Biochemical Pharmacology, Vol. 30, No. 12, pp. 1595–1604, 1981.
Printed in Great Britain.

0006-2952/81/121595-10 $02.00/0

INTERACTION OF 2-FORMYLPYRIDINE THIOSEMICARBAZONATO COPPER (II) WITH EHRLICH ASCITES TUMOR CELLS*

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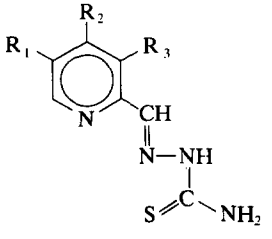
(*Received* 14 *July* 1980; *accepted* 17 *December* 1980)

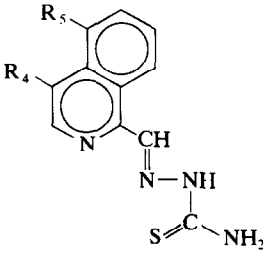
Abstract—The reaction of 2-formylpyridine thiosemicarbazonato copper (II) with Ehrlich cells was studied. The complex was readily taken up and bound by cells. Little efflux of copper was observed. Electron paramagnetic resonance studies were consistent with the complex having bound to thiol groups furnished by glutathione. The chelate was quite stable in cells, having a first-order rate constant for reaction of 4.5×10^{-5} sec^{-1}. However, this was apparently a reflection of a steady-state redox process in which thiols were being oxidized and oxygen reduced. A model reaction between the complex and reduced glutathione showed that the complex reached a steady state as oxygen was consumed in the process. Cellular DNA synthesis was inhibited at low concentration by this copper chelate, whereas RNA synthesis was much less sensitive. Although isolated mitochondria were inhibited by the complex, any cellular reaction was obscured by the rapid oxygen reduction that occurred in the thiol oxidation process.

In recent years several types of metal complexes have been shown to have cytotoxic or antineoplastic properties. The *cis* dichlorodiamine Pt(II) and bis(thiosemicarbazonato) Cu(II) complexes have been studied extensively [1,2]. More recently, some dimeric rhodium carboxylates [3] and copper and iron complexes of α-*N*-heterocyclic carboxaldehyde formyl thiosemicarbazones have drawn attention. Nevertheless, the possibility that the metal complexes may be a useful source of drugs is just beginning to be explored. In particular, there is a need to understand how such compounds interact with cells so that mechanistic information may be accumulated to facilitate the rational design of new materials.



In the study of α-*N*-heterocyclic formyl thiosemicarbazones, particular attention has been directed to the role of metal chelation in the mechanism of action of these compounds, which was hypothesized since the early work of French and his coworkers [4–8].

Among the important mechanistic conclusions emanating from the structural studies was the finding that an N N S tridentate ligand system is a common feature of all compounds with carcinostatic potency. It was found that active compounds of this series have the general features shown in the structural diagrams below:



Several studies have implicated iron binding in the mechanism of action of these compounds. Iron complexes of 5-substituted-2-formylpyridine thiosemicarbazone and 1-formylisoquinoline thiosemicarbazone have both *in vitro* and *in vivo* activity against tumor cells [9–11]. A principal site of inhibition, the enzyme ribonucleotide reductase, appears to be inhibited by the iron complexes of these various compounds in the standard assay system [11–13]. In addition, thiosemicarbazone ligands are known to sequester iron *in vivo* from the host organisms [6, 14, 15].

Complicating this picture is the finding that the copper complex of 2-formylpyridine thiosemicarbazone is also stable in biological systems [12] and has *in vitro* cytotoxic effects against Ehrlich cells [9, 11]. In this paper, we examine in more detail the

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interaction of this copper complex with Ehrlich cells. Our investigation of the nature of the interaction of the copper complexes of these ligands with tumor cells follows from our investigations of the chemical properties of these complexes [12, 16] and parallels the investigations on other copper-containing antineoplastic agents under investigation in our laboratory [17, 18].

MATERIALS AND METHODS

Reagents and materials. Many experiments with intact cells were performed in MEM,* obtained from the Grand Island Biological Co. (Grand Island, NY). The source of [methyl-^{3}H]thymidine (1 mCi/ml; 2 Ci/mmole) was Amersham/Searle (Arlington Heights, IL), and [5-^{3}H]uridine (1 mCi/ml; 1 Ci/mmole) was obtained from Research Products International (Elk Grove Village, IL). Sodium heparin solution (1000 units/ml) was obtained from Riker Laboratories, Inc. (Northridge, CA). The preparation of 2-formylpyridine thiosemicarbazone ligand is described by Antholine *et al.* [16]. Copper complexes of these ligands were prepared as described previously [16] and dispersed as freshly prepared solutions in DMSO. DMSO (gold-label quality), DTNB, and DMPO were supplied by the Aldrich Chemical Co., Inc. (Milwaukee, WI).

Animals and Ehrlich ascites cells. Routine procedures for the maintainance of our animal colony, transplantation of the Ehrlich ascites tumor line, and handling of the cells in our laboratory have been described previously [9, 11, 17].

Interaction of CuL with Ehrlich cells and mitochondria. Uptake of copper by cells was followed as a function of time by incubation of cells in MEM containing various concentrations of CuL. Cell pellets and supernatant fractions were analyzed for copper content by atomic absorption spectrophotometry using a Perkin–Elmer 360 instrument (Norwalk, CT). Pellets were digested in a 5:3:2 (v/v) $HClO_4$–HNO_3–H_2O acid mixture to solubilize them prior to analysis.

Intact cells preloaded with CuL were incubated at 25° and monitored at 380 nm (the absorption band of the complex) against a reference cuvette containing cell suspension only. These experiments were performed in a Beckman Acta V equipped with a dispersion attachment to minimize effects of sample turbidity. The entire visible absorption spectrum was also measured under these conditions. Similar results were obtained using 0.25 M sucrose or ascites fluid as the suspending medium.

