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Studies of the Reaction of 2-Formylpyridine Thiosemicarbazone and Its Iron and Copper Complexes with Biological Systems

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SUMMARY

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The interactions of 2-formylpyridine thiosemicarbazone with various biochemical systems have been studied to understand the potential behavior of this antitumor agent *in vivo*. This ligand removes iron from ferritin to form the iron(III) complex. The complex is rapidly reduced by hemoglobin and is only slowly reoxidized by oxygen in aqueous solution or plasma. Both the iron(III) and iron(II) chelates are stable in plasma. These results suggest that the iron(II) complex exists *in vivo*. However, the thermodynamic stability of the copper complex is also consistent with its formation in biological systems. Electron paramagnetic resonance spectra show that in plasma two adduct species of the copper complex form. One of these may involve histidine. Reaction of the ligand or its iron, copper, or zinc complex with the assay mixture used for ribonucleoside diphosphate reductase produces in each case the iron(II) complex of the ligand. These results are used to interpret a number of observations on the physiological effects of α-*N*-formyl heterocyclic thiosemicarbazones as well as some features of the inhibition of ribonucleoside diphosphate reductase by these compounds.

INTRODUCTION

α-*N*-Heterocyclic formylthiosemicarbazones comprise a class of compounds under active investigation as antineoplastic agents (1). They are unusual in that they strongly complex transition metals such as Fe^{2+}, Fe^{3+}, and Cu^{2+} with their common $N^\frown N^\frown S$ tridentate ligating structure, as shown in Fig. 1 (2, 3). This metal chelation property was recognized early and has become the foundation for a detailed hypothesis of mechanism of action (4). Based upon the known sensitivity of DNA synthesis to these compounds, together with the localization of this inhibitory effect on the conversion of ribonucleotides to deoxyribonucleotides, the following mechanism of action has been developed.

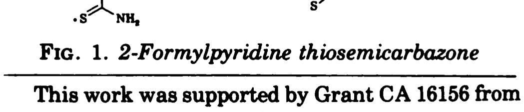

FIG. 1. *2-Formylpyridine thiosemicarbazone*

Moore has shown that this reaction, catalyzed by ribonucleoside diphosphate reductase, is markedly enhanced by the addition of Fe^{2+} to the assay mixture (5). By analogy with other metal-activated systems, the iron atom may bind in a coordinatively unsaturated way to the enzyme, generating the active site in which the vacant first coordination sites of the iron atom serve to bind and orient substrates (*S*) undergoing reaction.

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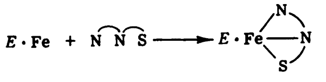
$$E + \mathrm{Fe}^{2+} \overset{K}{\rightleftharpoons} E\cdot\mathrm{Fe} \overset{S}{\rightleftharpoons} E\cdot\mathrm{Fe}\cdot S \rightarrow E\cdot\mathrm{Fe}\cdot P \qquad (1)$$

Inhibition by α-*N*-heterocyclic formylthiosemicarbazones then



(2)

involves binding to $E\cdot\mathrm{Fe}$ to prevent catalysis.

Observations on animals and humans exposed to such materials indicate that these compounds severely disrupt normal iron metabolism by complexing iron in these organisms, thereby placing the metal in a form which is excreted in large quantity (6–8).

Recent work from this laboratory has explored some basic features of the transition metal chemistry of a representative of these complexing agents, 2-formylpyridine thiosemicarbazone (3). In addition, both iron and copper complexes of 5-substituted 2-formylpyridine thiosemicarbazones and 1-formylisoquinoline thiosemicarbazone have been shown to be cytotoxic to tumor cells *in vitro* and, in the latter case, *in vivo* (9, 10).

A complex picture exists, therefore, in which ligands and their metal complexes have been used as cytotoxic agents. Are they separate, independently active compounds, or are they related by biological metal-ligand reactions which occur as inferred from the effect of ligands on iron metabolism? The present research was undertaken to begin the examination of these questions through the investigation of the interactions of the parent compound, 2-formylpyridine thiosemicarbazone, and its metal complexes with appropriate components of biological systems.

MATERIALS AND METHODS

Materials. The preparations of 2-formylpyridine and its iron(II), copper, and zinc complexes are described elsewhere (9). Horse spleen ferritin was obtained from Miles Laboratories. Human hemoglobin was prepared by hemolysis of red blood cells and used without removal of 2,3-diphosphoglycerate. Outdated whole blood and blood plasma were provided by the Milwaukee Blood Center. The mouse ascites fluid was the centrifuged supernatant of Ehrlich ascites tumor suspensions removed from Swiss mice bearing the tumor. Sperm whale myoglobin, batch 10, was obtained from Miles-Servac. Cytidine diphosphate, ATP, and dithiothreitol were purchased from P-L Biochemicals. Other chemicals were reagent grade.

