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CONCERNING THE ROLE OF ZINC IN THE ANTITUMOR ACTIVITY OF 3-ETHOXY-2-OXOBUTYRALDEHYDE BIS(THIOSEMICARBAZONATO) ZINC(II) AND RELATED CHELATES*

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Abstract—The pH-dependent stability constants for several bis(thiosemicarbazonato) zinc(II) and copper(II) complexes have been measured. The zinc chelates are very much less stable than the copper complexes. This is reflected in their stabilities in plasma and ascites tumor fluid. While the copper complexes are undissociated, 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) zinc(II) (ZnKTS) and its N^4-methylthiosemicarbazone derivative are nearly completely dissociated in plasma and only somewhat less in ascites fluid. However, 3-ethoxy-2-oxobutyraldehyde bis(N^4-dimethylthiosemicarbazonato) zinc(II) is stable in each medium in accord with its larger stability constant. The free ligands approach a similar final state of zinc chelation as their corresponding zinc chelates. In neither medium is copper unequivocally bound by the ligands. These results are used to assess the importance of ZnKTS in the mechanism of cytotoxicity *in vivo* and to rationalize the large variation in cytotoxicity observed *in vitro* with these zinc complexes.

THE COMPOUND, 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) (H_2KTS),† is an excellent antitumor agent in animals.[1] Experiments by Petering *et al.*,[2-4] in which dietary intake of copper was strictly controlled, demonstrated that, *in vivo*, H_2KTS is obligatorily activated by Cu^{2+} but not by Zn^{2+}. Furthermore, they demonstrated that CuKTS, the copper chelate of H_2KTS, was the highly cytotoxic entity.[5] Booth and Sartorelli[6,7] appeared to reach the conclusion that Cu^{2+} activates H_2KTS after examining the effects of Cu^{2+}, H_2KTS and H_2KTS + Cu^{2+}, upon Sarcoma ascites tumors. However, in later work attention was refocused on H_2KTS and ZnKTS as possible active agents.[8,9] Mihich and Mulhern[10] have also presented data which indicate that ZnKTS is active in ascites tumor systems. Recently, VanGiessen *et al.*[11] have carefully examined the influence of metal ions upon the cytotoxicity *in vitro* of a variety of bis (thiosemicarbazones). In addition to confirming the profound cytotoxicity of CuKTS and Cu^{2+} + H_2KTS *in vitro*, they, too, showed that Zn^{2+} + H_2KTS and ZnKTS have slight activity. However, they discovered other zinc bis-

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† Abbreviations: H_2KTSM = 3-ethoxy-2-oxobutyraldehyde bis(N^4-methylthiosemicarbazone); H_2KTSM$_2$ = 3-ethoxy-2-oxobutyraldehyde bis(N^4-dimethylthiosemicarbazone). Cu(II)KTS, Zn(II)KTS, CuKTSM, ZnKTSM, CuKTSM$_2$ and ZnKTSM$_2$ are the corresponding copper and zinc chelates of the above ligands.

(thiosemicarbazone) chelates with enhanced effectiveness. Taken together, these experiments present an ambivalent picture of the role of zinc in the mechanism of cytotoxicity of H_2KTS *in vivo*. It is the intent of this paper to re-examine this question in light of the chemical properties of H_2KTS, related bis(thiosemicarbazones), and their copper and zinc chelates.

MATERIALS AND METHODS

The bis(thiosemicarbazone) ligands were generously supplied by Dr. Harold G. Petering and Dr. Eugene Coats. Copper and zinc complexes were made by reacting the ligands with excess copper or zinc salts in aqueous solution at room temperature. The complexes, which formed immediately, were extracted into ether to remove metal ions and then reintroduced into appropriate aqueous solution. Fresh ethylene diamine (lot 072427), b.p. 116–117°, $n^{20}D$ 1·4565, from Aldrich Chemical was used. All other chemicals were reagent grade. Fresh human plasma was obtained from the Milwaukee Blood Center and was centrifuged before use. Ascites tumor fluid was provided by Dr. Harold G. Petering. It comprised the clear supernatant present after centrifugation of Ehrlich ascites tumor cell suspensions withdrawn from several Swiss mice.

