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The Role of Metals in the Antitumor Action of 1,5-Bisthiosemicarbazones

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SUMMARY

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A recent study of a new series of bisthiosemicarbazones showed that optimal inhibition of DNA synthesis in Leukemia 1210 cells was obtained with derivatives of 1,5-dicarbonyl compounds. In the present study these compounds were highly efficient scavengers of copper. Facilitation of uptake of Cu^{++} via chelation was associated with drug toxicity. Studies using a ^{35}S-labeled drug, $^{63}Ni^{++}$, and $^{64}Cu^{++}$ showed that metal chelates readily penetrated L1210 cells, where the metal was tightly bound. The drug then diffused from the cells to shuttle more metal ions inward. Chelation strongly potentiated the lipophilicity of both drug and metal, as shown by water/octanol partition studies. Some drugs were such potent scavengers of copper that drug toxicity was only abolished by addition of EDTA to "copper-free" media. Of the drugs studied, most could chelate both nickel and copper, and a few, only copper. The capacity for inhibiting synthesis of DNA was correlated with copper chelation and facilitation of uptake of Cu^{++}. The bisthiosemicarbazones of 1,5-dicarbonyl compounds were more effective agents for promotion of Cu^{++} uptake by L1210 cells than the analogous derivatives of α-ketoaldehydes, which have been extensively studied.

INTRODUCTION

The antitumor action of several 1,2-bisthiosemicarbazones, notably 3-ethoxy-2-oxobutyraldehyde bisthiosemicarbazone, has been described (1-3). KTS[2] is an effective agent in several experimental animal tumors. The compound is apparently ineffective unless metal ions are present, the most effective being copper (2-5). The nature of interactions between metal ions and KTS has been studied (5-9), as have the effects of the copper chelate of the drug on various metabolic processes in mammalian cells (1, 4, 10-13). We recently (14) described a series of bisthiosemicarbazones synthesized from 1,4-, 1,5-, and 1,6-dicarbonyl compounds (15, 16). These agents showed antitumor activity against murine Sarcoma 180 (15, 16). This series of compounds is structurally related to KTS (Fig. 1), but additional carbon or carbon and sulfur atoms are placed between the thio-

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[2]The abbreviations used are: KTS, 3-ethoxy-2-oxobutyraldehyde bisthiosemicarbazone; TES-E (N-tris-hydroxymethyl-2-aminoethane sulfonic acid buffer described in Ref 25); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid.

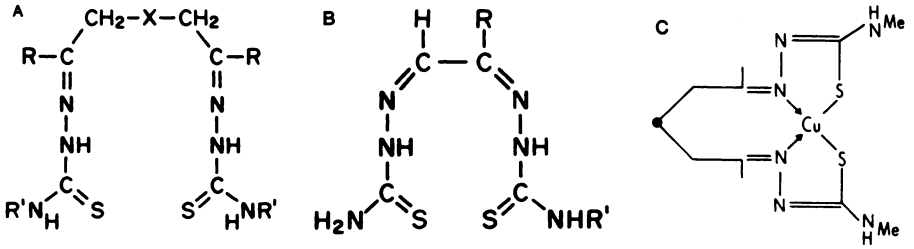

FIG. 1. *Structures of bisthiosemicarbazones*

A. 1,4-, 1,5-, and 1,6-Dicarbonyl derivatives B. 1,2-Dicarbonyl derivatives. For KTS, R = C_2H_5OCH—and R' = H. For B-1594, R = R' = CH_3. C. Copper chelate of B-1600 (● = CH_3) and B-2844 (● = S).

semicarbazone moieties. Unlike KTS, certain of the new series of bisthiosemicarbazones show a capacity for inhibition of DNA synthesis in "metal-free" media (14). These data suggest that 1,5-dicarbonyl bisthiosemicarbazones might be more potent scavengers of Cu^{++} than the 1,2-dicarbonyl analogues, such as KTS.

In the present study we have examined the properties of several bisthiosemicarbazones, including two labeled with ^{35}S. The kinetics of uptake and exodus of labeled drug, $^{63}Ni^{++}$, $^{64}Cu^{++}$, and various combinations of labeled and nonlabeled drugs and metal ions were studied using Leukemia 1210 cells. Determinants of rate of accumulation of metal ions and drugs were delineated. The capacity for chelation of copper into a lipid-soluble complex was a major determinant of drug action.

MATERIALS AND METHODS

Organic synthesis. Synthesis of these bisthiosemicarbazones has been described (15–18). Purity was tested by infrared and ultraviolet spectra and elemental analyses. Chelates of diketone derivatives were prepared by the addition of equimolar aqueous solutions of cupric acetate to bisthiosemicarbazone dissolved in *N,N'*-dimethylformamide, and by heating suspensions of bisthiosemicarbazones in aqueous methanolic alkali containing salts of nickel, cobalt, cadmium, and zinc.[3] The insoluble products were usually recrystalized from ethyl acetate. Elemental analyses showed that in every case 2 hydrogen atoms had been replaced by 1 metal atom. The structures revealed by x-ray crystallography may be analogous, as shown in Fig. 1C, to those established by X-ray studies for the nickel chelate of the corresponding 1,4-bisthiosemicarbazone (19, 20). Other preparations of metal chelates of bisthiosemicarbazones have been described (21–23).