Stability of compounds in biological media. Changes in the ultraviolet and visible spectra of compounds added to human plasma or mouse ascites fluid were used to detect reactions in these media. The visible–near ultraviolet bands of copper, iron, and zinc complexes change or disappear upon oxidation-reduction or substitution reactions and appear when HL[2] chelates these metals. The compounds were 10 μM in plasma. The details of this procedure are given elsewhere (11).

Hemoglobin titrations. Variously ligated hemoglobin and myoglobin were titrated with $\mathrm{Fe(III)}L_2^+$ in 0.015 M Tris-Cl at pH 7.3. The titrations of oxy and carbonmonoxy heme proteins were done in aerobic solutions with a set of rapid additions of titrant to minimize reoxidation of the product, $\mathrm{Fe(II)}L_2$. Deoxyhemoglobin was titrated in an anaerobic cell after oxygen had been removed slowly and replaced by an atmosphere of nitrogen.

Reaction of ferritin with HL. A 1.0-ml solution of 0.2 g atom of iron per milliliter of horse spleen ferritin was dialyzed without stirring at room temperature against 50 ml of 200 μM HL. The dialysis tubing had previously been treated with EDTA to remove extraneous metals. At intervals the composition of the external medium was examined spectrophotometrically for the presence of $\mathrm{Fe}L_2^+$.

Electron paramagnetic resonance spectra. Spectra were recorded on a Varian E-9 spectrometer, which was generously made available by Dr. Harold Swartz, Medical College of Wisconsin.

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[2] The abbreviations used are: HL, 2-formylpyridine thiosemicarbazone; ML_n, 2-formylpyridine thiosemicarbazonato metal complexes, in which M is the metal and *n* the number of ligands bound to metal; NTA, nitrilotriacetate; IQ-1, 1-formylisoquinoline thiosemicarbazone.

Reaction of HL with assay mixture of ribonucleoside diphosphate reductase. The components of the usual assay medium for ribonucleoside diphosphate reductase were made up fresh, adjusted separately to the given pH, and mixed prior to reaction. Both $Fe(NH_4)(SO_4)_2$ and $Fe(NH_4)_2(SO_4)_2$ were used as sources of iron, although the ferrous salt is typically used in enzyme assays (12). Ligand, HL, or $Fe(III)L_2$ was added, and the appearance of $Fe(II)L_2$ was followed spectrophotometrically over time. Results are displayed as the percentage of total HL present as $Fe(II)L_2$ plotted against time.

RESULTS

Reaction of HL and its metal complexes in human plasma and mouse ascites fluid. In other communications the thermodynamics of binding of Fe^{3+}, Fe^{2+}, Cu^{2+}, and Zn^{2+} to HL have been examined[3] (13). For the iron and copper complexes, large formation constants are observed, which exceed the pH-dependent binding constants for complexes of these metals involving amino acids and other typical cellular ligands. To gain further insight into the probable reactivity of HL and its iron and copper complexes in biological media, human plasma and mouse ascites fluid have been used as sources of a spectrum of biological ligands and metal complexes (11). When $Fe(III)L_2^+$, $Fe(II)L_2$ under anaerobic conditions, or CuL^+ is incubated in these media and their visible spectra are observed, no reaction occurs during a 30–60-min period. A similar result is observed with the ligand. However, when FeL_2^+ is added to plasma, a small difference spectrum between plasma in the sample and reference cells develops in the region of 400–430 nm. The nature of this effect is described later. In each case several different samples of plasma and ascites fluid were used. Hence these metal complexes are stable in typical biological environments, and the ligand finds no available metal ions for complexation within the sensitivity of the observation. The lower limit for observation of FeL_2^+ and CuL^+ is about 1.6 μM, based on an instrumental capability of detecting a change in absorbance of 0.01 in these difference spectra.

Reaction of HL with ferritin. Iron(III)-chelating agents such as nitrilotriacetate can slowly remove ferric iron from the iron storage protein ferritin (14). To probe the effect of HL on ferritin-bound iron, a similar dialysis experiment was employed. Figure 2 shows the results of this experiment, in which NTA was used as the standard for efficient metal complexation. The $Fe(III)L_2^+$ complex formed slowly, at a rate commensurate with the kinetics of iron binding by NTA.