The method of determination of pH-dependent stability constants, K(H), for bis-(thiosemicarbazonato) copper(II) complexes by competition with a ligand, ethylene diamine, of known stability with copper, has been described previously.[12]

$$K(H) = \frac{[\text{Cu chelate}]}{[Cu^{2+}]\,[\text{bis(thiosemicarbazones)}]_{\text{all forms}}}. \quad (1)$$

The theoretical equation expressing the pH dependence of K(H) has been shown to have the form

$$K(H) = 2\,pH + C \quad (2)$$

in which C is a constant.[12]

Because the zinc chelates have much smaller stability constants, protons effectively compete for the ligands according to the overall equation written below for H_2KTS.

$$2H^+ + ZnKTS \rightleftharpoons Zn^{2+} + H_2KTS. \quad (3)$$

There is no spectral evidence for the presence of multiple zinc ligand species in solution. However, the adequacy of equation (3) in describing the relevant equilibria is more firmly based on agreement of the equations describing the experimental data in Table 2 with the theoretical equation, equation (2).

Stability constants of the form of equation (1) can be calculated directly in the region of pH 4–7 as follows: Solid zinc chelate is placed into solution and its absorbance (A) measured. Then, after adding excess Zn^{2+} to a sample, its total concentration is measured spectrophotometrically at the peak of the visible absorbance band and is equal to A_0/ϵ, where ϵ is the molar absorbance. Following the form of equation (1),

$$K(H) = \frac{\epsilon A}{(A_0 - A)^2} \quad (4)$$

where A/ϵ = [Zn chelate] and $(A_0 - A)/\epsilon = [Zn^{2+}]$ = [bis(thiosemicarbazone)]$_{\text{all forms}}$ at a given pH.

The amount of zinc in solution present as $Zn(OH)^+$ is small and can be ignored in the calculation.[13] In one experiment, known nonstoichiometric amounts of $ZnCl_2$ and H_2KTS were mixed and the absorbance was measured as a function of pH. Here the equation is modified to

$$K(H) = \frac{A/\epsilon}{([Zn^{2+}]_i - A/\epsilon)([H_2KTS]_i - A/\epsilon)} \tag{5}$$

in which $[Zn^{2+}]_i$ and $[H_2KTS]_i$ represent initial concentrations. In all of these measurements a Radiometer PHM 26 pH meter, standardized at pH 7·00 and 4·00, was used. Readings were taken before and after each absorbance determination and showed a range of ±0·05 pH unit. All solutions were 0·1 M in KNO_3 to provide media of constant ionic strength.

The molar absorbances of the copper chelates were measured as described previously.[12] Those for the corresponding zinc chelates were determined secondarily, given the absorption spectrum of the zinc chelate in the presence of excess zinc to saturate the ligand and the corresponding spectrum when Cu^{2+} is added to convert the ligand entirely into its copper complex.

The reactions of bis(thiosemicarbazone) ligands and their metal complexes with plasma and ascites fluid were followed spectrophotometrically with a Beckman Acta V spectrophotometer equipped with a 0 to 0·1 absorbance scale which is sensitive to changes of 0·001 A. In order to balance the large background absorbance of the solutions, approaching one for plasma, both sample and reference cuvettes contained the biological fluid being examined. Under these conditions the background noise increased and the sensitivity was about 0·003 A. In actual runs, small random displacements in the curves over time are common, probably due to minute differences in the rate of precipitation of proteins as the fluids stand at room temperature. As long as there is no developing line shape to these changes, no further interpretation has been assigned to them.

In the handling of the fluids and in experiments involving them, care was taken to use glassware previously soaked in EDTA to remove extraneous metal ions, which might otherwise confuse the interpretations of the results.