Radioactive bisthiosemicarbazones. Labeled preparations of two drugs were made using an existing procedure (17). To prepare ^{35}S-labeled B-2844 (Fig. 1A; X = ^{35}S, R = R' = CH_3), a mixture of 20 μl of freshly distilled chloroacetone, 40 μl of benzene, and 10 μl of water was chilled to 0° in a 10 × 30 mm tube and mixed at this temperature with a 5-mm magnetic stirrer. A solution of 32 mg of $Na_2{}^{35}S$ (1 mCi) in 50 μl of water was added in 10-μl portions over 20 min. Four minutes after the last addition, 250 μl of water and 250 μl of benzene were added, the solution was mixed, and the benzene layer was removed. The aqueous layer was extracted with 250 μl of benzene, and the benzene extracts were shaken first with 500 μl of water and then with 500 μl of water saturated with NaCl. The benzene solution was dried over $MgSO_4$, the solvent was removed under vacuum, and the residue was taken up in 30 μl of methanol. A solution of 20 mg of 4-methylthiosemicarbazide (Aldrich Chemical Company) in 100 μl of water and 10 μl of glacial acetic acid was added. The product precipitated after chilling, and was recrystallized from 100 μl of warm dimethylformamide by addition of 200 μl of warm water. Yields were in the 8–10-mg range, and the product was characterized by its ultraviolet spectrum and mixed melting point determination with an authentic drug sample.

[3] M. Gannon, J. E. McCormick, and R. S. McElhinney, manuscript in preparation.

To prepare labeled B-2890 (Fig. 1A; X = ^{35}S, R = CH_3, R' = H), the procedure described above was followed except that 17 mg of thiosemicarbazide instead of the 4-methyl derivative were used.

Cell cultures. Murine L1210 cells were grown (24) in sealed flasks using Eagle's minimal essential medium (spinner modification) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Media were purchased from Grand Island Biological Company. Cells were collected by centrifugation when densities of 5–7 $\times$ 10^5/ml were reached. The cells were then suspended in fresh medium (7×10^6 cells/ml). Two isotonic solutions were employed for this purpose: the metal-free TES-E buffer (25), which approximates in ionic composition the ascitic fluid of the mouse and Eagle's minimal essential medium plus 10% fetal calf serum, with the bicarbonate replaced with HEPES buffer (26) at pH 7.3. Using either medium, cell suspensions could be held at 37° for at least 45 min with a decrease in pH of less than 0.02. The modified Eagle's medium[4] was used in all experiments except where specified.

Drug effects on incorporation of thymidine into DNA. This procedure has been described (14). Fresh cell suspensions in modified Eagle's medium, as described above, were held at 37° for 20 min. Drug or drug chelates, dissolved in dimethylformamide, were added, together with metal salts as indicated. The total addition of dimethylformamide did not exceed 10 μl/ml of suspensions, a level which did not affect incorporation of thymidine into DNA.

Ten minutes after addition of drug, [2-^{14}C]thymidine (final level, 0.5 μM; 0.02 μCi/ml of cell suspension) was added. For most experiments incubations were terminated 10 min later. The suspensions were chilled, and incorporation of radioactivity into material insoluble in 0.3 M $HClO_4$ was determined by liquid scintillation counting.

Cell cultures used in these studies consisted of 95% viable cells by dye exclusion studies. Suspensions of 6×10^6 cells incubated with 0.02 μCi of labeled thymidine in Eagle's modified medium incorporated 9000 $\pm$ 1000 cpm into DNA in 10 min. In TES-E medium incorporation was reduced to 2000 $\pm$ 200 cpm.

Uptake and exodus of labeled drugs and metals. Cells were incubated together with a specified level (usually 1–20 μM) of labeled drug or metal. Nonradioactive drug or metal was present as indicated. The rate of uptake of label was measured as a function of extracellular concentration of labeled compound, time, and temperature. We found that brief washing of cells at 0° with 0.9% NaCl did not remove detectable amounts of radioactive drug or metal ions. Cell pellets could thereby be freed from extracellular radioactivity. Cell pellets were suspended in 0.9% NaCl (0.5 ml), and radioactivity was measured by liquid scintillation counting.

To measure exodus of labeled materials, cells were loaded at 0° or 37° and then suspended in fresh medium at 0° or 37°. At intervals aliquots of the cell suspension were removed. The cells were collected, and intracellular radioactivity was measured.

Partition studies. An equilibrated mixture of 1-octanol and water was used. One-half-milliliter portions of each layer were placed in small tubes, along with 2-μl portions of labeled drug or metal (10 mM stock solutions). The tubes were shaken for 10 sec, and the layers were separated by centrifugation at room temperature for 30 sec. Samples (50 μl) from the layers were removed, and radioactivity was measured. In some experiments the chelation reaction was allowed to proceed in the aqueous layer before addition of octanol. The results are reported in terms of the ratio (P_c) of the concentration in the organic layer, to the concentration in the aqueous layer.

