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Evidence for a Copper:S-(Methylthio)-L-homocysteine Complex as a Glutamine Antagonist of Cytidine Triphosphate Synthesis in L1210 Murine Leukemia Cells

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SUMMARY

The mixed disulfide of methyl mercaptan and L-homocysteine, *S*-(methylthio)-L-homocysteine (L-SMETH), inhibits the growth of L-1210 leukemia cells in culture at micromolar concentrations. The inhibition is markedly promoted by added cupric ion, but not by ions of other metals, is stereospecific, and is competitive with glutamine. For example, at 10 μM each of L-SMETH and Cu^{2+}, almost complete growth inhibition was observed if cells were grown in 1 mM glutamine, 50% inhibition at 2 mM glutamine, and none at 4 mM glutamine. The inhibition is also completely relieved by cytidine in noncompetitive manner, but not by guanosine or uridine, indicating that the principal damage to the cellular economy resides in the amination of UTP to CTP. This was confirmed by high performance liquid chromatography analysis of cell extracts, which showed a marked decrease in CTP with *increases* in the levels of UTP, GTP, and ATP. A major swelling of cells leading to lysis accompanies the inhibition and increases in DNA and protein per cell confirms this unbalanced growth. The chemical basis for this biological interaction is presented.

SMETH was prepared to investigate its role as a prodrug for delivery of homocysteine to cells. It was modeled after the lower homolog, *S*-(methylthio)-cysteine (1), which upon intracellular reduction of the disulfide bond delivered cysteine to cells (2, 3). Similar delivery of homocysteine would promote the formation of *S*-adenosylhomocysteine, a potent inhibitor of cellular methylations (4) and projected end product for chemotherapy (5). Although SMETH was found to be a potent inhibitor of cellular proliferation, it did not function by the above anticipated mechanism. In this communication we describe its mode of action, together with copper, as a glutamine antagonist.

Materials and Methods

Preparation of SMETH. *S*-(Methylthio)-DL-homocysteine (DL-SMETH) was prepared from DL-homocysteine (Research Organics, Inc., Cleveland, OH) and methyl methanethiolsulfonate (Fairfield Chemical Co., Blythewood, SC) by a modification for the methylthiolation of L-cysteine as described by Smith *et al.* (6). The L-enantiomorph, L-SMETH, was prepared after reduction of L-homocystine (Sigma Chemical Co., St. Louis, MO) with sodium in liquid ammonia. The excess sodium was removed by addition of ammonium chloride and the ammonia was evaporated at room temperature under nitrogen. The residue was dissolved in oxygen-free water and the solution was neutralized to pH 6.5–7.0 with hydrochloric acid. A 1.25 molar excess of methyl methanethiolsulfonate in ethanol was added slowly and the product crystallized after 2 hr in an ice bath. The crystals were washed with ethanol and peroxide-free ether and dried over phosphorus pentoxide. Analysis (performed by Atlantic Microlab, Atlanta, GA) for SMETH; calculated, C, 33.13; H, 6.12; N, 7.73; S, 35.37; found, for DL-SMETH, C, 33.21; H, 6.12; N, 7.71; S, 35.28; for L-SMETH C, 33.19; H, 6.12; N, 7.68; S, 35.30. The racemate was resolved into two peaks and the chiral purity of L-SMETH was determined to be at least 99.9% by column chromatography with a resolving column. The assay was performed by Daicel Chemical Industries, (Himeji, Japan) through the courtesy of Dr. Hisao Nishimura of the New York office.

Growth and its inhibition in cell culture. The L1210 established murine leukemia line was maintained in RPMI 1630 medium (Quality Biologicals, Gaithersburg, MD) containing 16.5% fetal bovine serum (Advanced Biotechnologies, Silver Spring, MD) and gentamycin (Schering Corp, Kenilworth, NJ), 40 μg/ml. Cytotoxicity of SMETH was assessed as follows; cells were harvested at mid-log phase (8–10 × 10^5 cells/ml) washed with fresh growth medium and resuspended at 1 × 10^5 cells per ml as determined by a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Suspensions (7 ml) were added to 25-cm^2 Corning flasks and SMETH, 20 mM in water, with or without copper sulfate, 2 mM in water, were added as dilutions in water at no greater than 10 μl/ml of the cell suspension.