Electron paramagnetic resonance (e.p.r.) spectra of cells were obtained following treatment of cells with CuL. Cell suspensions were frozen in liquid N_2 and spectra were obtained at 77 K using a Varian E9 e.p.r. spectrometer (Palo Alto, CA). Visible-region difference spectra of cells suspended in CuL-containing media versus untreated cells were performed on an Acta V spectrophotometer equipped to measure turbid samples.

Mitochondria were prepared from ascites cells by the procedure of Thorne and Bygrave [19] using EGTA in place of EDTA, and from beef heart as described elsewhere [20]. Respiration of whole cells or mitochondrial suspensions was followed using a model 53 Yellow Springs Instrument Co. oxygen monitor (Yellow Springs, OH). Mitochondria and drug were incubated in a solution containing 0.25 M sucrose and 1 mM EGTA, pH 5.6. Oxygen uptake studies were performed with 0.2 ml of mitochondrial suspension in 2.5 ml of a medium containing 0.075 M Tris, 0.015 M KCl, 0.03 M KH_2PO_4, 0.045 M sucrose and 0.005 M $MgCl_2$ at pH 7.4 and 25°.

DNA synthesis, RNA synthesis, and precursor uptake. Incubations of Ehrlich ascites cells with [^{3}H]-thymidine to measure DNA synthesis and [^{3}H]-uridine to measure RNA synthesis were carried out in MEM by a procedure described previously [18]. In [^{3}H]-thymidine experiments, radioactivity in the acid-insoluble fraction was taken as a measure of DNA synthesis, and in [^{3}H]-uridine experiments radioactivity in the acid-insoluble fraction was interpreted as a measure of RNA synthesis. Uptake of either precursor into cells following drug treatment was evaluated from the counts in extracellular and acid-soluble fractions.

Reaction of copper complexes with cellular thiols. Thiol levels of ascites cell suspensions, ascites cell suspensions treated with various copper complexes, and ascites fluid were measured using the thiol reagent DTNB, by a modification of a procedure described previously [17]. Reagent grade DTNB dissolved in pure DMSO (20.7 nmoles/μl) was stored in a foil-covered vial at 4°, and was used only if no substantial yellow tinge was present. Suspensions of freshly isolated Ehrlich ascites cells in phosphate-buffered saline, pH 7.10 (10 mM potassium phosphate, 150 mM NaCl), were treated with various concentrations of CuL, CuKTS, or $CuKTSM_2$ complexes. The latter two materials served as positive and negative controls respectively [17, 18]. Suspensions were incubated at room temperature for 10–30 min and then disrupted on ice using a Branson Sonifier (Branson Sonic Power Co., Danbury, CT.) in the presence of excess DTNB. Cell sonicates were centrifuged at maximum speed in a clinical centrifuge to remove debris and diminish sample turbidity. Absorbance of the supernatant fraction at 412 nm was obtained on a Beckman Acta V spectrophotometer equipped with a dispersion chamber attachment for use with turbid solutions; identical incubations without DTNB were run to obtain the absorbance of blanks. Protein concentration of the cell suspension was measured by the biuret procedure [21].

Reduction of CuL *by glutathione and the consumption of* O_2. The kinetics of reduction were followed spectrophotometrically using a Beckman Acta V spectrophotometer equipped with a cell holder

* Abbreviations: MEM, Eagle's Minimum Essential Medium with Earle's salts; HL, 2-formylpyridine thiosemicarbazone, CuL, 2-formylpyridine thiosemicarbazonato copper (II), CuKTS, 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper (II), $CuKTSM_2$, ethoxy-2-oxobutyraldehyde bis (N^4-dimethyl-thiosemicarbazonato) copper (II), RDPR, ribonucleoside diphosphate reductase; DTNB, 5,5′dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis (β-amino ethyl ether)-*N*,*N*-tetraacetic acid; DMSO, dimethylsulfoxide; DMPO, 5,5-dimethyl-l pyrroline-*N*-oxide; and GSH, glutathione.

maintained at 25 ± 0.5°. The loss of absorbance of CuL over time was taken as a measure of the rate of reaction. Reactions were carried out at pH 7.8 in 0.1 M phosphate buffer. Initial thiol concentrations were 20–200 times that of CuL to provide pseudo first-order conditions for the reaction. Initial and final thiol concentrations were measured colorimetrically using DTNB [22]. Pseudo first-order rate constants were calculated for anaerobic reactions from log absorbance versus time plots. Reactions were carried out in aerated buffers or anaerobically in a closed cuvette in which the reactants were purged with nitrogen prior to mixing.

Oxygen uptake was measured in a Yellow Springs Instrument Co. model 53 with a Houston Instrument Co. recorder. The rate of O_2 consumption in two cells was monitored simultaneously, with one acting as control containing phosphate buffer (0.1 M, pH 7.8)+ GSH and the other, buffer + GSH + CuL. The slow control rate of O_2 reduction was subtracted from the rate in the presence of CuL to obtain the data.

Oxygen radical trapping. Buettner and Oberley [23] have shown that DMPO reacts with O_2^- and OH·radicals to form adducts that have distinctive free radical electron paramagnetic resonance signals. Their technique has been applied to the detection of radical species in the reaction of CuL with glutathione. Stock solutions of CuL, GSH, and the spin trap, DMPO, were mixed to yield final concentrations of 0.4 mM CuL, 10 mM GSH, and 2 mM DMPO. About 1 mM diethylene-triaminepentaacetic acid was added to suppress conversion of O_2^- to OH·by possible trace amounts of iron [23].