Specific activities. The initial specific activities of labeled drugs, ^{64}Cu, and ^{63}Ni were 10 mCi/mmole, 30 mCi/mg, and 3 mCi/mg, respectively.

RESULTS

Drug-induced inhibition of DNA synthesis in L1210 cells. Drug levels which inhibited DNA synthesis by 50% 10 min after

[4]We estimate the Cu^{++} content of current batches of this solution to be approximately 0.1 μM, using the procedure described in ref. 27.

their addition to cell suspensions are defined here as ID_{50} values. These values depended markedly on copper levels in the medium. In the present study the Cu^{++} level was held to 0.1 μM in the absence of added metal, and the results differ somewhat from data obtained previously (14), before the importance of this parameter was appreciated. Even in "copper-free" medium (TES-E buffer, metal-free components, deionized water), drug-induced inhibition of DNA synthesis was observed. It could, however, be prevented by the addition of EDTA (Table 1).

The reversal of drug toxicity by EDTA was demonstrable only when trace levels of Cu^{++} were present; EDTA at 10 μM did not protect DNA synthesis from the inhibitory effects of a mixture of 1 μM Cu^{++} and 1 μM drug (Table 1).

Effect of metals on drug-induced inhibition of DNA synthesis. Several transition metals altered the effects of different drugs on DNA synthesis. Drugs were selected from the group previously examined (14) and included compounds varying widely in capacity for inhibition of DNA synthesis in the absence of added metal. The data (Table 2) show that Cu^{++} was the most effective metal; Zn^{++}, Cd^{++}, and Ni^{++} were less effective, and Co^{++} antagonized at least one drug (B-2913). Nickel potentiated only the action of drugs with 1 or more divalent sulfur atoms at the X position (Fig. 1A), i.e., B-2844 and B-2913. Copper potentiated the action of all drugs, including some (B-2087 and B-1207) inactive at levels employed here when no metal was added.

Uptake and exodus of labeled drugs. These studies were carried out using two compounds readily synthesized in labeled form, B-2844 and B-2890. Their properties are summarized in Table 3. B-2844, with four substituent methyl groups, was the more effective inhibitor of DNA synthesis

TABLE 1

Effects of metal ions on drug-induced inhibition of DNA synthesis

Cells were incubated with drug and other components for 10 min; then labeled thymidine was added for an additional 10 min. For the structure of B-2844, see Fig. 1A (X = S, R = R' = CH_3). In the absence of added $CuCl_2$, the copper level of Eagle's modified medium was 0.1 μM, and the level in TES-E medium was undetectable.

Additions	DNA synthesis % of Control	
	Eagle's modified medium	TES-E medium
B-2844, 1 μM	75	85
B-2844, 1 μM; EDTA 10 μM	100	100
B-2844, 10 μM	35	45
B-2844, 10 μM; EDTA, 10 μM	97	100
$CuCl_2$, 1 μM	100	100
B-2844, 1 μM; $CuCl_2$, 1 μM	32	38
B-2844, 1 μM; $CuCl_2$, 1 μM; EDTA, 10 μM	34	40

TABLE 2

Promotion of drug-induced inhibition of DNA synthesis by transition metals

DNA synthesis was measured as incorporation of labeled thymidine in 10 min at 37°. Cells were incubated with 10 μM drug plus 10 μM metal ion as described in the legend to Table 1.

Drug	Structure			DNA synthesis					
	X	R	R'	None metal	Cu^{++}	Cd^{++}	Zn^{++}	Ni^{++}	Co^{++}
B-2844	S	Me	Me	20	1		6	15	25
B-1600	C	Me	Me	45	2	5	16	45	47
B-1207	—[a]	Me	Me	100	10	50	85	100	
B-3164	SO_2	Me	Me	47	3		42	45	50
B-2087	SO_2	H	Me	100	10		90	100	100
B-2913	SS	Me	Me	30	1		12	14	100

[a] No substituent at the X position.

TABLE 3

Comparison of two bisthiosemicarbazones labeled with ^{35}S

Structures are shown in Fig. 1A. The octanol/water partition coefficient, P_c, is shown. To obtain distribution ratios of drug (intracellular to extracellular level), cells were incubated with 1 μM drug for 1 min. Values were not altered over 10 min. ID_{50} values are drug levels required for 50% inhibition of DNA synthesis in Eagle's modified medium.

Drug	Structure			P_c	Distribution ratio		ID_{50}
	X	R	R'		0°	37°	
							μM
B-2844	S	CH_3	CH_3	25	2.4	1.1	7
B-2890	S	CH_3	H	5	4.2	2.9	22

in the absence of added metal. Octanol/water partition showed B-2844 to be the more lipophilic compound. B-2890, however, was more extensively accumulated by L1210 cells. Drug uptake is therefore not a major determinant of drug action. Uptake of both drugs was rapid, with equilibrium attained within 1 min. These data are also shown in Fig. 2. More drug was accumulated at 0° than at 37°. We interpret this to indicate a temperature-sensitive exodus of drug which is inoperative at 0°, while the uptake process is much less temperature-sensitive. In other experiments, we found that drug exodus from loaded cells was negligible at 0° but proceeded rapidly at 37° (Fig. 2). None of these results was altered by the presence of 10 mM EDTA.