Cells were grown at 37° for indicated times in tightly stoppered flasks and cell density was determined as indicated above. Cell volume distribution was monitored with a C1000 Channelyzer with standard size latex particles (Coulter) as reference. The results are expressed as growth fraction ($N - N_o/N_o$) or as per cent growth fraction compared with appropriate controls.

Analysis of nucleoside triphosphate pools. For analytical stud-

ABBREVIATION: SMETH, *S*-(methylthio)-homocysteine.

ies, the total incubation volume was increased to 100 ml in 175-cm² flasks, with the same proportions of cells and solutions described for the smaller incubation volumes. At the appropriate time, growth was terminated by shaking the flasks in ice and all further steps were performed with ice-cold reagents and under refrigerated conditions. On reaching 4°, the cells were pelleted in a refrigerated centrifuge and extracted with 500 μl of 10% trichloroacetic acid. The precipitate was sedimented in a microcentrifuge tube, the supernatant fluid was transferred to another such tube and extracted vigorously with an equal volume of Freon (1,1,2-trichloro-1,2,2,-trifluoroethane) containing tri-*n*-octylamine in 4:1 proportions by volume as described by Khym (7). The supernatant fluid was removed and 200 μl were analyzed by HPLC with the use of a Whatman 5SAX column (12.5 × 0.4 cm) and ammonium phosphate, pH 3.5, gradient (0.02 M to 0.7 M) over 40 min. The detection of components was made by UV absorbance at 254 nm.

Flow cytometric analysis. After an incubation, cells were fixed and stained as described by Crissman *et al.* (8). Samples were analyzed on a Becton-Dickinson FACS 440 flow cytometer (Mountain View, CA). The argon-ion laser (Coherent Innova 90-5, Palo Alto, CA) was tuned to 488 nm and operated at a power output of 200 mW in the light-stabilized mode. Fluorescein isothiocyanate fluorescence was determined with the use of a 535/15 band-pass filter and propidium iodide fluorescence with a 630/22 band-pass filter. Data from 2 × 10⁴ cells were collected from each sample and analyzed in a Becton-Dickinson Consort 40 computer system.

Results

SMETH toxicity and copper potentiation. SMETH was cytotoxic to L1210 cells in culture when present at a broad range of micromolar concentrations. The range of inhibitory concentration was both reduced and narrowed in the presence of copper ion (Fig. 1). This ion was very effective at micromolar concentrations in bringing a threshold level of SMETH (25 μM) to a completely inhibitory concentration (Fig. 2), while being nontoxic itself at much higher levels (Fig. 3 of Ref. 9). A concentration of 10 μM was more than adequate as a potentiating dose, but at this concentration other metal ions (Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Cr^{3+}) were ineffective (data not shown). Of interest is the fact that the lower homolog of SMETH, *S*-

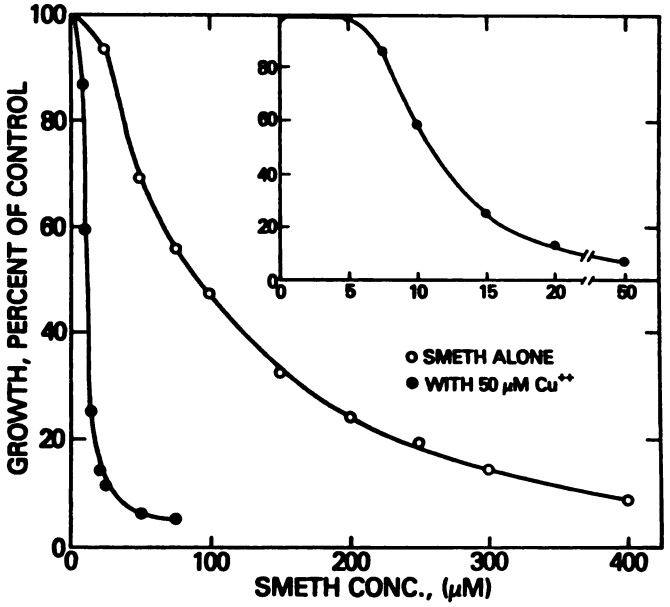

Fig. 1. Inhibition of growth of L1210 cells in culture by DL-SMETH and its potentiation by copper ion. The cells were incubated for 40 hr as described in Materials and Methods.

