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ASSOCIATION OF NUCLEIC ACIDS WITH COMPLEXES OF *N*-METHYL ISATIN-β-THIOSEMICARBAZONE AND COPPER

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Abstract – *N*-methyl isatin-β-thiosemicarbazone (M-IBT) and cupric ions form a stable complex with which nucleic acids associate firmly. A 50 μM M-IBT-Cu complex can bind 3·3 μM nucleic acid as nucleotide (1·1 μg/ml). The thiosemicarbazide side chain possesses full activity. Substitution of phenyl groups at the N-1 or N-4 positions of thiosemicarbazide does not interfere with nucleic acid binding ability. Of those cations tested, cupric ions are the most effective in complex formation, but ferrous ions are also active at high concentrations. Single- and double-stranded DNA and single- and double-stranded RNA are equally susceptible; there is no nucleotide base specificity. Aurin tricarboxylic acid is the most efficient agent which interferes with the association of the M-IBT copper complex with DNA. Other chelating agents such as EDTA, Tris-hydroxyaminomethane, 2-mercaptoethanol, histidine and diphenyl thiocarbazone inhibit the association also. The reaction between the chelate complex and the DNA occurs rapidly, is insensitive to ionic strength and temperature, and is optimal at pH 8·0. Nucleic acid association is reversed in the presence of organic solvents such as dimethyl sulfoxide, dimethyl formamide and dioxane.

Thiosemicarbazones possess a wide spectrum of antiviral, antibacterial, antifungal, and antitumor cell activity [1]. In view of this, their mode(s) of action is of considerable interest. The pyridine and isoquinoline derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones inhibit ribonucleotide reductase, which may account for their antitumor cell activity [2]. However, the mechanism of action of *N*-methyl isatin-β-thiosemicarbazone (M-IBT), which is valuable in the treatment of some complications of smallpox vaccination and in the prophylaxis of smallpox in exposed persons, is unclear [1]. Since the drug has no significant effect in vaccinia virus DNA synthesis [3], it appears that ribonucleotide reductase is not the principal target in the inhibition of vaccinia virus replication. Late, but not early, protein synthesis is inhibited, which is correlated with a decreased half-life of virus-specific polysomes at the late stage of replication [4]. No effect on cellular processes is detected at virucidal concentrations, although higher concentrations inhibit DNA and RNA synthesis primarily.

In addition to the intracellular inhibition just described, M-IBT can inactivate some viruses on direct contact. It has been shown that Rous sarcoma virus (RSV), other RNA tumor viruses, and herpesvirus are inactivated *in vitro* [5–7]. This inactivation appears to be a function of the chelating ability of the drug, since EDTA prevents the inactivation and the addition of cupric ions enhances it [6]. The loss of the ability of RSV to transform chick fibroblasts is probably due to the inhibition of the RNA-dependent DNA polymerase of the virus [6]. Since the activity of purified RNA-dependent DNA polymerase of RSV is inhibited by M-IBT, the locus of action of the drug appears to be either the nucleic acid template or the enzyme. In this paper, we report that nucleic acids directly associate with complexes of M-IBT and cupric ions. This interaction may be important in the chemotherapeutic activity of this group of drugs.

MATERIALS AND METHODS

Reagents and solutions

Drug stock solutions were prepared at millimolar concentration in 100% dimethylsulfoxide (DMSO) and applied at 1:500 dilution to minimize final DMSO content. The stock solutions were kept frozen; daily preparation of fresh solutions as previously recommended [4] was not necessary for reproducible results. Double-glass-distilled water was used to make the aqueous solutions.