Promotion of drug accumulation by metal ions. The observed uptake of labeled drug might be due in part to adherence of a lipid-soluble compound to the cell surface. It is not known whether such material would be removed by a brief wash with cold 0.9% NaCl.

The uptake of ^{35}S-labeled 8-2844 was strongly promoted by Cu^{++}. (The structure of the drug is shown in Fig. 1A; X = ^{35}S, R = R' = CH_3.) The effect was dependent on metal concentration, and was not detectable until the metal to drug ratio reached 2.5:1 (Table 4; see Fig. 8). Other metals were less effective, and Co^{++} appeared to antagonize drug uptake. The promotion of

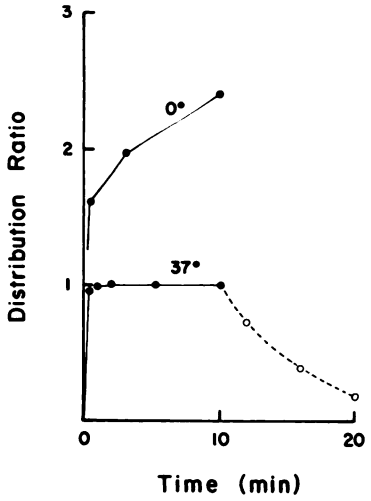

FIG. 2. *Uptake of ^{35}S-labeled B-2844 at 0° and 37°, and subsequent exodus of drug from cells at 37°*

The medium contained 1 μM drug; the intracellular to extracellular drug concentration ratio (●——●) is plotted against time. O- - -O, drug exodus at 37°. No drug was lost at 0°.

TABLE 4

Alteration of drug uptake by metal ions

Cells were incubated with 1 μM ^{35}S-labeled B-2844 (Fig. 1A; X = S, R = R' = CH_3) and metal salt for 10 min at 0° or 37°. Uptake is shown in terms of distribution ratio of drug between intracellular and extracellular water.

Metal ion	Incubation temperature	P_c at various drug to metal ratios				
		1:1	1:2.5	1:5	1:10	1:100
Cu^{++}	37°	1	1.7	3.6	14	50
	0°				3	10
Ni^{++}	37°				3	6
	0°				2	4
Zn^{++}	37°				4	7
Co^{++}	37°				0.8	0.7

drug uptake by Cu^{++} and Ni^{++} was temperature-sensitive (Table 4), but equilibrium was not rapidly established, and drug accumulation was increased when incubations were prolonged beyond 1 min (Fig. 3). Studies on subsequent drug exodus at 37° from cells loaded with ^{35}S-labeled drug and Cu^{++} showed that the rate of drug loss was slower (Fig. 3) than that of the exodus of drug from cells loaded with drug alone

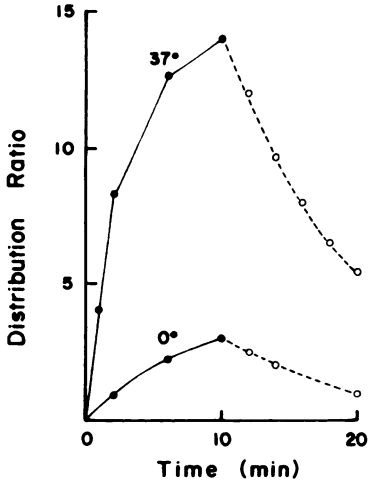

FIG. 3. *Uptake of ^{35}S-labeled B-2844 (1 μM) from medium containing 20 μM $CuCl_2$ at 0° and 37°*

After 10 min, the cells were suspended in fresh medium at 37° and drug exodus was measured (O- - -O) at the same temperature employed in the first incubation.

(Fig. 2). This may reflect the time required for dissociation of an intracellular chelate.

Promotion of uptake of $^{63}Ni^{++}$ and $^{64}Cu^{++}$ by bisthiosemicarbazones. When cells were incubated in Eagle's modified medium containing radioactive Cu^{++} or Ni^{++}, metal slowly entered cells via a process which was at least partly temperature-sensitive. The distribution ratio (intracellular to extracellular metal) attained was about 0.15 for either metal after 10 min at 37°. At 0° a distribution ratio of 0.09 was achieved after 10 min.

A study was made of the ability of substituted bisthiosemicarbazones to potentiate uptake of Ni^{++} and Cu^{++}. For one drug (B-2844, Fig. 1A; X = S, R = R' = CH_3) kinetic data are shown (Figs. 4 and 5). Drug-promoted uptake of $^{63}Ni^{++}$ and $^{64}Cu^{++}$ resulted in gradual accumulation of metal ion by the cell in a temperature-sensitive manner. In contrast, drug accumulation in the presence of metal ion (Fig. 3) was quite rapid, although also temperature-sensitive. Cu^{++} (Fig. 4) and Ni^{++} (not shown), once accumulated, were not readily lost from cells during subsequent washing at 37°.