The slow rate of complexation probably reflects the general difficulty in removing Fe(III) from its hydrolyzed polynuclear form, which exists in solution at pH 7 and which may be similar in structure to the iron core of ferritin (15). The qualitative interpretation given the ferritin-NTA reaction by Pape *et al.* is that the results are consistent with a metal chelation mechanism for the normal liberation of iron from ferritin *in vivo* (14). Following this view, it appears that such a reaction involving HL and ferritin provides a reasonable mechanism by which iron metabolism is disrupted by HL and by which FeL_2^+ can be formed *in vivo*.

Reaction of $Fe(III)L_2^+$ with hemoglobin and myoglobin. Having seen a difference spectrum in the 410–430 nm range after adding $Fe(III)L_2^+$ to plasma, which resembled the difference spectrum of the oxidized vs. reduced Soret bands of hemoglobin, we examined the possible reaction of $Fe(III)L_2$ with human hemoglobin and whole myoglobin. $Fe(III)L_2$ reacts rapidly with human oxy- and deoxyhemoglobin in an oxidation-reduction reaction. Methemoglobin is readily identified by the development of a 635 nm band in the spectrum of the reaction mixture. The spectrum of the sample between 500 and 600 nm is a superposition of hemoglobin species and $Fe(II)L_2$, which has a peak and shoulder at 600 and 540 nm. Because $Fe(II)L_2$ reoxidizes slowly in aerated solutions (see below), there was little diffi-

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[3] Antholine, W. E., and Petering, D. H., submitted for publication in J. Inorg. Chem.

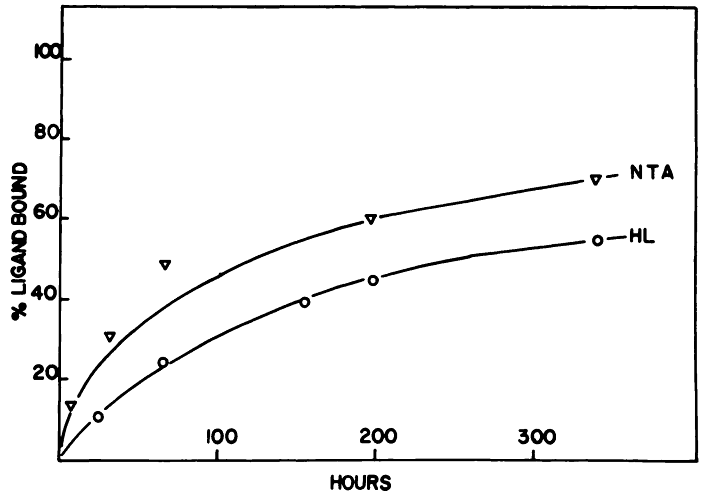


FIG. 2. *Reaction of HL and NTA with horse spleen ferritin*

Equilibrium dialysis of 1.0 ml of ferritin, 0.2 g atom in iron, against 50 ml of 200 μM HL in 0.1 M Tris, pH 7.6.

culty in examining this system with oxygenated hemoglobin or myoglobin. The stoichiometry of the reaction was determined in titration experiments using oxy- and deoxyhemoglobin and oxymyoglobin (Fig. 3). As expected, it is 1:1, leading to an over-all equation of reaction for myoglobin of

$$\mathrm{Fe(III)}L_2 + \mathrm{Mb(Fe(II))} \rightarrow \mathrm{Fe(II)}L_2 + \mathrm{Mb\text{-}Fe(III)} \qquad (3)$$

The complex does not reduce the carbonmonoxy derivatives of these proteins.

The reduction potentials of oxyhemoglobin, oxymyoglobin, and $\mathrm{Fe(III)}L_2{}^+$ are given in Table 1, together with the equilibrium constants for the 1-electron transfer between each of the heme protein species and $\mathrm{Fe}L_2{}^+$. It is clear that each reaction reported to occur here has a favorable equilibrium constant. However, as indicated by the small magnitude of the equilibrium constant for oxyhemoglobin plus $\mathrm{Fe}L_2{}^+$, the titration is not expected to be complete in the region of the stoichiometric end point of the reaction. This is shown in Fig. 3.