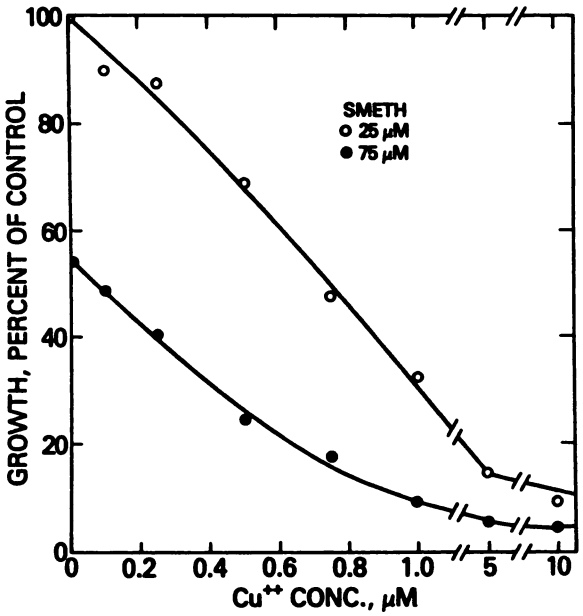

Fig. 2. Potentiation of the inhibitory activity of a threshold and ID_{50} concentration of DL-SMETH by copper ion. The cells were incubated as described in Fig. 1.

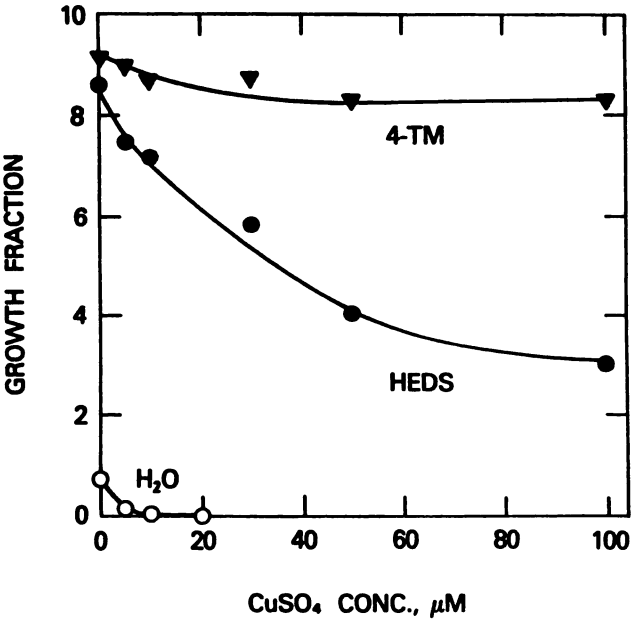

Fig. 3. The lower homolog of SMETH protects L1210 cells in primary culture from copper-induced toxicity. The incubation was performed as in Fig. 2, except that the L1210 cells were obtained directly from the mouse (10). Such cells fail to grow unless supplemented with an appropriate mercaptan or disulfide (○). Hydroxyethyl disulfide (*HEDS*), the oxidized form of mercaptoethanol, promoted growth, but this was inhibited by high copper concentrations (●). *S*-(methylthio)-L-cysteine, also termed 4-thiamethionine (*4TM*), at a concentration of 50 μM, supported growth that was independent of copper ion concentration (▼).

(methylthio)-L-cysteine or 4-thiamethionine, is not cytotoxic in the presence of copper and actually protected L1210 cells in primary culture from growth inhibition by copper (Fig. 3) due to depletion of cysteine (10). Thus, both the organic and inorganic moieties of this combination show high specificity in this inhibition.

Stereospecificity. The racemic form of SMETH was half

as active as L-SMETH (Fig. 4). D-SMETH was completely inactive at 50 μM with 50 μM copper ion.