Results of a survey of 23 compounds are shown in Table 5, along with supplementary data, discussed below.

Only compounds with 1 or more divalent sulfur atoms at X (Fig. 1A) markedly promoted uptake of Ni^{++}. Drugs with O, SO, or SO_2 at the X position did not promote nickel uptake. A hydrophobic group at R' was needed (compare B-2885, B-2908, and B-2909).

Uptake of Cu^{++} was strongly promoted by many drugs, including those with carbon atoms at the X position (Fig. 1A). A small hydrophobic group was also required at R' for optimal copper uptake. An SO or SO_2 group at X was tolerated if methyl groups were present at R and R'. Optimal promotion of Cu^{++} uptake was achieved

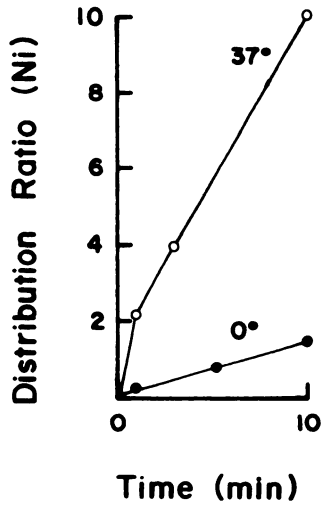

FIG. 4. *Uptake of $^{63}Ni^{++}$ at 0° and 37° from medium containing 10 μM Ni^{++} and 10 μM B-2844*

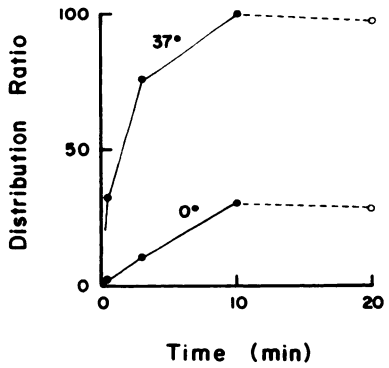

FIG. 5. *Uptake of $^{64}Cu^{++}$ from medium containing 10 μM metal and 10 μM B-2844 at 0° or 37°*

Distribution ratio = (Cu^{++} concentration in cells) / (Cu^{++} concentration in medium). After 10 min, cells were suspended in fresh medium and metal exodus was measured at 37° (O- - -O).

TABLE 5

Drug effects on nickel and copper uptake compared with drug ID_{50} values

Structures are shown in Fig. 1A except for B-1594 (Fig. 1B). Cells were incubated at 37° with 20 μM drug and 20 μM $^{64}Ni^{++}$ or $^{63}Cu^{++}$. Incubations were carried out for 10 min with nickel or for 3 min with copper. Drug-induced potentiation of metal uptake was calculated as the following ratio:

$$\frac{\text{cpm of intracellular metal with drug present}}{\text{cpm of intracellular metal with drug absent}}$$

The distribution of metal ions in the absence of drug was approximately 0.13–0.14 for both copper and nickel (intracellular to extracellular ratio).

Drug	Structure			Potentiation of uptake		ID_{50}[a]
	X	R	R'	Ni^{++}	Cu^{++}	
					μM	
B-1735	—[b]	H	Me	20		500
B-1207	—[b]	Me	Me	10	100	80
B-1604	C	Me	H	2	470	10
B-1549	C	H	Me	8	620	9
B-1600	C	Me	Me	6	700	7
B-1752	S	H	H	30	8	500
B-1712	S	H	Me	70	450	14
B-2999	S	H	Et	120		13
B-2890	S	Me	H	60	310	22
B-2844	S	Me	Me	75	665	7
B-2885	S	Me	Et	100		7
B-2908	S	Me	Pr	110	350	15
B-2909	S	Me	$CH\,CH_2$ OH	15	75	120
B-2913	SS	Me	Me	300	550	10
B-2104	SS	H	Me	150		5
B-3176	SO_2	H	H	5	13	400
B-3178	SO	Me	H	2.3	50	200
B-2087	SO_2	H	Me	3	10	500
B-3164	SO_2	Me	Me	3	250	30
B-2082	SO	H	Me	6		300
B-3215	SO	Me	Me	6	400	15
B-1929	O	H	Me	1	3.5	800
B-1594	—[c]	Me	Me	4	55	170

[a] The ID_{50} value is the drug concentration at which DNA synthesis is inhibited by 50% under standard conditions.

[b] No substituent at the X position.

[c] For structure, see Fig. 1B.

with bisthiosemicarbazones of 1,5-dicarbonyl compounds, although the derivative of B-2913, a 1,6-dicarbonyl compound, was also highly effective.

The ability of different drugs to promote Cu^{++} uptake appears to be correlated with their capacity to inhibit DNA synthesis in the absence of added Cu^{++}. A log-log plot of data from Table 5 illustrates this observation (Fig. 6).