Oxidation of $\mathrm{Fe(II)}L_2$ by oxygen. The thermodynamically stable form of the iron complex in the presence of air is the iron(III) species (Fig. 4). Upon exposure to solution equilibrated with air at 25°, previously formed $\mathrm{Fe(II)}L_2$ oxidizes slowly to $\mathrm{Fe(III)}L_2$. The isosbestic point at 492 nm is consistent with two interconvertible species in solution – $\mathrm{Fe(III)}L_2$ and $\mathrm{Fe(II)}L_2$ – with no other oxidation prod-

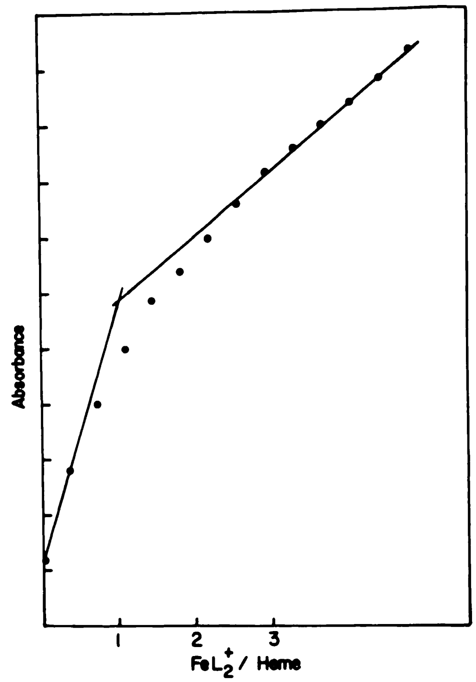


FIG. 3. *Titration of human hemoglobin with $\mathrm{Fe(III)}L_2{}^+$*

Hemoglobin concentration, 12.5 μM; absorbance $(A) = A_{540} - A_{520}$.

TABLE 1

Comparative half-wave potentials[a]

Species	$E_{1/2}$	$\Delta G^{\circ 1}$ [b]	log K_{eq} [b]
	mV	*kcal/mole* *Fe(III)L_2^+*	
Myoglobin	61 (pH 6)	−4.2	3.1
Hemoglobin	150 (pH 7.0)	−2.0	1.5
Fe(III)L_2^+	242 (pH 7.0)		

[a] From ref. 16.

[b] Fe(III)L_2^+ + heme protein ⇄ Fe(II)L_2 + heme protein$^+$, at 25°.

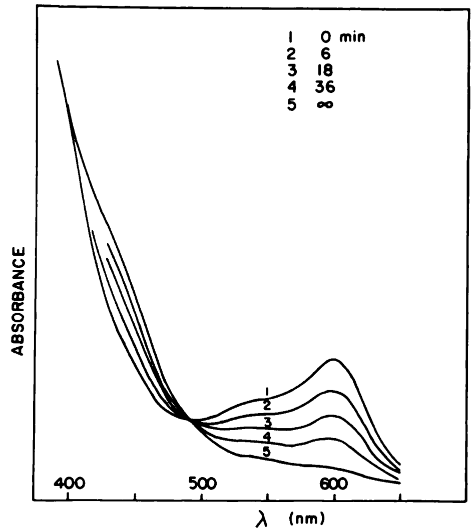


FIG. 4. *Autoxidation of Fe(II)L_2 in aqueous solutions at pH 7.4, in 0.1 M Tris-Cl*

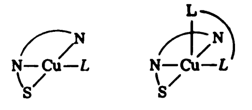
ucts observed (4). One may therefore conceive of the following oxidation-reduction system *in vivo:*

$$\text{Fe(III)}L_2 \underset{\text{O}_2,\ \text{slow}}{\overset{\text{hemoglobin, rapid}}{\rightleftharpoons}} \text{Fe(II)}L_2 \qquad (4)$$

Because of the prevalence of hemoglobin, the lower $p\text{O}_2$ of the tissues relative to air-equilibrated buffer, and the relative kinetics of the two processes, it is likely that the predominant form of the complex in the organism is Fe(II)L_2.

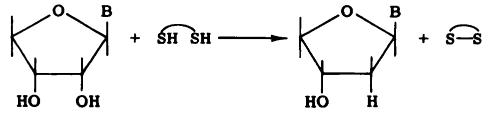
That the oxidation of Fe(II)L_2 in plasma is also slow and not catalyzed in this biological medium was confirmed by observing the reaction in plasma. Although complete oxidation to Fe(III)L_2 does occur, it is only about 70% complete in 20 min.

Forms of CuL^+ in vivo. The ligand HL forms a very strong, three-coordinate complex with Cu^{2+} (13). This chelate can then bind other mono- and bidentate ligands to constitute 1:1 adducts:



These adducts are observed by electron paramagnetic resonance spectroscopy in frozen solution. When CuL^+ is added to human plasma, two new EPR-detectable species are formed. One of these may be a histidine adduct, for the EPR parameters of the CuL · His adduct are very similar to one of the two signals present in plasma (Fig. 5 and Table 2). At a lower concentration, 10 μM, comparable to that used in studies of the stability of CuL^+ in plasma, a similar situation appears to exist. The $g_\perp$ region of the EPR spectrum of CuL^+ shifts, as occurs in Fig. 5b. However, because of instrumental noise, it is impossible to observe the $g_\parallel$ region of the spectrum.