SMETH toxicity is not due to homocysteine delivery. Although SMETH was originally synthesized as a prodrug to deliver homocysteine to cells, inhibition analysis indicated that cytotoxicity was not due to this mechanism. Such toxicity has been reported for homocysteine thiolactone, which in combination with added adenosine and an adenosine deaminase inhibitor such as deoxycoformycin can block growth due to adenosylhomocysteine formation (11). Adenosylhomocysteine is a potent inhibitor of cellular methylation processes and, as an endogenous product of methionine metabolism trapped intracellularly by added adenosine, is the reported basis for adenosine toxicity (12). We have evaluated this toxicity of adenosine across a concentration range of 5 to 40 μM. At 10 μM it was not toxic to L1210 cells but was when added together with a nontoxic concentration of L-homocysteine thiolactone. Adenosine, however, did not increase the potency of a toxic dose of SMETH (Table 1). The failure of adenosine to promote SMETH toxicity suggested that such toxicity was not due to adenosylhomocysteine formation.

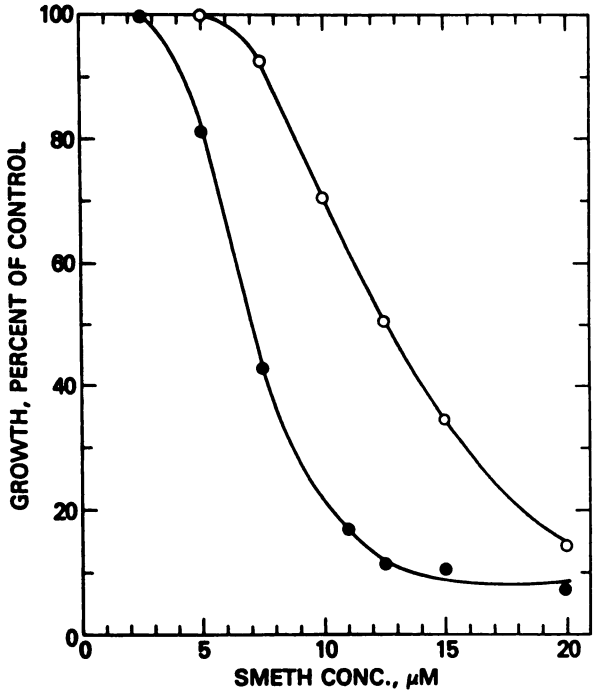

Fig. 4. The cytotoxicity of SMETH is stereospecific. The incubation was performed with copper ion at 50 μM as described in Fig. 1, with DL-SMETH (○) and with the pure L-enantiomorph, L-SMETH (●). The use of 10 μM copper sulfate gave nearly identical results.

TABLE 1

Adenosine does not potentiate SMETH cytotoxicity

Per cent inhibition is determined from the percentage of growth fraction obtained with and without the compounds indicated.

	Growth Inhibition
	%
L-Homocysteine thiolactone (200 μM)	
Alone	5
Plus adenosine (10 μM) and deoxycoformycin (20 μM)	39
DL-SMETH (75 μM)	
Alone	46
Plus adenosine (10 μM) and deoxycoformycin (20 μM)	46

Glutamine protection against SMETH toxicity. Glutamine, at millimolar concentrations, which supported growth, protected cells against SMETH and SMETH plus Cu^{2+} inhibition (Table 2). At these concentrations the full range from almost complete inhibition to complete protection is evident. This type of protection was not seen with other amino acids, some having a closer structural resemblance to SMETH. In fact, as can be seen in Table 3, such amino acids promoted SMETH toxicity. This promotion of inhibitory activity may be due to the phenomenon termed "trans-stimulation of uptake," which is common in the amino acid series (13). Such increased uptake would increase cytotoxity; further analysis of this problem would require radioactive material.

Amination of uridine to cytidine as site of inhibition. Among the several biochemical roles of glutamine, that involving the amination of UTP to CTP was the only locus blocked by copper-SMETH. This conclusion was sustained by two observations. 1) Cytidine alone protected the cells from growth inhibition and this protection was noncompetitive, equivalent concentrations of cytidine being equally effective at two concentrations of copper-SMETH that gave maximal growth inhibition (Fig. 5). Uridine and guanosine were ineffective in such protection. 2) HPLC analysis of cells inhibited in growth showed greater than a one-third diminution of CTP content but a 2-fold elevation of UTP, ATP, and GTP (Fig. 6). Other uncharacterized peaks were also elevated in the inhibited sample. Such elevated levels of cellular constituents may be due to the increased volume of inhibited cells as described below.