Concentration-dependent effect of metal ions on DNA synthesis. At an extracellular level of 1.6 mM, inorganic copper salts (acetate, chloride, sulfate) inhibited DNA synthesis by 50% in 10 min at 37°. In experiments using $^{63}Cu^{++}$, we found that the intracellular Cu^{++} level attained during this incubation was 240 μM. For Ni^{++} an extracellular level of 3 mM was required to inhibit DNA synthesis by 50%, corresponding to an intracellular Ni^{++} level of 450 μM. As shown below, the level of Cu^{++} required for similar inhibition of DNA synthesis, in the presence of 1 μM B-2844, was about 35 μM (see Fig. 9). In other studies we found that 50% inhibition of DNA synthesis was achieved with a combination of 3 μM B-2844 and 30 μM Ni^{++}, producing an intracellular nickel concentration of 90 μM. In both cases the inhibitory concentration of metal was markedly

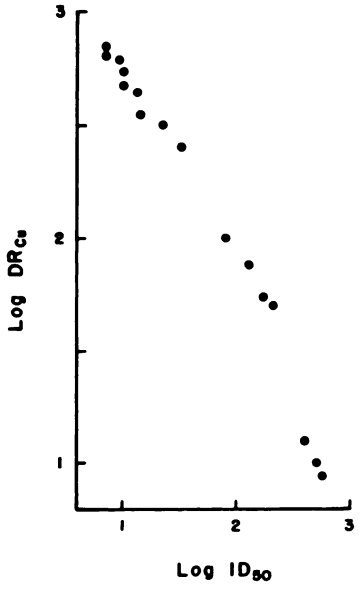

FIG. 6. *Log-log plot of data from Table 5*

Log ID_{50} values of different drugs (drug level required to inhibit DNA synthesis by 50% in medium) are plotted against log DR_{Cu} (drug-induced promotion of Cu^{++} uptake).

reduced when the metal was provided in the form of the chelate.

Effect of previously formed metal chelates on DNA synthesis. Addition of metal salts to solutions of bisthiosemicarbazones strongly potentiated drug action (Table 2). When crystalline chelates were employed, the ID_{50} value of the corresponding drug was also markedly decreased (Table 6). The previously formed chelates appeared to be as effective as freshly formed derivatives obtained by mixing drug and metal ions.

Monitoring of parameters involved in a drug action. Since metal ions enhanced drug uptake (Table 4), and drug enhanced metal uptake (Table 5), it was not immediately clear which phenomenon was responsible for the pronounced inhibitory effects of drug chelates on DNA synthesis. Aliquots of a suspension of L1210 cells were incubated in Eagle's modified medium containing 1, 2.5, 10, and 25 μM B-2844 (Fig. 1A; X = S, R = R' = CH_3) labeled with ^{35}S. Uptake of labeled drug was measured after 10 min. Identical samples of cell suspension were incubated with 1, 2.5, 10, and 25 μM nonlabeled drug for 10 min. Incorporation of labeled thymidine into DNA was then measured over the next 2 min. The time was kept short to minimize additional drug accumulation. The data (Fig. 7) show that inhibition of DNA synthesis by 50% (ID_{50}) occurred at 3 μM extracellular drug, which produced an estimated 2.9 μM intracellular drug concentration.

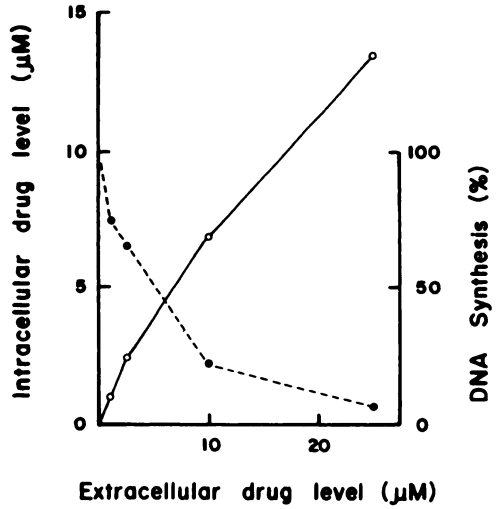

FIG. 7. *Plot of intracellular concentration of B-2844 as a function of extracellular drug level after 10 min at 37°*

Also shown (●- - -●) is DNA synthesis as a percentage of control at specified extracellular drug levels. Two-minute pulses of labeled thymidine, added after the initial 10-min incubation, were used.

Addition of Cu^{++} (final level, 1–5 μM) to medium containing 1 μM ^{35}S-labeled drug promoted drug uptake (Fig. 8); this effect was not detectable until the Cu^{++} level in the medium was increased above 1 μM. Even at 1 μM, however, the Cu^{++} caused a decrease in DNA synthesis to 32% of control. (In the absence of added Cu^{++}, the drug inhibited DNA synthesis to 75% of control.) At the highest Cu^{++} level tested, 5 μM, uptake of drug was increased to 4 μM. DNA synthesis was reduced to 10% of

TABLE 6

Inhibition of DNA synthesis by metal chelates

Cells were incubated for 10 min with drug or chelate; levels of these compounds required to inhibit DNA synthesis by 50% are reported.