Concentration determinations for zinc and copper in plasma and ascites fluid were carried out after dry ashing by differential pulse anode stripping voltammetry using Princeton Applied Research equipment.[14]

For human plasma and ascites fluid, respectively, zinc concentrations in μg/ml—molarity were 0·43 ± 0·10 6·5 × 10^{-6} (five determinations) and 0·44 ± 0·02—6·7 × 10^{-6} (two determinations), and copper concentrations were 0·6 ± 0·2—9·0 × 10^{-6} (two determinations) and 1·70 ± 0·15—2·7 × 10^{-5} (two determinations).

RESULTS

The pH-dependent formation constants for copper and zinc chelates of bis(thiosemicarbazones) related to H_2KTS have been determined and are summarized in Tables 1 and 2, together with the equations for the straight lines describing the data. Although CuKTS and CuKTSM have similar equations, $CuKTSM_2$ is a distinctly more stable complex. No reaction was observed when 1·00 M ethylenediamine or

TABLE 1. pH-DEPENDENT FORMATION CONSTANTS FOR COPPER CHELATES

Complex	pH*	log K(H)
CuKTS†	9·75	23·50
	9·37	22·70
	8·95	21·80
	8·70	21·30
	8·25	20·40
	7·95	19·70
	7·62	18·95
	log K(H) = 2·1 pH + 2·85	
CuKTSM‡	9·70	23·30
	9·45	22·65
	9·05	21·80
	8·75	21·20
	8·32	20·30
	8·05	19·70
	7·83	19·15
	7·55	18·45
	log K(H) = 2·2 pH + 1·90	

* Range = ±0·05.
† $\epsilon(469) = 6300 \pm 150$.
‡ $\epsilon(465) = 7950 \pm 150$.

$2{\cdot}34 \times 10^{-3}$ M 8-hydroxyquinoline-5-sulfonic acid competed with $CuKTSM_2$ for copper ion. It is estimated that K (pH 7·4) is greater than 5×10^{20}, assuming a small uncertainty in the absorbance measurement attributable to dissociation of the complex.

A striking trend in formation constants is seen with the zinc chelates. $ZnKTSM_2$ is substantially more stable than the other chelates. Furthermore, all three ligands exhibit much weaker binding of zinc than copper. Since Cu^{2+} also displaces zinc rapidly from these ligands (data not shown), the copper chelates are overwhelmingly favored to exist in solutions in which zinc and copper ions are both available for binding. It is also clear that a significant portion of ZnKTS is dissociated at pH 7·4, so that free Zn^{2+} and ligand will be present even in the absence of Cu^{2+}. For instance, for [ZnKTS] = 10^{-5} M, 25 per cent of the metal chelate will be dissociated.

Given these data, the stability and reactivity of the free ligands and their zinc and copper complexes in human plasma and mouse ascites fluid were investigated. In no case was a reaction between any of the copper complexes and ligands in these media observed. Chelate concentrations were less than 2×10^{-5} M and incubation times for CuKTS, CuKTSM and $CuKTSM_2$ in plasma were 24, 2 and 0·5 hr, respectively, and in ascites fluid 0·5, 4 and 4 hr respectively. This agrees with the prediction that ligand exchange reactions with CuKTS and related copper chelates are unlikely because of the very large thermodynamic stability of these complexes.[12] It is interesting to note that the spectra of these compounds in plasma and ascites fluid show a characteristic peak below 500 nm and a shoulder near 550 nm. The shoulder, in particular, indicates that the solution environment of the complexes has some lipid character.[12]

When the zinc chelates or their associated free ligands are added to plasma or ascites fluid, the interactions are more complex. Table 3 shows some results in