Cell expansion and unbalanced growth. Characteristic of SMETH and copper-SMETH growth inhibition is the progressive increase in cell volume observed over a 2-day period (Fig. 7). Ultimately the cells burst, as indicated by the accumulation of debris shown near the ordinate. Flow cytometric analysis of control and inhibited swollen cells showed increases in fluorescein isothiocyanate staining and propidium iodide

TABLE 2

Glutamine protects L1210 cells from growth inhibition by SMETH and cooper-SMETH in a competitive manner

Glutamine	Growth	
	L-SMETH (50 μM)	L-SMETH (10 μM) plus Cu^{2+} (10 μM)
mM	% of Control	
0.5	15	0
1.0	31	7.7
2.0	58	58
4.0	98	100

TABLE 3

Potentiation of growth inhibition by SMETH with amino acids

	Concentration for 50% additonalinhibition[a]
	mM
L-Leucine	1
S-Ethyl-L-cysteine	1
DL-Isopropionine	2
L-Methionine	2
L-Norleucine	2.5

[a] Estimated by interpolation. The DL-SMETH concentration was 75 or 100 μM.

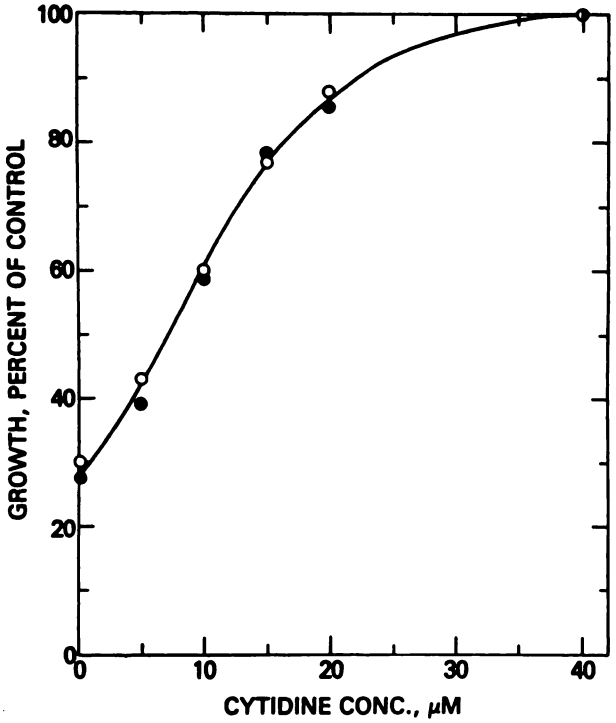

Fig. 5. Cytidine protects L1210 cells from the cytotoxicity of L-SMETH and copper ion. The cells were incubated with L-SMETH 15 μM (○) and 30 μM (●), together with their corresponding concentrations of copper sulfate.