Reaction of HL with ribonucleoside diphosphate reductase. Several studies have emphasized the sensitivity to HL of the conversion of ribonucleotides to deoxyribonucleotides in tumor cell systems and identified this as the molecular site of HL-induced cytotoxicity. Using 20–30-fold purified reductase, HL and many of its ring-substituted analogues have been shown to be remarkably strong inhibitors of the enzyme-catalyzed conversion (12, 17, 18):



(5)

Recently evidence was presented that the iron, copper, and zinc complexes of 1-formylisoquinoline thiosemicarbazone inhibit this reaction *in vitro* as strongly as the ligand alone (10). In order to clarify this complicated picture, a spectral analysis of the reaction of HL, FeL_2, FeL_2^+,

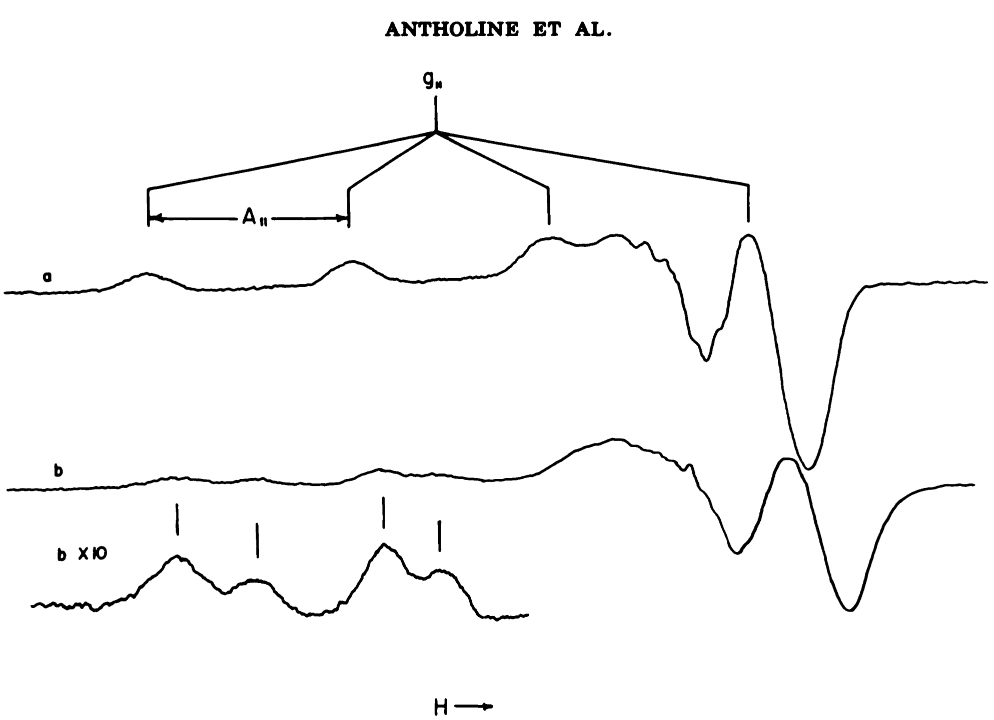


FIG. 5. *EPR spectra of CuL*$^+$

a. Cu*L* acetate in water. b. Cu*L*$^+$ in human plasma. b × 10. Ten-fold expansion of the $g_{\parallel}$ region of b. All spectra were taken at 77°K; microwave power, 5 mW; modulation amplitude, 6 G; modulation frequency, 100 kHz; microwave frequency, 9.089 GHz. The concentration of Cu*L*$^+$ was 60 μM.

TABLE 2

EPR parameters for CuL in frozen solutions

Species	$g_{\parallel}$	$A_{\parallel}$
		G
Cu*L*	2.206	185
Cu*L* + His	2.188	183
Cu*L* + plasma[a]	2.152, 2.181	167, 188
	2.160, 2.187	170, 184

[a] Two values are shown for each of two different samples of human plasma.

Cu*L*$^+$, and Zn*L*$^+$ with the assay mixture excluding enzyme was carried out. Some of the results are summarized in Fig. 6. Using H*L* and a freshly made assay mixture containing Fe^{3+} adjusted to pH 6.9, Fe(II)L_2 forms in a biphasic manner. At pH 5.2 the over-all reaction is considerably faster, leading to complete complexation of iron. If Fe(III)L_2^+ is added directly at either pH, reduction occurs within the time of mixing. When $Fe(NH_4)_2(SO_4)_2$ is used instead of the ferric salt, complete complexation of the limiting amount of H*L* occurs immediately at pH 5.2 and within 4 min at pH 7.35.