TABLE 2. pH-DEPENDENT FORMATION CONSTANTS OF ZINC COMPLEXES

Complex	pH*	log K(H)
ZnKTS†,‡	6·81	4·67
	6·73	4·75
	6·60	4·34
	6·30	3·64§
	6·26	3·75
	6·10	2·87§
	5·90	2·80
	5·90	2·73§
	log K(H) = 2·1 pH − 9·6	
ZnKTSM‡,‖	6·20	4·42
	6·18	4·64
	5·93	4·10
	5·90	3·93
	5·65	3·62
	5·63	3·47
	5·32	3·04
	log K(H) = 1·7 pH − 6·2	
$ZnKTSM_2$‡,¶	5·98	7·10
	5·62	6·76
	5·44	6·52
	5·20	6·02
	5·10	5·85
	4·89	5·62
	4·80	5·29
	4·52	4·84
	4·18	4·45
	log K(H) = 1·7 pH − 2·85	

* Range = ±0·05.
† $\epsilon(417) = 10{,}800 \pm 200$.
‡ Determined by equation (4).
§ Calculated by equation (5).
‖ $\epsilon(423) = 13{,}000 \pm 200$.
¶ $\epsilon(445) = 12{,}800 \pm 100$.

TABLE 3. INTERACTION OF BIS(THIOSEMICARBAZONE) COMPOUNDS WITH PLASMA*

Compound	Initial concn (M)	Time (hr)	[Zn chelate]	[Cu chelate]	$\frac{[\text{Zn chelate plasma}]}{[\text{Zn chelate } H_2O \text{ pH } 7{\cdot}3]}$†
H_2KTS	$8{\cdot}8 \times 10^{-6}$	4	9×10^{-7}	None‡	
ZnKTS	$9{\cdot}7 \times 10^{-6}$	4	$1{\cdot}1 \times 10^{-6}$	None	0·14
H_2KTSM	$5{\cdot}7 \times 10^{-6}$	4	None‡	None	
ZnKTSM	$7{\cdot}0 \times 10^{-6}$	4	None	None	
H_2KTSM_2	$8{\cdot}8 \times 10^{-6}$	4	$8{\cdot}0 \times 10^{-6}$	None	
$ZnKTSM_2$	$4{\cdot}1 \times 10^{-6}$	4	$3{\cdot}2 \times 10^{-6}$	None	0·8

* Fresh human plasma from The Milwaukee Blood Center, pH = 7·30.
† Ratio of [Zn chelate] in plasma to that calculated for aqueous solution at pH 7·3 knowing K(H) and C_t.
‡ Estimated lower limit of copper complex detected by this method: 1×10^{-6} M.

plasma. H_2KTS, ZnKTS, H_2KTSM and ZnKTSM may be grouped together, because over time little if any zinc ion is or remains bound to these ligands or zinc chelates respectively. Using three samples of fresh plasma and one of plasma from outdated whole blood, the amount of undissociated ZnKTS remaining ranged between 0 and 10 per cent of the total analytical concentration of ZnKTS placed in solution ($\sim 10^{-5}$ M). When similar initial concentrations of H_2KTS were present in plasma, 0–10 per cent became associated with zinc ion. It is clear that plasma ligands dissociate ZnKTS beyond what is expected at pH 7·30 in aqueous solution. ZnKTSM dissociated completely in plasma in each experiment and H_2KTSM consistently showed no association with zinc. Within the uncertainty of the experiment, no copper complex forms in any case. In some experiments, reaction mixtures were incubated at room temperature for 24 hr with no change in the spectrum.

Considering the metal complexes, within the time of mixing them with plasma, metal-ligand dissociation occurs, leaving the free ligands which are detected in the 330–350 nm region of the spectrum.

The behavior of H_2KTSM_2 and $ZnKTSM_2$ was strikingly different. Using either compound, significant amounts of the zinc complex were present in solution over time. The free ligand rapidly acquired zinc, and the preferred chelate was stable in plasma, with 80–100 per cent of its initial concentration observed spectrophotometrically over time in several experiments. The absorbance maximum is shifted to longer wavelength relative to aqueous solution and is consistent with the metal chelate existing in an environment with lipid caharacter.