The materials and the companies from which they were purchased are as follows: 2-keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) (KTS), 2-keto-3-ethoxybutyraldehyde bis(*N*-4-methylthiosemicarbazone) (KTSM), pyruvaldehyde bis(thiosemicarbazone) (PTS) and pyruvaldehyde bis(*N*-4-methylthiosemicarbazone) (PTSM) were from Nutritional Biochemicals. *N*-methylisatin-beta-thiosemicarbazone (M-IBT) was from K & K Laboratories. Semicarbazide (SC), thiosemicarbazide (TSC), 1-phenylthiosemicarbazide (1-φTSC), 4-phenylthiosemicarbazide (4-φTSC) and 4-methylthiosemicarbazide (4-MeTSC) were from Eastman Kodak. Aurin tricarboxylic acid (ATA) was from Fisher Scientific. Diphenylthiocarbazone (DφTC) was from J. T. Baker. (DMSO) (spectroquality) was from Matheson, Coleman & Bell. 1-Formylisoquinoline thiosemicarbazone (IQ-1), 2-formylpyridine thiosemicarbazone (PyTSC) and 5-hydroxy-2-formyl pyridine thiosemicarbazone (5-OH Py TSC) were kind gifts of Dr. Frederick French, Palo Alto, Calif. Preformed purified M-IBT-Cu complex was prepared by Dr. William Kaska, University of California, Santa Barbara. 2-Mercaptoethanol (BME) and piperazine-*N*,*N*-bis-(2-

ethane sulfonic acid) (PIPES) buffer were from Calbiochem. Nitrocellulose filters (0·45 μm pore) were from Millipore. The tritiated compounds, uridine, thymidine and ribo-homopolymers, were from Schwartz Mann. Unlabeled ribohomopolymers, polyvinyl sulfate and calf thymus DNA were from Sigma.

Nucleic acid binding assays

Filtration. The drug, metal ion and nucleic acid to be tested were added to 0·5 ml buffer and incubated at 0° for 10 min. The reaction mixture was diluted with 3 ml buffer and passed through a 0·45-μm pore nitrocellulose filter. After washing with 10 ml buffer, the filters were dried and the radiolabeled nucleic acid content was determined by liquid scintillation. A typical reaction contained M-IBT, $CuSO_4$ and [^{3}H]thymidine-labeled HeLa double-stranded DNA in 0·05 M phosphate buffer, pH 8·0, incubated for 10 min prior to filtration. One hundred per cent retention of DNA on the filter represents 95–100 per cent of acid-precipitable counts.

Low-speed centrifugation. M-IBT, $CuSO_4$ and [^{3}H]thymidine-labeled DNA were added to buffer and incubated at 0° for 10 min. A sample was applied to a glass-fiber filter and the remaining solution was centrifuged for 30 min at 4500 *g*. Another sample was taken near the top of the tube, applied to a glass-fiber filter, dried and counted by liquid scintillation. Centrifugation assays were done with 2-ml volumes in narrow tubes to allow a separation of the precipitate from the sampling zones; no dilution of the original reaction solution was made. Filter retention and centrifugation assays gave identical results in all cases. The centrifugation assay was employed particularly with a solvent which damaged the filter, such as DMSO in high concentrations, and with a nucleic acid which bound strongly to nitrocellulose, such as single-stranded DNA.

RESULTS

Complex formation by M-IBT and $CuSO_4$

Optical effects. The spectrum of M-IBT in aqueous solution is shown in Fig. 1. Upon addition of cupric ions, the spectrum shifts from a discrete maximum at 354 nm to a broad maximum between 420 and 460 nm. Visually, a bright yellow color develops in contrast to the faint yellow color of the original 50 μM solution. Similar spectra are seen in 50% DMSO, in which nucleic acid association, as judged by pelleting of DNA in the centrifugation assay, does not occur (see below).

Crystal formation. Microscopic examination of the solution containing both M-IBT and $CuSO_4$ reveals yellow crystals 1–2 μm in diameter. These can be sedimented (7000 *g*) and recovered as large aggregates ranging to more than 50 μm in diameter. On filtration, the aggregates are seen as a yellow precipitate on the filters. Addition of DNA (550 ng/ml) did not change the appearance of the aggregates. IQ-1 and $CuSO_4$ form similar aggregates.