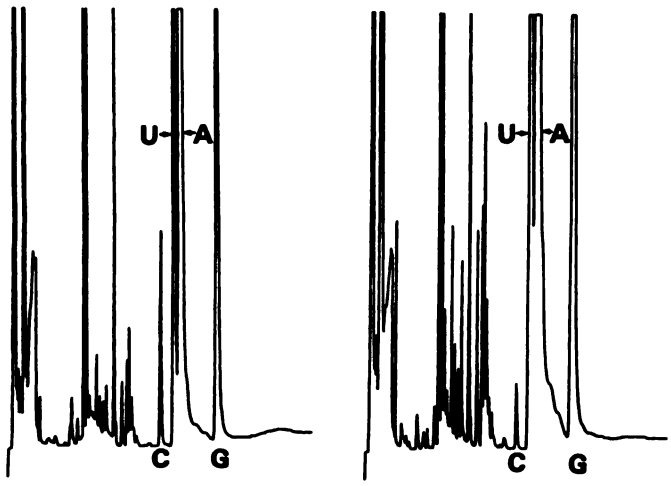

Fig. 6. Analysis of the nucleoside triphosphate content of cells incubated without and with L-SMETH and copper ion. The cells were incubated for 14 hr in the medium described under Materials and Methods but containing only 1 mM glutamine, half the normal concentration. The inhibited culture also contained 15 μM each of SMETH and copper sulfate. At the end of the incubation, the cell density in the inhibited culture was determined, and the entire population was centrifuged for analysis. An aliquot containing an equal number of cells from the control culture was removed for comparison, and both samples were processed and analyzed as described in Materials and Methods. *Left*, controls; *right*, inhibited culture. *Letters* under their corresponding peaks indicate the separated nucleotide triphosphate shown below, followed by retention times and the effect of copper-SMETH. *C*, cytidine triphosphate, 20.0 min, decreased to 29% of control; *U*, uridine triphosphate, 21.3 min, increased to 233% of control; *A*, adenosine triphosphate, 22.6 min, increased to 196% of control; *G*, guanosine triphosphate, 27.5 min, increased to 186% of control. The modal volume of the control cells was 820 μm^3 and that of the inhibited cells 1140 μm^3.

staining in the latter, which are indicative of increases in protein and DNA per cell, respectively (Fig. 8).

Discussion

Homocystine and metal binding. Cecil and McPhee (14) observed that homocystine reacted with silver ion at a rate 267 times that of cystine and that this increased rate was dependent upon the presence of unsubstituted amino groups. They indicated (15) that the metal ion was bound by ammine formation and therefore brought closer to the disulfide bond. Appropriate configuration required that two methylene groups were spaced between the unsubstituted amino group and the disulfide, possibly to allow hydrogen bond formation in the unsubstituted amino acid. Thus, a similar reaction rate did not occur with cystine. It is noteworthy that SMETH has the same amino to disulfide configuration as homocystine and that the cuprous ion has nearly identical reactivities to those of the silver ion (16).

Copper-disulfide bonding and reactivity. The coordination between the cuprous ions and a symmetrical disulfide as described by Ottersen *et al.* (17) involves a lengthening of the disulfide bond and thus its weakening.

$$Cu^{+} + RSSR \rightarrow RS\overset{+}{\underset{|}{S}}R \quad (Cu)$$

The $-\overset{+}{\underset{|}{S}}-R$ (Cu) moiety thus becomes a much better leaving group than the original -SR. The sulfur-sulfur bond becomes more susceptible to nucleophilic attack, and the structure may be considered an example of an intermediate in concomitant electrophilic and nucleophilic catalysis of the scission of this bond as described by Kice (18, 19).

$$Nu^{-} + RS\overset{\curvearrowright}{\underset{|}{S^{+}}}R \rightarrow RSNu + RSCu \quad (Cu)$$

Relation to reaction with the enzyme active site. The positioning of glutamine in CTP synthetase relative to its reactive thiolate anion can be represented diagramatically as indicated by Levitzki (20) in Fig. 9, *left*. In this representation the glutamine subsequently loses its amide group and reacts to form a thio-ester. A corresponding positioning of copper-SMETH is shown in the Fig. 9, *right*. This positioning is dependent upon the "natural" L-configuration of SMETH and places the nucleophilic thiolate ion in close proximity to the sulfonium moiety of the copper disulfide function. In accordance with the reactions described above, the cuprous sulfide of homocysteine would act as the leaving group and the enzyme would be methylthiolated. The possibility that migration of the copper to the sulfur of the methylthio-moiety of SMETH would make the methylthio-moiety the leaving group cannot be ignored at this stage of the investigation.