In ascites fluid, this picture is complicated further. However, as in plasma, ligand and corresponding zinc complex behave similarly over time, and H_2KTSM_2 and $ZnKTSM_2$ react differently from the other compounds. The results are illuustrated in Fig. 1(a–f). With H_2KTS, ZnKTS, H_2KTSM or ZnKTSM, significant amounts of zinc complexes are observed in ascites fluid after a brief incubation. Here 25–35 per cent of the ZnKTS or ZnKTSM, introduced at about 10^{-5} M initial concentration at pH 7·7, exist in the medium, contrasted with the nearly complete dissociation of the chelates observed in plasma. The ligands, H_2KTS and H_2KTSM, consistently acquire zinc from the fluid to reach 10–20 per cent of chelate saturation.

The spectra clearly indicate that little if any CuKTS forms in ascites fluid exposed to H_2KTS or ZnKTS. Unfortunately, the results with H_2KTSM and ZnKTSM are ambiguous, for Fig. 1(c,d) shows that some long wavelength absorbance in the region consistent with CuKTSM formation develops over time. However, the ill-defined line shape with no maximum near 470 nm does not identify the source of the absorbance as the copper complex.

When H_2KTSM_2 or $ZnKTSM_2$ is placed in ascites fluid, the ligand immediately becomes or remains saturated with zinc. The resulting spectrum of $ZnKTSM_2$ exhibits a long wavelength shift in absorbance maximum from its position in aqueous solution, suggesting that it is in a lipid environment. Both findings are similar to the results in plasma. Small changes in the spectra of these compounds occur with time, changes which are consistent with the slow conversion of some $ZnKTSM_2$ to $CuKTSM_2$.

DISCUSSION

Studies on the pharmacology of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) have focused in part on the role of its copper and zinc complexes in the antitu-

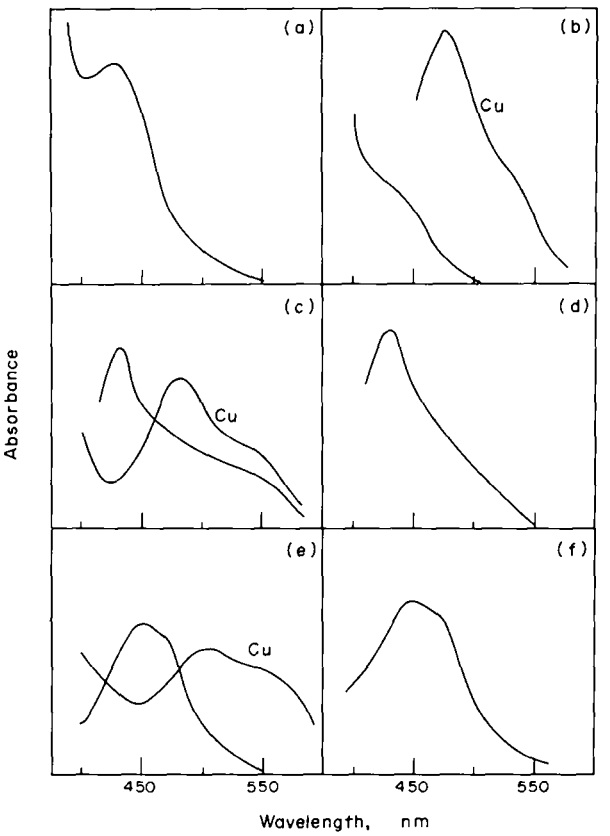

FIG. 1. Spectra of bis(thiosemicarbazones) in ascites fluid. (a) ZnKTS; (b) H_2KTS; (c) ZnKTSM; (d) H_2KTSM; (e) $ZnKTSM_2$; (f) H_2KTSM_2. Corresponding copper complexes in ascites fluid are listed as Cu. Absorbance scales for each chelate were adjusted arbitrarily.