Association of DNA with copper complexes of M-IBT and other thiosemicarbazones

Various thiosemicarbazide derivatives were tested for their ability to form complexes which cause the retention of DNA on a filter. M-IBT, IQ-1, the TSC side chain alone and side chain derivatives 1-phenylTSC, 4-phenylTSC and 4-methylTSC were shown to be active at approximately equal concentrations using 50 μM $CuSO_4$ (Table 1). No retention of DNA by 50 μM $CuSO_4$ alone was observed (Table 2). The nucleic acid binding activity of 1-phenylTSC alone may be due to the presence of metal ions in this drug preparation, since the addition of 400 μM EDTA caused a reversal of nucleic acid binding. An atomic adsorption spectrophotometric analysis for copper revealed approximately one molecule of copper/10,000 molecules of 1-phenyl TSC. It appears unlikely, therefore, that copper is the adventitious metal ion. The preformed, purified M-IBT Cu complex is active at 40 μM, which indicates that the DNA retention is due to the complex *per se* rather than to the M-IBT and the copper acting synergistically but separately. The addition of 400 μM EDTA interferes with the activity of the preformed complex. The kethoxal and pyruvaldehyde-bis-(thiosemicarbazone)'s and their 4'-methyl derivatives were active only when the cupric ion concentration was increased 10-fold to 500 μM $CuSO_4$. Semicarbazide, 2-formylpyridine TSC and 5-OH-2-formylpyridine TSC were inactive at the highest cupric ion concentration, which did not by itself precipitate DNA. Inactive drugs were also tested with ferrous ions, the only other metal ion which formed active complexes with M-IBT (see below), but ferrous ions did not activate any compounds which were inactive with cupric ions. The strong Co^{2+} binding properties of 5-OH-2-formylpyridine TSC [8] prompted an examination of nucleic acid binding with this complex. 2-Formylpyridine TSC, but not the 5-OH derivative, showed appreciable nucleic acid binding activity at 160 μM concentrations with 500 μM $CoCl_2$ (data not shown).

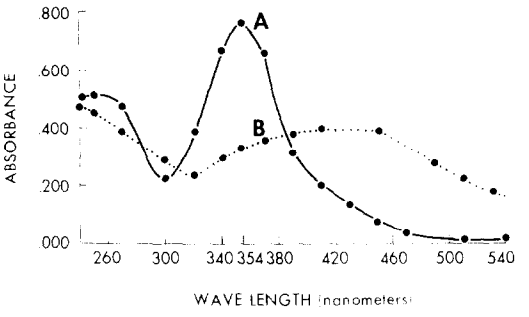

Fig. 1. Absorption spectrum of *N*-methyl isatin-β-thiosemicarbazone (λ_{max} = 354 nm) at 25° in 0·05 M phosphate buffer, pH 8·0. (A) 50 μM M-IBT; (B) 50 μM M-IBT with 50 μM $CuSO_4$. Identical patterns were obtained in 50% DMSO.

The formation of a chelate precipitate is not sufficient for the retention of nucleic acids. Both diphenylthiocarbazone (DϕTC) and aurin tricarboxylic acid (ATA) form stable, visible precipitates with cupric ions, but nucleic acids are not removed from solution by them. This indicates that the retention of the DNA on the filter is not due to nonspecific trapping in the precipitate.

The dependence of DNA retention on the concentration of M-IBT is illustrated in Fig. 2. In the pres-