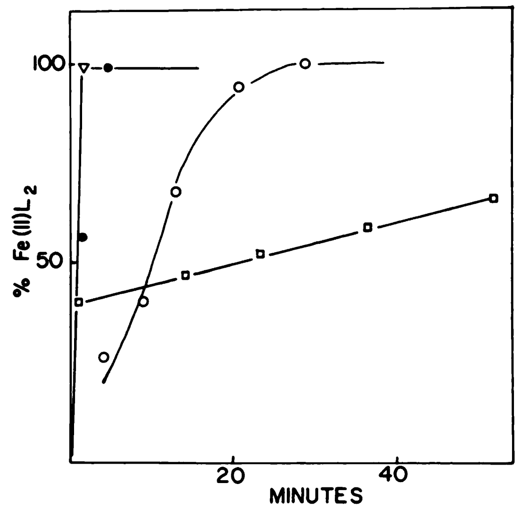


FIG. 6. *Reaction of HL with assay mixture for ribonucleoside diphosphate reductase*

The percentage of total ligand as Fe(II)L_2 is plotted against time. Concentrations were 8.3 mM potassium phosphate buffer (pH 7.0), 4.2 mM ATP, 4.2 mM $Mg(C_2H_3O_2)_2$, 10 mM NaF, 6.3 mM dithiothreitol, 23 μM $Fe(NH_4)(SO_4)_2$, 0.17 mM CDP, and 16 μM H*L*. ▽, Fe(III)L_2 added instead of H*L* or medium containing Fe^{2+}, pH 5.2; ●, Fe^{2+}, pH 7.35; ○, Fe^{3+}, pH 5.2; □, Fe^{3+}, pH 6.9.

The addition of $\mathrm{Cu}L^+$ or $\mathrm{Zn}L^+$ to the assay medium containing either Fe^{3+} or Fe^{2+} leads to their quantitative conversion to $\mathrm{Fe(II)}L_2$. Dithiothreitol reduces $\mathrm{Cu(II)}L^+$ and presumably removes its copper as the copper(I) mercaptide, leaving $\mathrm{H}L$ to form $\mathrm{Fe(II)}L_2$. The zinc complex is partially dissociated in the absence of competing ligands, so that it is readily converted to the more stable $\mathrm{Fe(II)}L_2$ (13). With 2-formylpyridine thiosemicarbazone, therefore, ligand and metal complexes are qualitatively similar, in that the introduction of each into the assay system generates $\mathrm{Fe(II)}L_2$. Since the standard assay mixture contains Fe^{2+}, it is clear that these metal-ligand reactions are rapid and go to completion (12).

DISCUSSION

A substantial research effort has followed the initial observation by Brockman *et al.* (19) that monothiosemicarbazones with the $\mathrm{N}\frown\mathrm{N}\frown\mathrm{S}$ functional grouping have antitumor properties. Included in this work have been the synthesis and testing by French and co-workers of a large number of such compounds. Significant structure-function correlations have not been found among these materials (20, 21). In complementary studies, Sartorelli's group has focused on the biochemical mechanisms by which compounds of this general type destroy tumor cells (17, 18). Central to the thinking of each of these two lines of inquiry has been the finding that the $\mathrm{N}\frown\mathrm{N}\frown\mathrm{S}$ constellation of functional groups can act as a tridentate metal-binding ligand which is hypothesized to have cytotoxic activity because of its binding to a metal, such as Fe^{2+}, located at a sensitive site in the cell, namely, ribonucleoside diphosphate reductase (4, 22). Lacking thus far have been relevant facts about the metal-binding chemistry of these ligands which can be used to evaluate this work. Hence the present work focuses on the bioinorganic chemistry of the parent ligand, 2-formylpyridine thiosemicarbazone, as a model for the study of comparative properties of variously substituted thiosemicarbazones. It is recognized, of course, that ring substituents will modify the chemistry of the parent compound, perhaps to a large extent. Therefore extrapolation of results of this work to related ligands can only be based on the supposition that substituent effects are secondary perturbations of the chemical properties of 2-formylpyridine thiosemicarbazone.

The ligand 2-formylpyridine thiosemicarbazone ($\mathrm{H}L$) reacts with horse spleen ferritin to remove iron from its core as $\mathrm{Fe(III)}L_2$. Its efficacy in this chelation process is similar to that of nitrilotriacetate. The results with NTA have been used previously as a basis for postulating the physiological mobilization of iron from ferritin by a chelation mechanism (14). In both cases the rates of reaction are slow, and direct comparisons with the movement of iron from ferritin stores *in vivo* are difficult. Nevertheless, this result suggests a biochemical site for the chelation of iron by monothiosemicarbazones.