RESULTS

Uptake of CuL *by cells.* Studies of the cellular uptake and efflux of CuL confirmed the binding of the complex within the cell. As shown in Fig. 1, there was a biphasic accumulation of copper by Ehrlich cells treated with CuL (final DMSO concentration of 9%) which was dependent upon the external concentration of the drug. When cells exposed to the complex were resuspended in fresh medium after centrifugation and washing, only a small difference in intracellular copper would be detected. Whether this represented efflux or was a minor artifact of the resuspension procedure could not be distinguished. The uptake experiments were repeated at 37° using 2.5 mg cell protein/ml and CuL concentrations varying between 11 and 69 nmoles/mg cell protein. DMSO content of these incubations never exceeded 1 per cent. In all cases, greater than 77 per cent of the complex was taken up in 1 hr (data not illustrated).

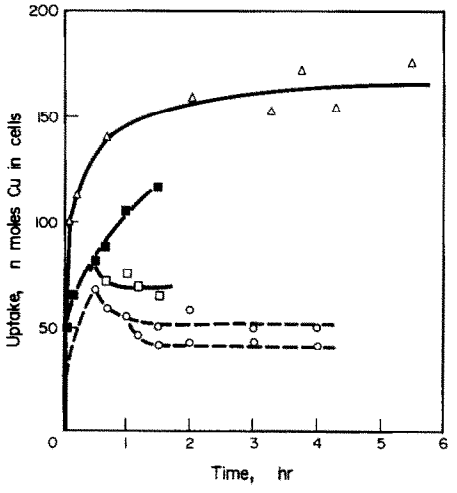

Fig. 1. Kinetics of uptake of CuL by Ehrlich cells. Key: (△) 26 nmoles CuL/mg cell protein, 6.7 mg protein/ml; (■) 13 nmoles/mg CuL, 13 mg protein/ml; (○) 9 nmoles/mg CuL, 18.5 mg protein/ml; and (□, ○) cells removed from incubations, washed thoroughly and placed in fresh medium. All runs were carried out in MEM at 25°. Suspensions contained 9% DMSO, used originally to dissolve CuL.

Reaction of CuL with cells. The reaction of CuL with Ehrlich cells was monitored in several ways. The 380 nm absorbance band of CuL was followed as a function of time by visible spectrophotometry. A very slow decrease in absorbance was noted over the course of 3 hr. Plotting the time-dependent absorbance changes as a first-order decay process, an average first-order rate constant of 4.5×10^{-5} sec^{-1} was obtained for 1.08 to 1.35×10^{-4} M CuL, using cell concentrations ranging from 1.2 to 14.7 mg protein/ml. During theses kinetic runs, the visible spectrum of the complex was virtually unchanged, showing that the 380 nm absorbance was from CuL.

Samples of cells were removed during these incubations, washed, and then packed into quartz tubes for e.p.r. spectral analysis. The resulting spectra, obtained at 77K, also indicate that the complex was undissociated (Fig. 2b). Furthermore, there were significant perturbations of $g_\parallel$ and $A_\parallel$ from values obtained with the control (Fig. 2a) [16]. These spectral shifts are indicative of adduct formation between CuL and Lewis bases [12, 16]. This was definite evidence at low temperature for the binding of CuL within the cell. In addition, a distinct superhyperfine structure from bound nitrogen couplings was clearly resolved in the $g_\perp$ region of the spectrum. These spectral features are consistent with the formation of an adduct between CuL and thiol as described below.

Reaction of CuL with cellular thiols. Table 1 presents the results of DTNB determination of cellular free thiol concentration after reaction of cells with copper complexes. Both CuL and CuKTS diminished the intracellular thiol concentration about 0.7 nmole/mg for each nmole of drug added after a 15- or 30-min incubation of cells with drug. Thiol concentrations of the cell suspension after disruption (including extracellular and surface thiol as well as intracellular thiols) were calculated with respect to the biuret protein determination. Starting cellular thiol levels were approximately 50–70 nmoles RSH/mg cell protein and 3–7 nmoles RSH/mg ascites fluid protein. As in previous results, the large majority of cellular thiol was intracellular and not bound to the exterior of the plasma membrane of these cells [17]. Intracellular thiol levels were diminished significantly as a result of CuL or CuKTS treatment, but they were not decreased after treatment with $CuKTSM_2$, in agreement with previous work [17, 18].