Table 1. Effect of thiosemicarbazone–copper complexes on DNA*

	Compound	Concn (μM)	$CuSO_4$ (μM)	Retention of DNA (%)
1.	M-IBT	30		10
		30	50	100
2.	M-IBT–Cu complex (purified)	40		100
	+ 400 μM EDTA, pH 8·0	40		20
3.	Semicarbazide	400		15
	(SC)	400	5000	5
4.	Thiosemicarbazide	20		5
	(TSC)	20	50	60
		40	50	100
5.	4-MethylTSC	20		10
		20	50	85
6.	1-PhenylTSC	20		100
		20	50	100
	+ 400 μM EDTA, pH 8·0	20		20
7.	4-PhenylTSC	20		10
		20	50	100
8.	2-Formylpyridine	80		5
	TSC	80	50	5
		80	500	10
		80	5000	10
9.	5-Hydroxy-2-formyl-pyridine TSC	80		5
		80	500	5
		80	5000	10
10.	1-Formylisoquinoline	40		5
	TSC (IQ-1)	40	50	85
11.	Kethoxal bis(thiosemicarbazone)	60		0
	(KTS)	60	500	60
		60	5000	100
12.	4'Methyl KTS	60		0
	(KTSM)	60	500	70
		60	5000	100
13.	Pyruvaldehyde	60		0
	Bis(thiosemicarbazone)	60	500	80
	(PTS)	60	5000	100
14.	4'Methyl PTS	60		0
	(PTSM)	60	500	40
		60	5000	100

* Reactions contained 500 ng ^{3}H-DNA/ml in 0·05 M phosphate buffer, pH 7·0; drugs and $CuSO_4$ were added and the solution was passed through 0·45-μm pore nitrocellulose filters, dried and counted in a Beckman liquid scintillation spectrometer. $CuSO_4$ alone did not cause DNA retention at the highest concentration used. One hundred per cent retention of DNA on the filter represents 95–100 per cent of acid-precipitable counts.

Table 2. Effect of various metal ions on DNA retention by M-IBT*

	Metal ion	Concn (M)	DNA retention (%)	DNA retention with 50 μM M-IBT (%)
1.	Cu^{2+}	10^{-6}	0	0
		10^{-5}	0	100
		10^{-4}	0	100
		10^{-3}	0	100
2.	Fe^{2+}	10^{-4}	0	15
		10^{-3}	0	60
		10^{-2}	0	30
3.	Fe^{3+}	10^{-3}	0	0
4.	Ca^{2+}	10^{-3}	5	10
5.	Mg^{2+}	10^{-3}	0	25
6.	Mn^{2+}	10^{-3}	0	15
7.	Zn^{2+}	10^{-3}	0	10
8.	Co^{2+}	10^{-3}	0	15
9.	Ni^{2+}	10^{-3}	0	25

* Reactions contained 550 ng DNA/ml in 0·05 M phosphate buffer, pH 8·0. Metal ions and M-IBT were added and the solution was passed through 0·45-μm pore nitrocellulose filters. IBT (50 μM) alone causes 10–15 per cent DNA retention.

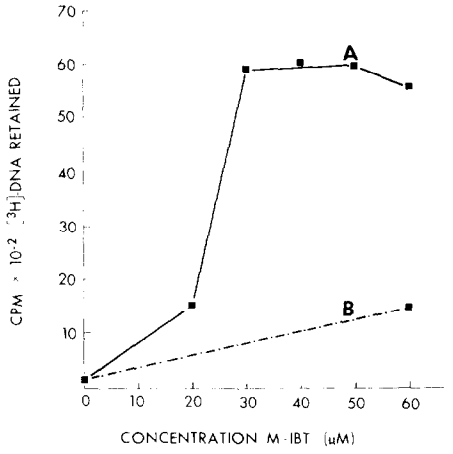

Fig. 2. Formation of a nucleic acid binding complex by *N*-methyl isatin-β-thiosemicarbazone. Association of nucleic acid with the complex was measured by retention of ^{3}H-thymidine-labeled DNA on a 0·45 μm pore filter. (A) M-IBT with 10 μM $CuSO_4$; (B) M-IBT without purposefully added metal ions. Reactions contained 550 ng/ml of DNA in 0·05 M phosphate buffer, pH 8·0.