Drug	Structure			Inhibition of DNA synthesis by					
	X	R	R'	No. metal	Cu^{++}	Cd^{++}	Ni^{++}	Zn^{++}	Co^{++}
					μM drug				
B-1600	C	Me	Me	7	0.5	1.7	6	3	8
B-2844	S	Me	Me	6.5	0.55	1.0	5.5	2.5	
B-1207	—[a]	Me	Me	80			60	6.3	

[a] No substituent at the X position.

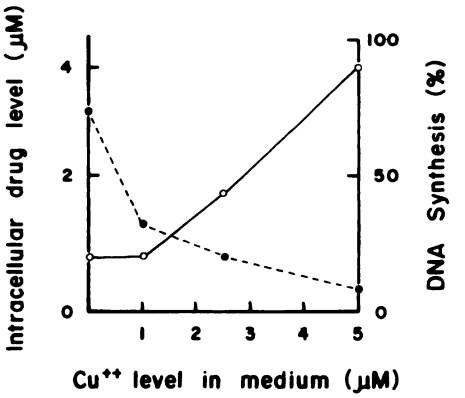

FIG. 8. *Intracellular level of ^{35}S-labeled B-2844 produced by addition of different amounts of Cu^{++} to medium containing 1 μM drug*

Also shown (●- - -●) is DNA synthesis as a percentage of control at specified extracellular Cu^{++} levels in medium containing 1 μM drug.

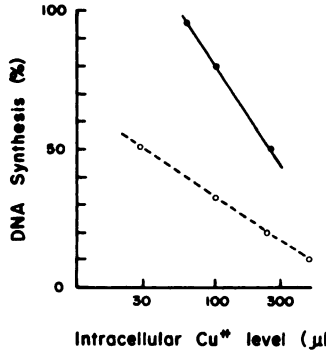

FIG. 9. *DNA synthesis (as percentage of control) as a function of intracellular Cu^{++} concentration*

●——●, cells were incubated in medium containing graded amounts of $CuCl_2$. O- - -O, medium contained 1 μM B-2844 and graded amounts of $CuCl_2$.

control. When the intracellular drug level was elevated to 4 μM solely by addition of more drug to the medium (Fig. 7), we found that an intracellular level of 4 μM would decrease DNA synthesis to 45% of control. It is clear that the observed effect of added metal on DNA synthesis cannot be explained solely on the basis of enhanced uptake of drug. Drug may, however, reach previously inaccesible sites when chelated to Cu^{++}.

In Fig. 9 we compare the inhibition of DNA synthesis as a function of intracellular Cu^{++} concentration obtained by incubation of cells in medium containing inorganic copper salts with the inhibition observed at different levels of Cu^{++} in the presence of 1 μM drug (B-2844). The drug strongly promoted the inhibitory effect of a given intracellular level of Cu^{++}.

Effect of chelation of partition of drug and metal between octanol and water. Addition of metal ions strongly promoted movement of drugs from water to octanol, although the drugs alone were more soluble in the latter. This effect required a high metal to drug ratio for Zn^{++}, but a low metal to drug ratio for Cu^{++}. Cobalt, a metal antagonistic to drug action (Table 2), inhibited movement of drug from water to octanol. These data are summarized in Table 7. Also striking was the effect of drugs on partition of metals between these solvents (Table 8). Copper partition was changed about 250-fold by addition of an equimolar amount of certain agents (B-1600, B-2844).

The rate of chelate formation plays a role here. We found that the concentration of Ni^{++} in the octanol phase could be increased by delaying addition of octanol to the aqueous solution of $^{63}Ni^{++}$ and drug for 2 hr (Table 8). As seen before (Table 5),

TABLE 7

Metal effects on partition of a bisthiosemicarbazone between octanol and water

Labeled B-2844 (1 μM) was partitioned between octanol and water; specified metal concentrations were also present. Data represent values obtained at 20° after 1 min of equilibration and are the ratios of drug concentration in octanol to drug concentration in the aqueous phase.

Metal ion	Concentration	P_c	Log P_c
	μM		
None		24	1.38
Cu^{++}	1	52	1.72
	10	29	1.46
Ni^{++}	1	27	1.43
	10	28	1.45
	100	23	1.36
Zn^{++}	1	29	1.46
	10	42	1.62
	100	55	1.74
Co^{++}	1	18	1.26
	10	3	0.70
	100	2	0.30

TABLE 8

Effect of drugs on partition of metal ions between octanol and water

Structures are shown in Fig. 1A except for B-1594 (Fig. 1B). Labeled metal ion (10 μM) was partitioned between octanol and water; a 10 μM concentration of the specified drug was also present. Mixtures were partitioned at once except in the final column, where the nickel drug chelate was allowed to stand for 2 hr in the aqueous phase before addition of octanol. Data are reported as the following ratio:

$$\frac{\text{cpm in organic phase}}{\text{cpm in aqueous phase}}$$

Drug	Structure			Partition coefficient		
	X	R	R'	Ni^{++}	Cu^{++}	Ni^{++} (2 hr)
None				0.003	0.014	0.015
B-1600	C	Me	Me	0.007	3.6	0.03
B-2844	S	Me	Me	0.02	3.6	0.08
B-1929	O	H	Me	0.004	0.15	0.2
B-1594	—[a]	Me	Me	0.005	1.42	0.01

[a] For structure, see Fig. 1B.

drugs with a central sulfur atom were more potent effectors of Ni^{++} movement from water to octanol (e.g., B-2844) than drugs with all-carbon skeletons (B-1600, B-1594).