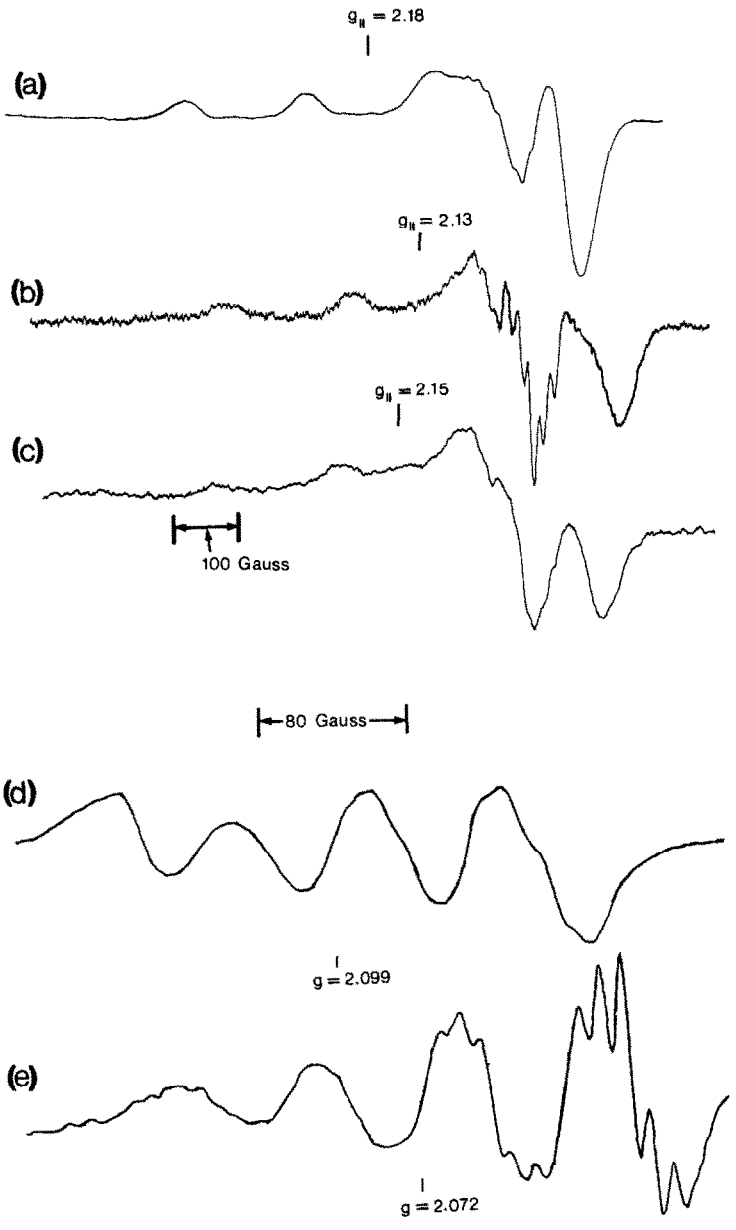

Fig. 2. Electron Paramagnetic resonance spectra of CuL and Ehrlich cells at 77K. Spectra (a, b and c) show e.p.r. spectra of solutions frozen in liquid nitrogen: (a) CuL^+ in 0.1 M KCl and 0.25 M sucrose; (b) CuL^+ in Ehrlich ascites tumor cells; and (c) CuL^+ plus excess glutathione. Spectra (d and e) show e.p.r. spectra of solutions run in a flat cell at room temperature for CuL^+ in KCl at pH 7 in the absence (d) and presence (e) of glutathione. Spectrometer conditions: modulation frequency, 100 kHz; modulation amplitude, 5 G; (a) gain 10×10^2, time constant 1 sec, scan time 4 min, power 5 mW; (b) gain 5×10^3, time constant 3 sec, scan time 8 min, power 5 mW; (c) gain 10×10^3, time constant 10 sec, scan time 1 hr, power 5 mW; (d) gain 5×10^3, time constant 1 sec, scan time 4 min, power 200 mW; (e) gain 5×10^3, time constant 1 sec, scan time 4 min, power 200 mW.

Respiration studies. Previous studies with 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper (II) and Ehrlich cells were consistent with the view that Cu(II) is initially cyclically reduced and reoxidized, leading to an increase in oxygen consumption by the cell suspension [17, 20]. This was followed by a slow inhibition of respiration of the cells relative to untreated controls. Experiments summarized in Fig. 3 show that CuL also stimulated oxygen consumption. There was a concentration-dependent stimulation of oxygen consumption in cells exposed to complex relative to untreated controls. The rate increased and then fell over time. Only in the 60-min points at high concentration,

Table 1. Effect of copper complexes on intracellular thiol concentrations

Experiment and sample	Condition of preincubation	(nmoles Drug/ mg cell protein)	(nmoles RSH/ mg protein)	nmole RSH lost/ nmole Drug used
Ascites fluid	15 min, 25°		5.4	
Ascites cells	15 min, 25°		49.8–62.9*	
Ascites cells	15 min, 25°		47.6	
CuL	15 min, 25°	11	37.8	0.9
CuL	15 min, 25°	55	33.0	0.3
CuKTS	15 min, 25°	10.9	36.1	1.1
CuKTS	15 min, 25°	54.6	28.8	0.4
Ascites cells	30 min, 37°		42.5	
CuL	30 min, 37°	28.6	20.7	0.8
CuKTS	30 min, 37°	28.4	23.9	0.7
$CuKTSM_2$	30 min, 37°	24.4	40.1	0.09
$CuKTSM_2$	30 min, 37°	48.7	41.8	0.02

* Range of values for five cell concentrations was approximately 0.6 to 6.5 mg protein per incubation (approximately 2.5×10^6 to 2.8×10^7 cells per incubation).

however, did one see evidence for significant inhibition of respiration.

Figure 4 shows concentration-dependent effects of CuL upon cell mitochondria and demonstrates that, rather than stimulating mitochondrial respiration, the complex acted as a potent inhibitor of state 3 NADH-linked respiration. Succinate and pyruvate–malate dependent state 4 oxygen consumption was much less sensitive to the complex, as in state 3 respiration with succinate as the electron donor. Similar results were obtained using beef heart mitochondria. Thus, the stimulation of respiration by CuL seen with intact cells was apparently not due to the uncoupling of mitochondria.