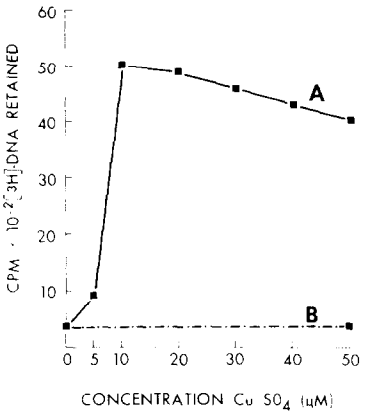

Fig. 3. Effect of $CuSO_4$ on the ability of *N*-methyl isatin-β-thiosemicarbazone to form a nucleic acid binding complex. Nucleic acid association with the complex was measured by retention of ^{3}H-thymidine-labeled DNA on a 0·45 μm pore filter. (A) $CuSO_4$ with 30 μM M-IBT; (B) $CuSO_4$ alone. Reactions contained 550 ng/ml of DNA in 0·05 M phosphate buffer, pH 8·0.

ence of 10 μM $CuSO_4$ (empirically derived; see below), 30 μM M-IBT is required for the retention of a maximum amount of DNA. The level of retention is the same for any order of M-IBT, $CuSO_4$ or DNA addition.

Metal ions effective for nucleic acid association

The concentration of cupric ion required to form an active chelate complex was investigated. Using the filter retention assay, 6 μM $CuSO_4$ yielded a complex with 50 μM M-IBT which could completely remove 550 ng DNA/ml from solution (Fig. 3). In subsequent experiments, 10 μM $CuSO_4$ was generally used as the minimum concentration.

As can be seen in Table 2, increasing the copper concentration as high as 10^{-3} M did not result in the formation of another type of M-IBT complex which was unable to cause retention of DNA. In addition, it was of interest to determine whether other metal ions, particularly those of the first transition series, could combine with M-IBT and cause filter retention of DNA. Using a standard concentration of M-IBT and DNA, metal ions were tested over the range of concentrations which did not by themselves retain the DNA. Only ferrous ion (10^{-3} M) caused significant DNA retention in the presence of 50 μM M-IBT. Other metal ions including Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} were ineffective at concentrations ranging from 10^{-6} to 10^{-3} M (Table 2).

Table 3. Effect of competing chelating agents on DNA retention by M-IBT and $CuSO_4$*

	Compound	Concn (μM)	M-IBT (μM)	$CuSO_4$ (μM)	Retention of DNA (%)
1.	Control phosphate, pH 8·0	0·05 M	50	10	100
2.	Tris, pH 8·0	0·01 M	50	10	100
		0·02 M	50	10	0
3.	EDTA, pH 8·0	400		10	0
		10	50	10	100
		20	50	10	0
4.	2-Mercaptoethanol	280		10	0
	(BME)	30	50	10	100
		40	50	10	10
5.	Diphenylthiocarbazone	100		100	0
	(DϕTC)	10	50	10	60
		30	50	10	0
6.	Aurintricarboxylic acid	200		50	0
	(ATA)	1	50	10	100
		4	50	10	10
7.	Histidine	2000		10	0
		20	50	10	50
		50	50	10	5

* Reactions contained 550 ng DNA/ml in 0·05 M phosphate buffer, pH 8·0. The order of addition to the DNA solution was: competing agent, $CuSO_4$, M-IBT.

Effect of competing chelating agents on retention of DNA

It was of interest to determine whether certain chelating agents, which by themselves or with added copper had no ability to cause DNA retention, could inhibit the activity of the M-IBT–Cu complex. As can be seen in Table 3, Tris, EDTA, BME, DϕTC, ATA and histidine can prevent the effect of the M-IBT–Cu complex. None of the compounds either alone (data not shown) or with 10 μM $CuSO_4$ can cause retention of DNA on the filter. The effect of histidine was tested, since it had been observed previously that histidine can prevent the inactivation of herpesvirus by M-IBT [9].

In addition to these chelating agents, the effect of poly rA and poly vinyl sulfate (PVS) on DNA retention was determined. It was found that 250 ng poly rA added to the M-IBT–copper complex prior to ^{3}H-HeLa cell DNA inhibited over 90 per cent of the binding of DNA to the filter (data not shown). The addition of 400 ng PVS inhibited the filter binding of the DNA by 50 per cent. Surprisingly, the addition of more PVS, up to as high as 4 μg, did not significantly decrease the filter binding below 50 per cent (data not shown). The finding that PVS competes with DNA in this reaction indicates that the purines and pyrimidines in DNA do not play a major role; rather, it appears that the charged backbone is more important.