Once formed, $\mathrm{Fe(III)}L_2$ does not readily react with typical biological ligands, such as amino acids, which are present in these media. However, it does react rapidly with hemoglobin and myoglobin in oxidation-reduction reactions, which produce the met-heme proteins and $\mathrm{Fe(II)}L_2$. This reduction process should facilitate the movement of the complex across lipid membranes, for $\mathrm{Fe(II)}L_2$ has a net charge of zero and consequently has substantial solubility in nonpolar solvents.

These results and their proposed extensions to the situation *in vivo* are consistent with the thermodynamic stability of $\mathrm{Fe(II)}L_2$, the kinetic inertness of $\mathrm{Fe(II)}L_2$, and some of the observed responses of organisms to 5-hydroxy-2-formylpyridine thiosemicarbazone (12, 6–8). From other work the value of K_1K_2 at pH 7.4 for the pH-dependent equilibria

$$\mathrm{Fe}^{2+} + \mathrm{H}L \underset{\substack{k_{-1}\\ K_1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Fe}L^+ \underset{\substack{k_{-2}\\ K_2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{Fe}L_2 \qquad (6)$$

is 6.3×10^{15}, with $K_2 \gg K_1{}^3$. This is a large constant for an iron(II) complex (12). Likewise $\mathrm{Fe(III)}L_2$ is stable in biological fluids both because of its thermodynamic stability and because it is extremely inert to dissociation or to ligand substitution.[3] Apotransferrin is typically present in human plasma in the concentration range of 1–2 μM. Present experiments are not sensitive enough to decide whether apotransferrin at these concentrations can remove iron(III) from the complex. Such competition is thermodynamically favorable, because log K (pH 7.4) for Fe(III)·transferrin under physiological conditions of pH and $p\text{CO}_2$ is 10^{24} (23–25). However, the kinetic inertness of Fe(III)L_2 in ligand substitution processes would probably prevent this metal exchange.[3] For instance, the rate constant for ligand exchange between Fe(III)L_2 and 200–300 μM EDTA is extrapolated to be 6×10^{-4} hr^{-1} at pH 7.4.

Toxic manifestations of the intravenous administration of 5-OH-HL to humans include intestinal toxicity, the excretion of large quantities of iron, anemia, and hemolysis (7, 8). Assuming a similarity in behavior between HL and 5-OH-HL in the biochemical systems studied here, some of these responses may be rationalized as follows. 5-OH-HL encounters available iron in the ferritin of intestinal mucosa. The Fe(III)L_2 formed, carrying a 1+ charge, tends to be localized within these cells. Since FeL_2 may be a toxic material itself, as suggested by its probable inhibition of ribonucleoside diphosphate reductase, adverse effects may occur at this site. Iron, obtained from ferritin here and perhaps elsewhere and bound as Fe(III)L_2, is reduced in the blood to Fe(II)L_2 and excreted as the blue complex, accounting for the intense dark green urine of patients given this drug.

Recently Peisach *et al.* (26) have described the events leading to lysis of red cells exposed to acetylphenylhydrazine. The initial reactions lead to the oxidation of hemoglobin. Later Heinz bodies develop and increased hemolysis occurs. Possibly a similar mechanism is involved in the hemolytic process associated with exposure of humans to the 5-hydroxy compound, given the results described here for the oxidation of hemoglobin by Fe(III)L_2.

Although there is no direct evidence for the formation of CuL^+ *in vivo*, the magnitude of its stability constant at pH 7.4, 2.0×10^{13}, suggests the possibility (13). It is clearly large enough to remain stable in the presence of typical biological ligands found in plasma or Ehrlich ascites tumor fluid. Since CuL has been shown to be cytotoxic to tumor cells when incubated *in vitro*, the finding that it is stable in plasma argues that it can exist *in vivo* as a potential antitumor agent (9).

In other work the pH-dependent binding constant for ZnL^+ in aqueous solution was determined (13). At pH 7.4 it is 4.0×10^5, so small that ZnL is partially dissociated at this pH. In the presence of other ligands, such as found in plasma, it will be completely dissociated. Therefore this metal complex is not expected to be important *in vivo*.

Having described the thermodynamic behavior of metal complexes of HL, which supports the known existence of FeL_2 and possibly of CuL *in vivo*, we now ask: What is the proximate reactive molecule in the cytotoxic reaction, HL or one of its complexes? Because a variety of monothiosemicarbazones inhibit the cellular conversion of ribonucleotides to deoxyribonucleotides, the molecular basis of cytotoxicity has been identified with this lesion in the synthesis of DNA (17, 18).