[^{3}H]-*Thymidine and* [^{3}H]-*uridine utilization by* CuL-*treated cells*. CuL inhibited short-term DNA synthesis after brief exposure of cells to the complex, with a sharp dose-dependent inhibition at levels on the order of 1 nm drug administered/mg cell protein (Fig. 5). It was 20-fold less effective against RNA synthesis under similar conditions (Fig. 6). That this inhibition was not an artifact of diminished precursor uptake was illustrated by the lack of effect of drug on the accumulation of radioisotope by cells. A similarly sharp dose-dependent inhibition of DNA synthesis has been observed for CuKTS, whereas inhibition by $CuKTSM_2$ is a result of diminished precursor uptake by cells [18]. Cu bleomycin, an

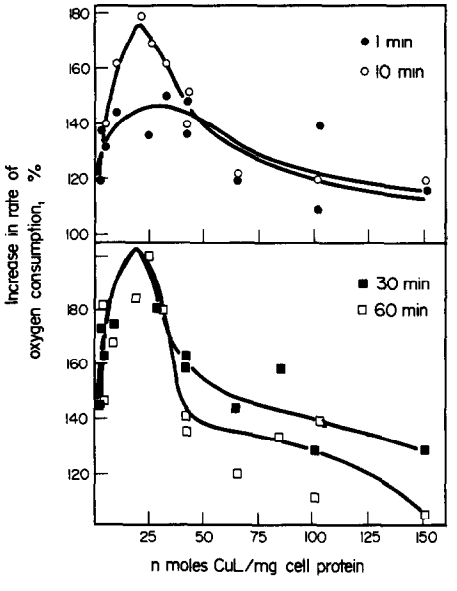

Fig. 3. Increase in oxygen consumption of Ehrlich cells incubated with CuL. Per cent increase in oxygen consumption of CuL-treated cells was measured relative to controls as a function of CuL concentration and time. All runs were carried out in MEM at 25°. The DMSO (used to dissolve CuL) concentration in both CuL and control samples ranged between 1 and 3 per cent.

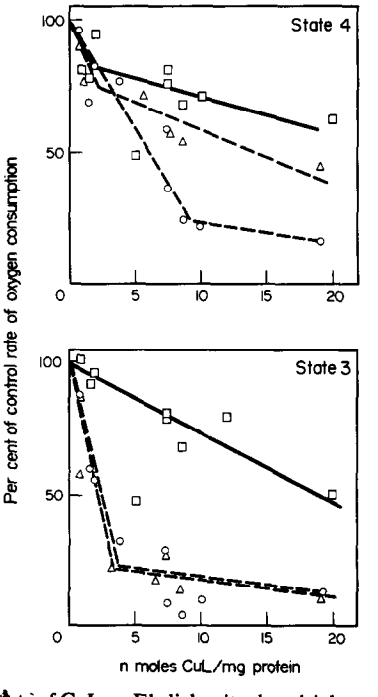

Fig. 4. Effects of CuL on Ehrlich mitochondrial respiration. Substrates used: (□) succinate; (△) pyruvate-malate; and (○) α-oxoglutarate.

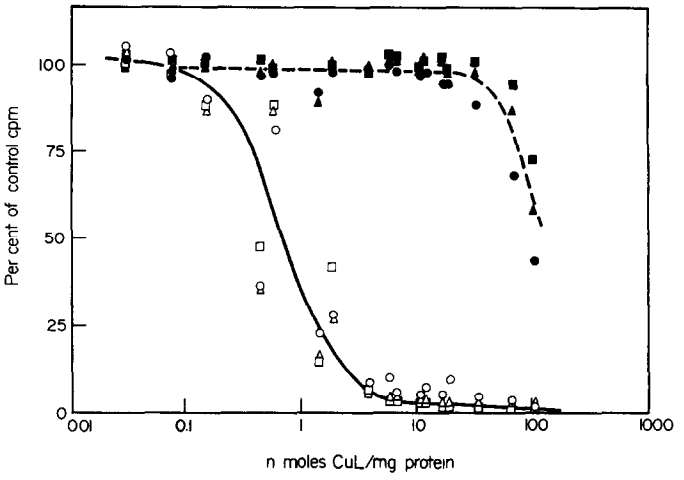

Fig. 5. Dependence of [^{3}H]-thymidine uptake by Ehrlich cells and incorporation into DNA upon CuL concentration. Key: (solid symbols) uptake, and (open symbols) incorporation; circles, triangles, and squares represent 5, 10, and 20-min incubation of precursor with cells after initial 15-min incubation of cells with CuL.

effective antitumor agent *in vivo*, was much less inhibitory to DNA synthesis at these concentrations and showed a much broader dose-dependent response than did CuL (D. H. Petering, unpublished data).

Model reactions of CuL *with thiols*. Attempts have been made to model the e.p.r. spectrum of Fig. 2b using a variety of Lewis bases. Figure 2c shows the e.p.r. spectrum at 77K of CuL in the presence of reduced glutathione, which is presumed to interact with the complex via its sulfhydryl group. One can see that the major features of spectrum 2b are matched well. Although the excellent resolution of the $g_\perp$ superhyperfine structure from bound nitrogens is missing, there are inflections in this region which suggest the presence of nitrogen superhyperfine structure.

In contrast, the room temperature e.p.r. spectrum of CuL and GSH clearly reveals resolved superhyperfine splittings which are absent in the copper complex [Fig. 2 (d and e)]. The splittings in the furthest upfield line in spectrum 2e appear to be due to two nearly equivalent in-plane nitrogens, giving a 1:2:3:2:1 intensity pattern. The non-equivalent nitrogens in the parent structure could become more electronically equivalent if another sulfur ligand enters the coordination plane of the thiosemicarbazone to yield an N_2S_2 set of ligating atoms for the copper in place of the N_2SX set for CuL in solution. The shift of the isotropic g value from 2.099 to 2.072 seen here in spectra 2d and 2e is consistent with the addition of a sulfur atom to the in-plane coordination sites according to previous studies [24, 25]. Thus, there is evidence for significant adduct formation between CuL and glutathione at room temperature. Given these results and those shown in spectra 2b and 2c, CuL may well react with thiols such as glutathione in the Ehrlich cells.