Nucleic acids which associate with the M-IBT-Cu complex

In order to characterize the nature of this interaction more fully, we tested a variety of nucleic acids for their ability to associate with the M-IBT–Cu complex. The data in Table 4 indicate that single- and double-stranded HeLa cell DNA, poliovirus RNA, which is predominantly single stranded, and Rous sarcoma virus tRNA [10], which has a significant amount of double-stranded structure, are retained on filters in the presence of the complex. However, oligo dC (12–18 nucleotides) and deoxynucleoside triphosphates are not retained. There does not appear to be any base specificity, since homopolymers of rA, rI, rC and rU are equally retained. Reactions were also carried out with limiting concentrations of

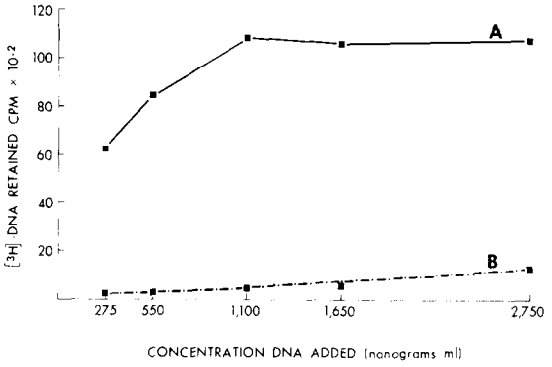

Fig. 4. Nucleic acid binding capacity of an *N*-methyl isatin-β-thiosemicarbazone and $CuSO_4$ complex. Nucleic acid association was measured by retention of ^{3}H-thymidine-labeled DNA on a 0·45 μm pore filter. (A) 50 μM M-IBT with 10 μM $CuSO_4$; (B) 10 μM $CuSO_4$ alone. Reactions were performed in 0·05 M phosphate buffer, pH 8·0.

M-IBT and copper in order to detect small differences in affinity among the various nucleic acids used, but none was observed.

The stoichiometry of the association was investigated also. As depicted in Fig. 4, it was found that a mixture of 50 μM M-IBT and 10 μM $CuSO_4$ can bind a maximum of 3·3 μM DNA (calculated as nucleotide) or 1·1 μg/ml. The addition of larger amounts of DNA did not reduce the amount of DNA bound; the addition of $CuSO_4$ over a wide range of concentrations (10–5000 μM) did not alter the interaction.

Effect of pH, ionic strength, solvent, temperature and time on the association of DNA

Variations in some of the environmental factors which might affect the interaction of M-IBT, $CuSO_4$ and nucleic acid were studied to evaluate the basis of the association. Filter retention of DNA was optimal at pH 8·0 (Fig. 5). Appreciable retention was seen at pH 7 to 12; efficiency decreased steadily below pH 7. The correlation between the loss of nucleic acid association below pH 7 and the p*K* of 6·5 for the secondary phosphoryl group indicates that this group may be the site of interaction.

Retention of DNA occurred in H_2O, phosphate and other weak Cu^{2+} chelating buffers such as PIPES

Table 4. Effect of M-IBT–copper complexes on different nucleic acids*

	Nucleic acid	Polymer size	Nucleic acid association with 50 μM IBT-Me + 10 μM $CuSO_4$ (%)
1.	HeLa dsDNA	Large	100†
2.	HeLa ssDNA	Large	100‡
3.	Polio ssRNA	7000 Nucleotides	100‡
4.	Poly(rA); poly(rU); poly(rC); poly(rI)	300 Nucleotides	100†
5.	RSV 4s RNA	75 Nucleotides	100†
6.	Oligo(dC)	12–18 Nucleotides	0‡
7.	dATP; dTTP; dCTP; dGTP	Mononucleotide	0‡

* Reactions contained 500 ng nucleotide/ml or less in 0·05 M phosphate buffer, pH 8·0.
† Filtration assay.
‡ Centrifugation assay.

