	208*
1 m*M*	DA_8			898	N.Q.†	
1 m*M*	$_3TA_3$			864		271
1 m*M*	$_3TA_4$		420	1177		
1 m*M*	$_4TA_4$			1001		582
1 m*M*	$_3TA_5$			968		649
1 m*M*	$_3TA_6$			681		253
1 m*M*	$_3TA_7$			936		N.Q.†
1 m*M*	$_3TA_8$			669		N.Q.†
1 m*M*	Spermine		411	1650		

*Chromatographed identically with $_3TA_5$, $_3TA_6$, or $_3TA_7$ in samples supplemented with these same homologs. †N.Q., not quantitated. Homolog or diamine was detectable as a flattened peak that could not be reliably quantitated.

Table 3. Effects of polyamines or their homologs on DFMO-induced cytostasis of cultured L1210 cells. Cells were treated for 48 hours with 1 m*M* DFMO and 1 or 10 μM putrescine or spermidine homolog. The final untreated cell number was 8.6×10^5. The experiments were repeated three times in duplicate. Experimental variation was usually not greater than 10 percent.

Homolog	Percent of control growth*			
	Without DFMO		+1 m*M* DFMO	
	1 μM	10 μM	1 μM	10 μM
None	100	100	55	55
DA_3	91	93	56	51
DA_4 (putrescine)	101	98	98	94
DA_5	96	92	52	75
DA_6	92	96	60	74
DA_7	98	98	52	50
DA_8	94	93	54	51
$_3TA_3$	70	43	56	40
$_3TA_4$ (spermidine)†	101	84	105	85
$_4TA_4$	94	94	87	81
$_3TA_5$	95	92	83	87
$_3TA_6$	96	96	66	82
$_3TA_7$	112	85	60	44
$_3TA_8$	98	53	55	23
Spermine	98	61	99	66

*Percent of control growth was derived as the final treated viable cell number minus initial inoculum divided by the final untreated viable cell number minus initial inoculum × 100. †At 0.1 μM, spermidine did not support growth in this system.

when the latter are elevated during DFMO treatment. Exogenous spermine, however, may gain access to the enzyme, perhaps through the lysosomal system. Alternatively, high intracellular levels of spermine achieved during treatment with DFMO and spermine could inhibit spermine synthase and thereby lead to an accumulation of spermidine.

Of the putrescine homologs, only DA_5 and DA_6 afforded some growth in the presence of DFMO, but this may be attributable to their conversion by the cell to $_3TA_5$ and $_3TA_6$, respectively. Apparently, the substrate specificity of spermidine synthase is broader than the structural specificity for biological function since the enzyme converted DA_7 to $_3DA_7$ (Table 2), but the latter did not support growth (Table 3). In no instance was there indication of further biological conversion of spermidine homologs to spermine homologs at a detection limit $\sim$ 5 pmole per 10^6 cells. The putrescine homolog DA_8 was not converted to a spermidine homolog. Despite having a chain length similar to that of spermidine, DA_8 did not support growth, suggesting that the central nitrogen is essential in spermidine function.

Similar data for prokaryotes has been obtained by measuring the growth of *Haemophilus parainfluenzae* (*13*) or polyamine auxotrophs of *Escherichia coli* (*14*) in media supplemented with spermidine homologs. As we have found with eukaryotes, the structural stringencies of prokaryotes for spermidine are not absolute, and limited variation in the tetramethylene moiety still permits biological function as indicated by growth. One striking difference between eukaryotes and prokaryotes is that norspermidine ($_3TA_3$) was not toxic to bacteria and substituted for spermidine in supporting growth (*14*).

Although polyamines influence various cellular reactions, particularly in vitro, many of these are nonspecific ionic interactions that probably would not impose structural constraints on the spermidine molecule. The interaction of spermidine with DNA, however, is potentially structure dependent. The spacing of the positively charged nitrogen atoms in the polyamines is particularly well suited to stabilize double-stranded regions of nucleic acids. Molecular models based on x-ray diffraction studies (*6*) indicate that the tetramethylene portion of spermidine can bridge the narrow groove of the helix, thus binding together phosphate groups on opposite double helical DNA strands, whereas the trimethylene moiety can bridge adjacent phosphate groups on the same strand. If this represents a critical biological function of polyamines, our study demonstrates that DNA exhibits unexpected tolerance for variation in the structure of spermidine. However, rotations of the cationic groups around the carbon–carbon and carbon–nitrogen bonds impart considerable conformational flexibility on these polycations, and this could account for the apparent allowances in the structural variability of spermidine.

Other intracellular functions of spermidine may also be essential for cell proliferation. Cellular protein synthesis, for example, is affected by polyamines at the transcriptional and translational levels (*14*), as are the structure and activity of transfer RNA (*15*). It may now be possible to obtain an indication of which of these or other spermidine functions are essential for cell proliferation by mapping the structural specificity of each and comparing it with that described in this report.

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9. Cells were treated for 24 hours with 1 μM MGBG in the presence and absence of 10 μM spermidine or spermidine homolog. Spermidine and spermidine homologs that competed effectively with [^{3}H]spermidine for uptake prevented MGBG-induced cytotoxicity. The putrescine homologs were not tested.
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Discrete Visual Defects in Pearl Mutant Mice

Abstract. *The mutant mouse pearl, characterized by its hypopigmentation, has a specific functional defect in a sensory system—the retina. The intact pearl mouse has reduced sensitivity in the dark-adapted condition. Normal sensitivity is restored by isolation and superfusion of the retina with bicarbonate-buffered Ringer solution, suggesting that the retinal expression of the pearl mutation depends on a diffusible substance. The pearl phenotype is described as a possible model for human congenital stationary night blindness.*

Successful analysis of neurological mutants of the mouse (*1*) depends on the ability to recognize defective phenotypes. Mutants with gross malformations or abnormalities of locomotion are easily detected; however, mutants with sensory defects are often indistinguishable from normal mice. Consequently, mutants such as reeler (*2*), dystonia musculorum (*3*), and retinal degeneration (*4*), as well as the coat-color mutants (*5, 6*), have been extensively examined, but mutants with sensory defects (*7*) have received little attention. We report here a mouse mutant that has no gross malformations or abnormalities of locomotion