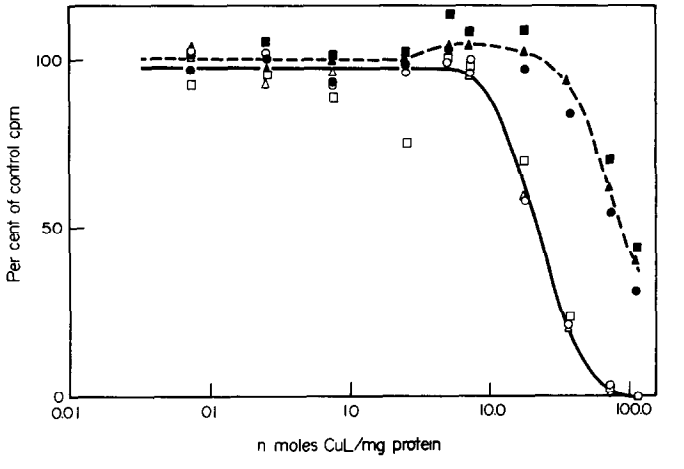

Fig. 6. Dependence of [^{3}H]-uridine uptake by Ehrlich cells and incorporation into RNA upon CuL concentration. Key: (solid symbols) uptake, and (open symbols) incorporation; circles, triangles, and squares represent 5, 10, and 20-min incubation of precursor with cells after initial 15 min incubation of cells with CuL.

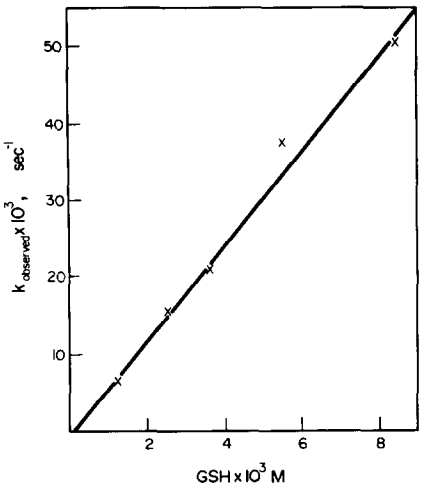

Fig. 7. Summary of kinetics of reaction of CuL with GSH under anaerobic conditions. Pseudo first-order rate constants are plotted versus GSH concentration. All kinetic runs were done in 0.10 M KCl and 0.1 M phosphate at pH 7.8 and 25.0°.

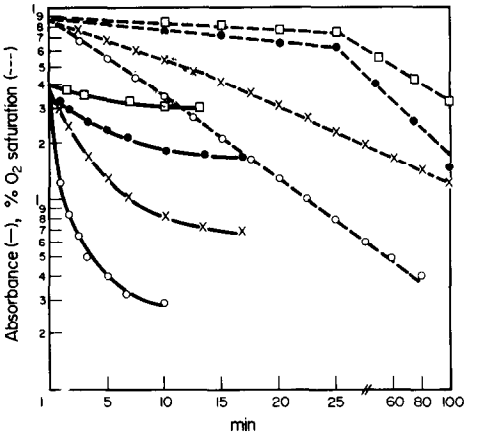

Fig. 8. Kinetics of reaction of CuL with GSH and the oxygen consumption of this reaction under aerobic conditions. Reaction mixtures contained 0.034 mM CuL and 0.1 M phosphate at pH 7.8 and 25°. Initial GSH concentrations for absorbance measurements: 0.63 (□), 1.29 (●), 3.47 (X), and 5.71 mM (○). Initial GSH concentrations for O_2 measurements: 0.77 (□), 1.37 (●), 3.49 (X), and 5.68 mM (○).

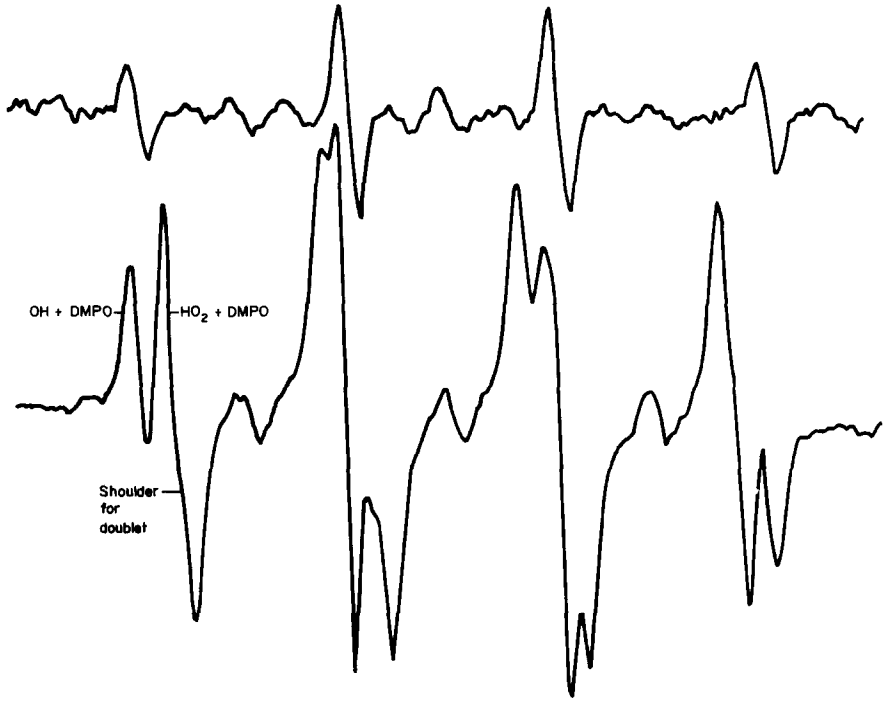

Fig. 9. Electron paramagnetic resonance spectra of the spin-trapped radicals generated in the aerobic reaction of CuL with GSH. The components described in Materials and Methods were mixed at room temperature at t = O, transferred to a flat cell, and their e.p.r. spectrum run repetitively at t = 1 min and every 4 min thereafter at room temperature. The upper spectrum shows the control reaction omitting CuL in which GSH reacts directly with O_2. The lower panel shows the complete reaction mixture.