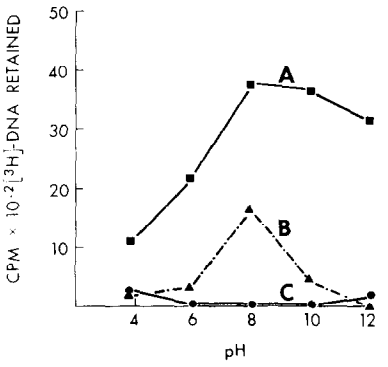

Fig. 5. Effect of pH on association with an *N*-methyl isatin-β-thiosemicarbazone and $CuSO_4$ complex. Nucleic acid association was measured by retention of ^{3}H-thymidine-labeled DNA on a 0·45 μm pore filter. (A) 50 μM M-IBT with 50 μM $CuSO_4$; (B) 50 μM M-IBT alone; (C) 50 μM $CuSO_4$ alone. Reactions contained 550 ng/ml of DNA in 0·05 M phosphate buffer.

and barbital. Tris buffer must be specifically excluded due to its strong binding of cupric ions (Ref. 11 and Table 3). Salt neither facilitated nor impeded the association of DNA with the M-IBT–Cu complex up to 1·5 M NaCl, which indicated that the involvement of salt linkages is unlikely.

The pelleting of DNA in the centrifugation assay was sensitive to the presence of several organic solvents. Assays performed with DMSO (25% v/v) dimethylformamide (25% v/v) and dioxane (10% v/v) prevented pelleting of the DNA. These concentrations of solvents solubilized the M-IBT–Cu complex, since no yellow precipitate was seen at the bottom of the tube. The M-IBT–Cu complex was not dissociated in the solvents, since the absorption spectrum resembled that of the complex described in Fig. 1. The association of DNA with the M-IBT–Cu complex occurred to the same extent at 0° and 37°. The interaction was rapid and complete within 5 min, the shortest time possible for the filtration assay.

Binding of M-IBT to DNA

Although M-IBT alone does not cause appreciable filter retention of DNA (Table 1), it was of interest to determine whether binding of M-IBT to DNA occurred. We tested this possibility by exposing coliphage λ-DNA to ^{35}S-labeled M-IBT and centrifuging the DNA in a rate zonal 5–20% sucrose gradient, at 270,000 *g* for 2·5 hr in an SW 41 rotor. No ^{35}S-M-IBT associated with the λ-DNA. In addition, we could demonstrate no change either in sedimentation rate of ^{3}H-λ-DNA or in the density (in 1·7 g/ml of CsCl equilibrium gradients run at 147,000 *g* for 64 hr in an SW 50·1 rotor) of ^{3}H-HeLa cell DNA treated with 30 μM M-IBT or with 50 μM $CuSO_4$. It appears that M-IBT alone neither associates with nor significantly affects the structure of DNA. In contrast, when either ^{3}H-λ-DNA or ^{3}H-HeLa cell DNA was exposed to both M-IBT and copper, and centrifuged in sucrose or CsCl gradients, respectively, both DNA's pelleted. However, when treated ^{3}H-HeLa cell DNA was centrifuged in CsCl gradients containing 7% dioxane, the DNA did not pellet, but banded at the same density as did the untreated sample.

DISCUSSION

We have demonstrated that M-IBT, thiosemicarbazide and several thiosemicarbazide derivatives form stable complexes with cupric ions, with which nucleic acids associate firmly. The activity of the side chain, TSC, alone is in accord with the previous observation that it possesses the activity to inactivate RSV [6]. An earlier report by Bhuyan and Betz [12] indicated that 2-keto-3-ethoxybutyraldehyde-*bis*(thiosemicarbazone) (KTS) and its copper complex did not associate with nucleic acids. However, this study utilized Tris buffer, which adversely affects association of complex to DNA. This may explain why no association was seen. Even if a nonchelating buffer had been used, interaction with nucleic acids may not have been detected, since a high concentration of copper is required for this compound (Table 1).