Comparison with other glutamine amidotransferase inhibitors. The activity of copper-SMETH differs from that of the natural amidotransferase inhibitors, azaserine, diazo-oxo-norleucine, and acivicin, in that only one critical cellular pathway is blocked, the amination of UTP to CTP. The other

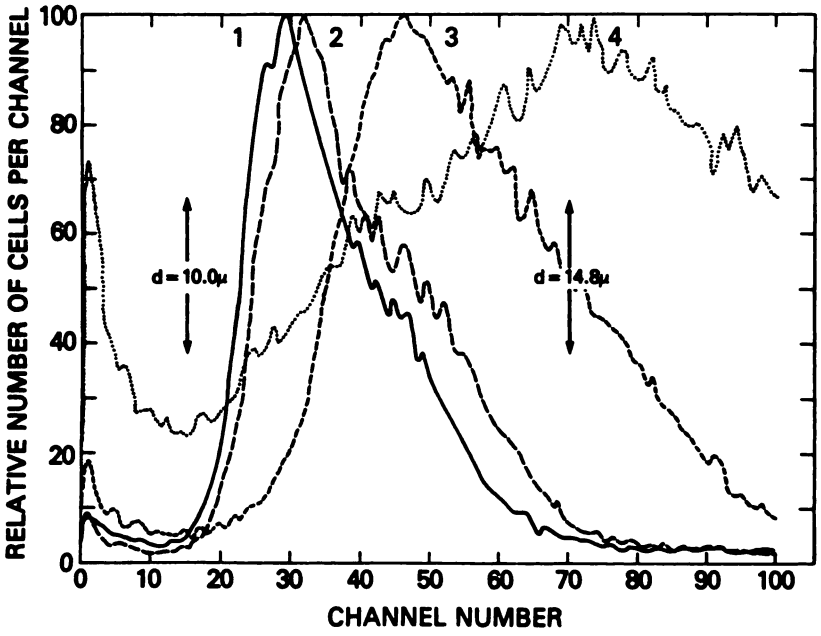

Fig. 7. Progression of volume increase and lysis of L1210 cells from inhibited cultures. Cells were incubated under standard conditions with L-SMETH and copper sulfate, each 15 μM, and cell volume was monitored during the course of the incubation as described in Materials and Methods. *1*, control cells, modal volume in μm^3, 870; *2*, with inhibitor for 16 hr, 915; *3*, for 24 hr, 1270; and *4*, for 40 hr, 1820.