For the drugs examined, the potentiation of octanol solubility of metal ions was roughly proportional both to the extent of drug-induced promotion of metal ion uptake and to the drug-induced promotion of metal toxicity. This is discussed below.

DISCUSSION

These studies describe the properties of a group of substituted bisthiosemicarbazones which are structurally related to a familiar analogue, the bisthiosemicarbazone of 3-ethoxy-2-oxobutyraldehyde, shown in Fig. 1. KTS is a derivative of a 1,2-dicarbonyl compound; the most potent agents of the present group are 1,5- and 1,6-dicarbonyl derivatives. We have reported (14) that the latter compounds can inhibit DNA synthesis in L1210 cells in the absence of added metal ions. Serum was, however, present. Petering and Van Giessen (1) found that the copper requirement for KTS toxicity could only be shown using serum-free medium. The present data (Table 1) show that a metal cofactor in toxicity could only be demonstrated by addition of EDTA to "copper-free" media. This observation suggests that certain compounds considered here may be more potent scavengers of copper than KTS. Although KTS was not available for study, an analogue, B-1594, was examined.

The present data do not implicate the rate of drug uptake as an important determinant of drug response; uptake apparently depends on the relative lipophilicity of drug analogues. The major determinant of the capacity of different compounds for inhibition of DNA synthesis was their ability to promote uptake of Cu^{++} by L1210 cells. Copper-induced promotion of drug uptake could also be shown, but only at metal to drug ratios far higher than the equimolar ratio required to enhance significantly drug inhibition of DNA synthesis. Furthermore, addition of EDTA had no effect on drug uptake, but abolished the effect of a model compounds, B-2844, on DNA synthesis.

These data do not rule out the possibility of access of the drug component of a drug-metal chelate to intracellular regions not accessible to the drug alone. Bhuyan and Betz (12) also considered this situation in their discussion of KTS-copper interactions.

Since the toxicity of the different drugs examined depended strongly on the capacity of each for potentiation of Cu^{++} uptake by cells, it seems far more likely that it is the metal, rather than the drug component of the chelate, which exerts ultimate toxicity. The possibility that the chelate itself, inside the cell, plays a role in the toxicity of the combination cannot yet be eliminated.

The present model, as established by studies here, is one of an essentially nonconcentrative process of drug accumulation, coupled with a temperature-sensitive exodus process. When extracellular Cu^{++} (or Ni^{++}) is supplied, rapid chelate formation produces the substrate for a temperature-sensitive uptake system. Accumulation of metal inside the cell then occurs, since metal is "bound" to intracellular components, while the drug component of the chelate readily leaves the cell. Repeated shuttling of metal ions into the cell

must eventually result in transfer of the bulk of the intracellular metal into the cell.

Partition studies, suggested by the work of Hansch and co-workers (28, 29), show that the lipophilicity of both drug and metal ion is increased upon chelation. The effect is far more striking in the case of the metal. Furthermore, as noted before (5, 10, 12), intracellular metal carried as a bisthiosemicarbazone chelate is considerably more toxic than an equivalent concentration of intracellular metal deposited from an external inorganic salt. Penetration of a lipophilic metal complex to sensitive intracellular regions, followed by dissociation of metal, appears to be the most likely explanation of the mode of action of the toxic chelates examined here. There are alternative possibilities: increased lipophilicity of drug in the chelate complex might expose new intracellular sites to the drug, or the chelate itself might be the ultimate toxic agent.

A comparison with earlier studies on KTS seems appropriate. The copper chelate of KTS readily enters cells (1, 5, 10, 12), and dissociation occurs, with the metal being retained (10, 12). KTS appears to be inactive in the absence of added Cu^{++} (1). The present study shows broad areas of agreement with the earlier data, with certain details regarding modes of uptake of the various components of the system resolved by the use of radioactive tracers. Some compounds studied here appear to be more potent scavengers of Cu^{++} than KTS. Whether this property will be reflected in enhanced antitumor activity, or merely in increased toxicity, remains to be determined. It does seem clear that future development of new bisthiosemicarbazones should involve a careful study of metal-drug synergism and of drug-induced potentiation of metal uptake by model tumor cell systems. A potentially useful compound might be overlooked if the appropriate metal ion were not simultaneously supplied.

Studies on the interactions between copper chelates of the bisthiosemicarbazones and intracellular components should resolve questions concerning the mode of action of these compounds. Recent work by D. H. Petering (7), describing reactions between the copper chelate of KTS and biological thiol groups, marks a significant beginning in this regard.

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