The mammalian enzyme present in a crude preparation from the Novikoff hepatoma is iron-activated, according to the work of Moore (5). This has been confirmed recently by Hopper (27, 28) with the highly purified protein from rabbit bone marrow. Hence in the enzymatic assay mixture ferric or ferrous iron has been added to stimulate catalysis, presumably by replacing iron lost during purification. In other studies, using a partially purified preparation from Ehrlich tumor cells, no iron was added to obtain activity (29). Nevertheless, in all of the work on inhibition by monothiosemicarbazones, the assay mixture has contained iron (4, 21).

Both Sartorelli and French have envisioned the general mechanism of inhibition of the reductase by representative monothiosemicarbazones as follows. The enzyme loosely binds iron, which further coordinates components of the reaction in the active site and perhaps participates in the oxidation-reduction reaction itself (4, 22). The inhibitor also can bind to the free first coordination sites of bound iron to prevent catalysis. Alternatively, FeL can react with the enzyme to form an inactive ternary complex. Reactions 7–10 formally describe this mechanism.

$$E + \mathrm{Fe} \overset{K_1}{\rightleftharpoons} E\cdot\mathrm{Fe} \qquad (7)$$

$$E\cdot\mathrm{Fe} + S \overset{K_2}{\rightleftharpoons} E\cdot\mathrm{Fe}\cdot S \rightarrow \text{products} \qquad (8)$$

$$E\cdot\mathrm{Fe} + \mathrm{H}L \overset{K_3}{\rightleftharpoons} \underset{\text{inactive ternary complex}}{E\cdot\mathrm{Fe}\cdot L + \mathrm{H}^+} \qquad (9)$$

$$E + \mathrm{Fe}L \overset{K_4}{\rightleftharpoons} E\cdot\mathrm{Fe}\cdot L \qquad (10)$$

The assay used to examine catalysis by ribonucleoside diphosphate reductase is itself a complicated set of iron-binding ligands together with the strong reducing agent dithiothreitol ($E_0' = -340$ mV). In particular, the mixture contains phosphate, cytidine diphosphate, adenosine triphosphate, acetate, dithiothreitol, and hydroxide, all of which can bind ferrous and ferric iron. Hence the set of reactions presented above is, itself, oversimplified.

When the very strong iron(II,III)-binding ligand HL is added to the assay mixture, which was made up with either Fe^{3+} or Fe^{2+} salts in the presence of the reducing agent dithiothreitol, Fe(II)L_2 is observed to form. Using Fe^{2+}, as is done in the actual enzyme assays, the reaction at 7.35 is so rapid that all of the ligand is converted to Fe(II)L_2 within a fraction of the total time used for incubation of the enzyme with the medium. Hence inhibition of ribonucleoside diphosphate reductase by HL in this assay system appears to involve principally the interaction of the enzyme with Fe(II)L_2.

That ligand-bound iron is the active inhibitor is further supported by two brief reports that the previously formed ion chelate of 1-formylisoquinoline thiosemicarbazone inhibits the reductase as efficiently as the ligand (10, 30). This picture has been complicated by the report that the zinc and copper complexes of this thiosemicarbazone also prevent catalysis by this enzyme, to a degree similar to the iron chelate (10). In view of the results with HL presented here, what surely occurs with the zinc complex is that it is dissociated by ligands in the assay mixture to release free IQ-1, which then functions as Fe(II)(IQ-1)$_2$. Similarly, the copper chelate is probably reduced by dithiothreitol to Cu(I)(IQ-1), which reacts with thiol to yield Cu(I)SR and free IQ-1.

What remains for investigation is the actual mode of inhibition. Previous quasi-steady-state kinetic studies on the crude enzyme from Novikoff hepatoma present a complicated array of results with different monothiosemicarbazone inhibitors (1, 31). Nevertheless, in view of the recent chemical studies of Fe(II)L_2, the following general comments can be made. Assuming that an iron complex does comprise the inhibitory form of HL, that species must be either FeL_2 or Fe(II)L^+. It is known that $K_2 \gg K_1$ in reaction 6, in which $\log K_1K_2$ at pH 7.4 is 6.3×10^{15}.[3] Hence, for the enzyme to form an inactive ternary complex $E\cdot\mathrm{Fe}\cdot L$, the equilibrium constant for the following reaction must be very large indeed:

$$E + \mathrm{Fe}L_2 \rightleftharpoons E\cdot\mathrm{Fe}\cdot L + L \qquad (11)$$

If FeL_2 is the active form, then a mechanism which does not involve the direct binding of E and Fe must be considered. Only studies with the enzyme in pure form with a convenient assay technique will resolve this interesting question.

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