mor effects of this compound. Petering *et al.*[4,5,11] have provided conclusive evidence *in vivo* and *in vitro* that CuKTS is the active form of this material. H_2KTS is only active *in vivo* when the animal receives dietary copper, irrespective of the zinc status of the animal.[4] In tumor cell systems *in vitro*, the same pattern emerges, with the difference that Zn^{2+} activates H_2KTS to a small degree.[11]

Similar results were obtained in the treatment of the Sarcoma 180 tumor.[6] Although neither H_2KTS nor Cu^{2+} injected into the peritoneal cavity alone is active, the combination is cytotoxic. These data indicate that the fluid bathing the ascites cells has an insufficient quantity of copper free to complex with H_2KTS ("available" copper) to form a detectable amount of the active drug. This contrasts with the situation for solid tumors treated orally or intraperitoneally with H_2KTS, in which the drug encounters available copper as it moves to the site of the neoplasm.[1,4]

Various studies show ZnKTS to be active *in vivo* in solid tumors and in Sarcoma ascites tumors when injected intraperitoneally.[5,9,10] However, since dietary copper was present in the first instance and because none was available in the second, the mode of action in these two cases may differ, as will be considered below.

In order to provide a basis for understanding these results, a survey of the physicochemical properties of CuKTS and ZnKTS has been initiated.[12,15] One early finding

confirmed here was that thermodynamically CuKTS is a remarkably stable complex, with a formation constant at 25° and pH 7·4 of $10^{18\cdot6}$. Hence, in the presence of copper free to react, H_2KTS will be converted to CuKTS. In the current work, the formation constant at 25° and pH 7·4 for ZnKTS is $10^{6\cdot0}$, some 12 orders of magnitude less than that for the copper complex. Furthermore, at pH 7·4 a significant amount of the zinc complex will be dissociated into Zn^{2+} and H_2KTS. Since H_2KTS reacts rapidly with other small copper complexes such as bis(ethylenediamine) copper(II) to remove the metal ion, the conversion of partially dissociated ZnKTS to CuKTS in the presence of available copper is expected to be rapid as well as virtually complete.[12]

In order to extend these conclusions to biological systems, the interaction of H_2KTS and its zinc and copper complexes with human plasma has been examined, for all of these compounds will be exposed to this fluid as they move from the point of administration to the tumor. CuKTS is completely stable over long periods of time. However, ZnKTS is rapidly dissociated to Zn^{2+} and H_2KTS so that between 0 and 10 per cent of the chelate remains. The concentrated array of plasma ligands, such as amino acids, complete with H_2KTS for Zn^{2+} to cause almost complete dissociation. The dissociated ligand chelates little or no copper over time. According to the error limits of the experiment (discussed previously), less than 10^{-6} M CuKTS can be formed.

As expected from the results with ZnKTS, H_2KTS is added to plasma, little if any zinc complex or CuKTS forms. Hence, in human plasma, ZnKTS is rapidly converted to H_2KTS, which then, by analogy to other work, must chelate copper to be active.[4,5] If the general similarity of rodent and human plasma is granted with respect to copper distribution and presence of zinc-binding ligands, then the finding that ZnKTS is active *in vivo* in animals receiving adequate dietary copper is equivalent to the earlier observations that H_2KTS is active under such conditions.[1,4,5]

Two views may be derived from the difficulty in binding plasma copper by H_2KTS. Either, a small undetected amount is chelated, which would be enough to activate H_2KTS as an antitumor agent, or, the activation process occurs elsewhere, such as in the liver before Cu^{2+} is fixed in ceruloplasmin.