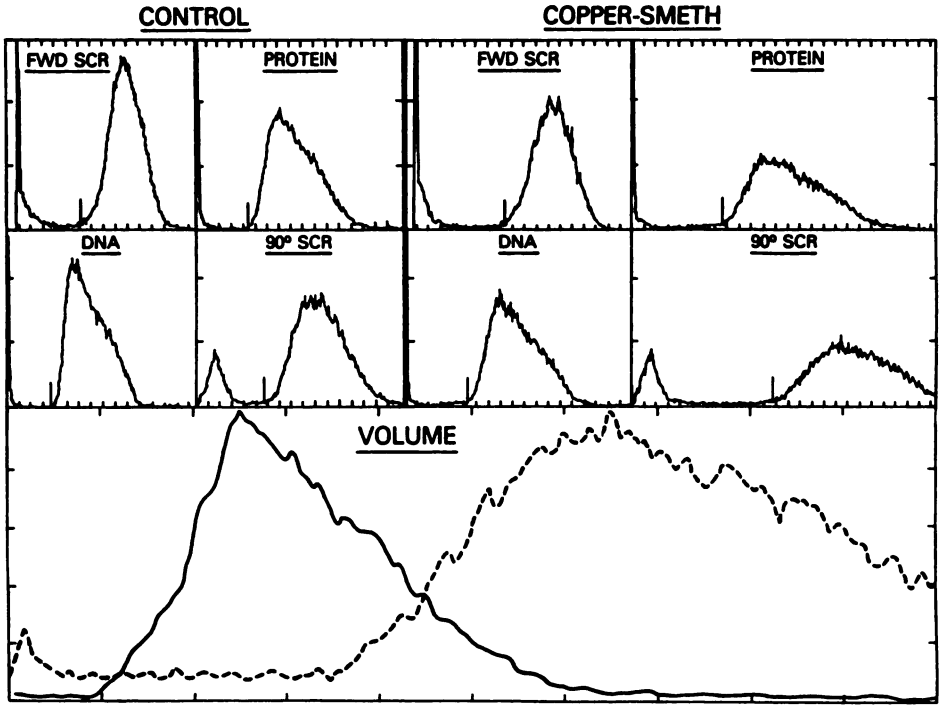

Fig. 8. Flow cytometric analysis of L1210 cells from cultures inhibited with SMETH and copper ion. The cells were incubated for 24 hr without and with L-SMETH and copper sulfate as described in Fig. 6. At the end of the incubation, cell number and cell volume distribution (*lower*) were determined and an equal number of cells were removed for staining and analysis as described in Materials and Methods. Over 1×10^4 cells were analyzed and inhibited cells showed a 60% increase in protein, a 35% increase in DNA, and a 100% increase in volume. *FWD SCR*, forward scatter; *90° SCR*, 90° scatter of light. *Abscissa*, magnitude of item shown in panel; *ordinate*, relative number of cells. The correspondence of protein distribution and 90° scatter with volume distribution of the cells is particularly striking.

inhibitors also block some sites in purine biosynthesis (21), including the amination step for the synthesis of guanosine monophosphate (22, 23). We have no explanation for this difference. It should be noted, however, that the reactive center of copper-SMETH is internal (Fig. 9) whereas in the other glutamine analogs the reactive site may be considered terminal. This introduces the concept of bulk tolerance in inhibitor specificity, for the methylthio- and copper moieties must be acceptable within the reactive site of the enzyme. This factor could be evaluated by determining the potency of copper-SMETH as an inhibitor for the other glutamine amidotransferases. Also, groups larger than methyl can be introduced in the mixed disulfide, to yet further challenge the bulk tolerance of the enzymes from different tissues.

Possible relationship between the biochemical and cellular lesions. Homocysteine has been shown to promote endothelial cell damage via copper-catalyzed hydrogen peroxide generation (24) and to be toxic by blocking methionine metabolism via its adenosyl derivative (25); homocysteine thiolactone may be toxic due to acylations of cellular constituents by the reactive thiolactone moiety (26). Because cells can be protected from SMETH and copper-SMETH inhibitions by glutamine and cytidine, these possible alternative mechanisms of cytotoxicity may be considered inoperative in our system.

The marked swelling and lysis from the SMETH- and copper-SMETH-promoted CTP deficiency may be a consequence of a block in phospholipid biosynthesis for plasma membrane generation (27). This appears probable because we report that

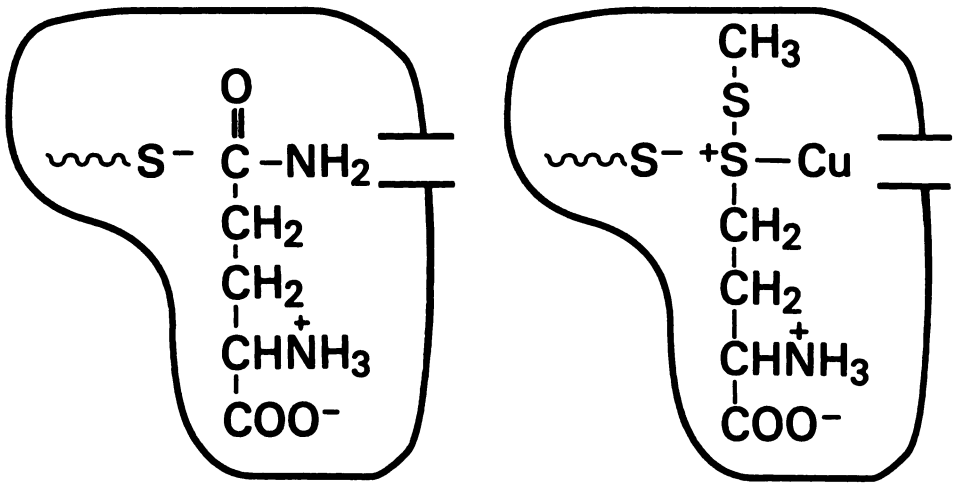

Fig. 9. Diagrammatic representation of glutamine and copper-SMETH at the enzyme reactive site. *Left*, glutamine; *right*, copper-SMETH.

DNA and protein synthesis had continued in the inhibited swollen cells and because of the observation (27) that the K_m of the reactions involving CTP in phospholipid biosynthesis are higher than those of nucleic acid formation. Thus, as the availability of CTP becomes limiting, a block in phospholipid biosynthesis would be the first to become evident and could result in unbalanced growth, membrane pathology, swelling, and lysis.

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