Besides the possibility of binding of GSH to CuL, one must also consider a redox reaction between the Cu(II) complex and the thiol. When CuL and the tripeptide were mixed, the complex was reduced as shown by the loss of its characteristic absorption spectrum and the disappearance of its e.p.r. signal. The characteristic spectral features of the ligand appeared during this process. Under anaerobic conditions, reactions carried out in the presence of more than a 10-fold excess of thiol followed first-order kinetics in the loss of the 380 nm absorbance band and went to completion. Figure 7 shows the dependence of the pseudo first-order rate constants upon the concentration of GSH. The second-order rate constant calculated from these data was 6.2 sec^{-1}.

In the presence of oxygen, however, similar reaction mixtures go to a steady state leaving CuL in solution, which was detected as the glutathione adduct by e.p.r. spectroscopy in Fig. 2 (Fig. 8). The kinetics of oxygen consumption by the same aerobic solutions of CuL and GSH are shown in Fig. 8. Oxygen reduction continued long after CuL had reached a steady state.

Finally, the nature of the intermediate reduced products of oxygen in the aerobic reaction of CuL with glutathione was investigated. When DMPO, a spin-trapping reagent, was added to a mixture of CuL, GSH, and a saturated phosphate buffer, both $O_2^{\cdot-}$ and OH· spin adducts of DMPO were generated (Fig. 9). Repetitive scans of the electron paramagnetic resonance properties of the trap indicated an apparent steady-state concentration of the $O_2^{\cdot-}$–DMPO adduct for longer than 40 min. This adduct has been reported to have a half-life of 80 sec at pH 6 [23]. Thus, the continuous presence of the adduct is consistent with a constant flux of $O_2^{\cdot-}$. Often CuL was dissolved in 10% DMSO to increase its solubility for these experiments. In the presence of DMSO, the reaction mixture also produced a carbon-like radical, presumably due to the attack of reduced oxygen radicals upon the methyl groups of DMSO.

DISCUSSION

Past studies by Saryan *et al.* [11] have shown CuL and related monothiosemicarbazonato copper complexes to be potent cytotoxic agents against cells exposed to compounds *in vitro* and then injected into host mice. This study addressed the question of the mode of interaction of CuL with intact tumor cells. Because of the importance of the copper in this process, comparisons of CuL with other copper-containing antineoplastic agents, such as CuKTS and Cu bleomycin, will be of interest.

Uptake of CuL by Ehrlich cells was largely irreversible over the time periods examined and occurred readily despite the 1+ charge on the complex. Uptake may have been facilitated by amino acid ligands in the MEM which can form adducts with the CuL species [12, 16]. The observation of minimal efflux of CuL suggests that the drug was bound within cells. Electron paramagnetic resonance spectra of CuL in Ehrlich cells at low temperature support the formation of an adduct between CuL and cellular ligands. These spectra were modelled by CuL plus glutathione, a principal thiol-containing constituent of these cells.

Another striking feature of the interaction of CuL with Ehrlich cells was that very little net breakdown of the complex was observed. Unlike CuKTS, which is rapidly broken down with concomitant loss of the characteristic visible absorption and e.p.r. spectra [17], the spectrum of CuL in cells decayed very slowly. Electron paramagnetic resonance spectra also confirmed the stability of the CuL complex in cells.

The apparent stability of CuL in cells represented a steady state rather than an equilibrium for some time after addition of the drug to cells. While there was little change in the CuL spectrum, intracellular thiol levels fell, presumably because of their oxidation to disulfides, which do not react with DTNB. Concomitantly, non-mitochondrial oxygen consumption was greatly stimulated.

Except for the lack of breakdown of CuL, these results are very similar to those obtained for CuKTS [17, 18, 20]. The difference between these two complexes appears to reside in the reaction of O_2 with the complexes in the presence of thiols. With CuKTS, oxygen had no effect either on the second-order rate constant or on the extent of reaction in the presence of excess thiol. Similarly, the *in vivo* reduction of CuKTS occurred much more rapidly than the stimulation of O_2 consumption [17, 20]. For this reaction, Minkel and Petering [17] postulated an initial reaction in which Cu(II) is reductively dissociated from the complex.

$$\underset{\text{Cu(II)KTS}}{} + 2\text{RSH} \rightarrow \underset{\text{Cu(I)SR}}{} + \underset{(\text{RS}\cdot)}{1/2\text{RSSR}} + \underset{}{\text{H}_2\text{KTS}} \qquad (1)$$

which precedes the further redox processes involving O_2 summarized as

$$4\text{RSH} + \text{O}_2 \xrightarrow{\text{Cu}} 2\text{RSSR} + 2\text{H}_2\text{O} \qquad (2)$$

in which copper, either as Cu(I)SR or another copper complex, serves as a redox catalyst.

This separation of reactions can no longer be maintained with CuL. The binding of the N N S thiosemicarbazone ligand to Cu^{2+} leaves an in-plane as well as axial coordination site(s) that can interact with thiols and oxygen differently than the S N N S·Cu(II) structure of Cu(II)KTS. One obvious difference was the stronger interaction of CuL with Lewis bases including thiols. To assess the importance of this difference will require further study. What is clear is that the tridentate ligand that was bound to copper facilitated reoxidation of the reduced complex relative to dissociation to yield free ligand and Cu(I)SG.