Turning to experiments with ascites fluid, in certain respects the Ehrlich ascites tumor is like a cell suspension *in vitro* in that the drug is injected into a closed volume. Furthermore, the medium in each case is fixed so that the trace metals and ligands to which the drug is exposed are restricted for a time in comparison with oral or intravenous injection. Since some conclusions about the cytotoxicity of ZnKTS have derived from studies on ascites tumors, it is important to see how ZnKTS and H_2KTS behave in this system.[9,10]

CuKTS is stable over time. However, when ZnKTS is added at low concentration ($\sim 10^{-5}$ M) to mouse ascites fluid, extensive chelate dissociation occurs. Nevertheless, significant amounts of ZnKTS remain, in contrast to the situation in plasma. Here, then, the system contains H_2KTS. ZnTKS and Zn^{2+}-fluid ligands. No CuKTS is formed during lengthy incubation, in agreement with the previously stated findings of Booth and Sartorelli.[6,7]

A final state containing somewhat less ZnKTS is reached with H_2KTS in ascites fluid. Since some ZnKTS forms in this reaction, it is clear that ascites fluid is more delicately balanced than plasma between the availability and chelation of zinc. How-

ever, an important difference between H_2KTS and ZnKTS in ascites fluid is that endogenous zinc is limited (7×10^{-6} M zinc in fluid by polarographic analysis, 1×10^{-5} M by reaction with an excess of H_2KTSM_2). Hence in previous experiments in which 100 mg/kg of H_2KTS and 25 mg/kg of ZnKTS were injected intraperitoneally, approximate concentrations of $2{\cdot}5 \times 10^{-3}$ M and $4{\cdot}5 \times 10^{-4}$ M for H_2KTS and ZnKTS, respectively, are present in 4 ml of ascites fluid for a 25 g rodent.[10] The endogenous zinc level is only about 2 per cent of that introduced by ZnKTS. At these concentrations, therefore, the effects of ZnKTS, which supplies large quantities of zinc to the system, may be quite different than those for H_2KTS.

In another investigation with ascites tumors, the metabolic lesions due to $ZnCl_2$ (20 mg/kg) and ZnKTS (50 mg/kg) could not be readily distinguished.[9] Given the facile dissociation of ZnKTS and its lipid solubility, ZnKTS may be considered a carrier of Zn^{2+} through the tumor cell membrane, facilitating the transport of zinc along a concentration gradient from outside to inside the cell. Then the major difference between systems containing $ZnCl_2$ and ZnKTS is the relative rates of transport of zinc into the cell. Although under these conditions ZnKTS may be cytotoxic, the results have limited value for the consideration of mechanisms of activity *in vivo*. In plasma the same situation does not exist. The system is not closed and plasma ligands strongly compete for zinc, to free H_2KTS to scavenge for available copper.

The view developed here of the roles of copper and zinc in bis(thiosemicarbazone) cytotoxicity needs to be expanded in light of the recent findings that the degree of thiosemicarbazone methylation in the 4-position profoundly affects the cytotoxicity of these compounds *in vitro* in the presence of copper or zinc.[11] The ligand, H_2KTS, is activated by Cu^{2+}, H_2KTSM_2 by Zn^{2+} and H_2KTSM by either metal. An examination of the formation constants for these copper complexes does not readily distinguish $CuKTSM_2$ from CuKTSM or CuKTS. All have extremely large formation constants. The variation in reactivity and cytotoxicity *in vitro* with structure of the copper complexes will be detailed elsewhere.

However, considering the variation in activation by zinc with structure, H_2KTSM_2 is different form H_2KTS or H_2KTSM, for its zinc complex is considerably more stable at pH 7·4 in aqueous solution and in plasma and ascites fluid. Because $ZnKTSM_2$ is more stable and is readily formed from H_2KTSM_2 by reaction with available zinc, it may be expected to have biological properties qualitatively different from those of ZnKTS or ZnKTSM. For example, it should be much more efficient in transporting zinc into the cell and perhaps then in reacting specifically to disrupt cellular activities.

The greater cytotoxicity of $H_2KTSM + Zn^{2+}$ relative to $H_2KTS + Zn^{2+}$ might be due to the larger formation constant for ZnKTSM. However, this difference may be an aritifact of the assay system *in vitro*, which contains few competing ligands for zinc.[11] In plasma and ascites fluid, ZnKTS and ZnKTSM dissociate to similar extents and cannot be distinguished.

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