It is interesting that the purified, preformed M-IBT–Cu complex and the M-IBT and $CuSO_4$ solution are equally active. This indicates that the M-IBT and the copper act as a chelate complex. The alternative hypothesis that the two compounds act in sequence, perhaps even at different sites, appears to be incorrect.

The evaluation of the results presented in this paper, especially regarding the requirements of different drugs for exogenous copper, must take into consideration the possible presence of copper in the experimental system itself. For example, the finding that 1-phenyl TSC alone causes retention of DNA can be explained by the presence of metal ions in the drug preparation. Other components, such as nucleic acids, particularly those isolated from natural sources, buffers and laboratory-distilled water, are possible sources of metal ions.

The effectiveness of EDTA in preventing and reversing RSV inactivation and nucleic acid retention indicates that the continued presence of a divalent cation is required and that the binding affinity for the cation to EDTA is greater than that for M-IBT. Since BME has the same effect as EDTA in this regard and since BME is known to chelate heavy metals such as copper [13], we believe that BME is acting as a competing chelating agent rather than as a reducing agent, which might change M-IBT to an inactive form.

The feature common to the nucleic acids tested which is responsible for their interaction with the drug metal complexes is unclear. It appears that binding is not due to a specific nucleotide base composition or to the type of sugar residue. An interaction with phosphate groups in the nucleic acids may be significant, since a decrease in binding at hydrogen ion concentrations below the p*K* (6·5) of the secondary phosphoryl group was observed. The competition observed with polyvinyl sulfate supports this possibility. However, since the salt concentration could be varied without influencing retention, it seems unlikely that only salt linkages are involved. The inhibition of filter retention by solvents which weaken hydrogen and hydrophobic bonds suggests that these bonds may play a role. The finding that the homopolymer oligo dC (12–18 nucleotides) was not retained yet transfer RNA and the homopolymer poly rC (300 nucleotides) were, implies that chain length is an important criterion, perhaps through multiple weak bonds.

The strength of Cu^{2+} binding by TSC ($K_{diss} = 10^{-11}$) and by KTS ($K_{diss} = 10^{-18}$) has been reported [14, 15]. No similar information is available for M-IBT. In view of the lack of correlation between K_{diss} and nucleic acid binding activity for TSC and KTS, we speculate that, while a strong copper binding activity is desirable, the insolubility of the chelate complex is of greater importance. However, the insolubility of the complex is not the sole determinant, since the ATA copper complex precipitates but does not cause binding of DNA to the filter. A specific interaction between complex and nucleic acid must, therefore, occur. We view nucleic acid binding by chelate complexes to be the result of the formation of chelate 'lakes' or aggregates. If this is correct, the complexes could be acting as a resin for nucleic acid binding through multiple weak bonds yielding the nucleic acid precipitate.

In view of the ability of 40 μM preformed M-IBT–copper complex to interact with DNA (Table 1) and its ability to inhibit the RNA-dependent DNA polymerase of Rous sarcoma virus as well as M-IBT itself, e.g. by greater than 90 per cent at that concentration (Ref. 6 and W. E. Levinson, unpublished results), we hypothesize that the mode of inhibition of the enzyme activity is due to the binding of the ligand–metal complex to the nucleic acid rather than to the enzyme. It is known that the RNA-dependent DNA polymerase of avian tumor viruses is a zinc metalloenzyme [16, 17], which has led to the proposition that chelating agents such as 1,10-phenanthroline inhibit enzyme activity by binding to the zinc in the active site [16–18]. However, the stability constants at pH 7·4 of the KTS–copper complex is $10^{18\cdot6}$, whereas for the KTS zinc complex it is $10^{6\cdot0}$ [19]. The extraordinarily greater stability of the thiosemicarbazone copper complex makes it unlikely that the ligand in the complex would bind to the zinc in the enzyme. In view of this and the evidence for nucleic acid interaction reported herein, we propose that the nucleic acid, not the enzyme, is the site of inhibition.

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