The e.p.r. experiments and oxygen consumption kinetics of CuL-glutathione reactions suggest that CuL was cycling between oxidation states, being reduced by thiol and reoxidized by O_2, without significant dissociation of Cu and ligand. Thus, it appears that CuL serves as a catalyst and that the qualitative features of the *in vitro*, aerobic reduction of CuL with thiols—adduct formation, approach to a steady state for Cu(II)L, enhanced oxygen

uptake—match those seen for the interaction of CuL with Ehrlich cells.

Further information about the transformations of oxygen in the reaction of CuL with cells is suggested by the *in vitro* e.p.r. spin-trapping experiments that revealed that O_2 was reduced to species such as O_2^- and the reactive hydroxyl radical (Fig. 9). This reaction was shown to generate larger quantities of these reactive intermediates than were seen in a comparable reaction using Fe(III) bleomycin in the presence of glutathione and O_2 [26, 27]. This is significant, since it has been postulated that the DNA strand scission activity of bleomycin is based on the radical generating capacity of its iron (II) complex in the presence of O_2 [27]. Downey *et al.* [28] have obtained data suggesting that 1,10-phenanthroline will degrade DNA in the presence of cupric salts and thiol reducing agents. Similar experiments are underway to assess the reactivity of CuL thiol mixtures toward DNA in order to ascertain whether the mechanism of radical-induced degradation of biomolecules may be implicated in the case of CuL as well.

Thus, the reaction of CuL with cells appears to produce at least three effects: (1) CuL is bound to cellular ligands, perhaps glutathione; (2) cellular thiol groups are oxidized; and (3) reduced oxygen radicals are generated. The relationships of these chemical events to cell death have yet to be established. Recently, however, the production of oxygen radicals has been implicated in the mechanism of quinone antitumor agents such as streptonigrin and for Fe-bleomycin [27, 29]. Antholine *et al.* [30] have recently demonstrated an additive cytotoxic effect of CuL and X-rays on CHO cells, lending further support to the hypothesis of the radical damage in the mechanism of CuL.

Turning to the biological effects of CuL, the complex strongly inhibits the incorporation of thymidine into DNA and does so with approximately the same concentration dependence as CuKTS in short-term assays [18]. The lack of effect of CuL on RNA synthesis at concentrations that result in 90 per cent or greater, inhibition of DNA synthesis implies a strong specificity of CuL for reactions involved exclusively in the DNA pathway. Similar conclusions were reached using thiosemicarbazone ligands tested *in vivo* against sarcoma 180 [31, 32]. The lack of inhibition of uptake of thymidine or uridine confirms that the inhibition of DNA and RNA synthesis in this system is not an artifact of diminished precursor uptake.

A comparison of the concentration dependence of inhibition of DNA synthesis by CuL (1 nmole/mg) with its cytostatic effect (50 nmoles/mg) shows a significant disparity, with the concentration required for effective tumor inhibition being many-fold greater than that required for DNA synthesis inhibition [11]. This result differs from that of CuKTS, in which approximately equivalent concentrations were effective against both processes.

These results show that short-term exposures of unsynchronized Ehrlich cells to CuL are sufficient to inhibit DNA synthesis and tumor growth. However, CuL has also been shown to inhibit the cell cycle of CHO cells in G_1[30]. Hence, there are multiple biological responses to CuL. It has yet to be determined how the initial chemical reactions described here relate to these responses and to irreversible cytotoxicity.

Acknowledgements—This work was supported by NIH Grant CA 16156. Electron paramagnetic resonance spectra were run at the National Biomedical EPR Center at the Medical College of Wisconsin in Milwaukee. L. A. Saryan is a recipient of NIH Postdoctoral Fellowship CA 05528, under which a portion of this work was performed.

REFERENCES

1. G. R. Gale, in *Antineoplastic and Immunosuppressive Agents* Part II (Eds. A. C. Sartorelli and D. G. Johns), pp. 829–40. Springer, Berlin (1975).
2. D. H. Petering and H. G. Petering, in *Antineoplastic and Immunosuppressive Agents* Part II (Eds. A. C. Sartorelli and D. G. Johns), pp. 841–9. Springer, Berlin (1975).
3. A. Erck, L. Rainen, J. Whileyman, I. M. Chang, A. P. Kimball and J. Bear, *Proc. Soc. exp. Biol. Med.* **145**, 1278 (1974).
4. F. A. French, A. E. Lewis, A. H. Sheena and E. J. Blanz, Jr., *Fedn. Proc.* **24**, 402 (1965).
5. F. A. French and E. J. Blanz, Jr., *J. med. Chem.* **9**, 585 (1966).
6. F. A. French and E. J. Blanz, Jr., *Cancer Res.* **26**, 1638 (1966).
7. E. J. Blanz, Jr. and F. A. French, *Cancer Res.* **28**, 2419 (1968).
8. E. J. Blanz, Jr., F. A. French, J. R. DoAmaral and D. A. French, *J. med. Chem.* **13**, 1124 (1970).
9. W. E. Antholine, J. M. Knight and D. H. Petering, *J. med. Chem.* **19**, 339 (1976).
10. K. C. Agrawal, B. A. Booth, E. C. Moore and A. C. Sartorelli, *Proc. Am. Ass. Cancer Res.* **15**, 289 (1974).
11. L. A. Saryan, E. Ankel, C. Krishnamurti, D. H. Petering and H. Elford, *J. med. Chem.* **22**, 1218 (1979).
12. W. E. Antholine, J. Knight, H. Whelan and D. H. Petering, *Molec. Pharmac.* **13**, 89 (1977).
13. E. C. Moore, K. C. Agrawal and A. C. Sartorelli, *Proc. Am. Ass. Cancer Res.* **16**, 639 (1975).
14. R. C. DeConti, B. R. Toftness, K. C. Agrawal, R. Tomchick, J. A. R. Mead, J. R. Bertino, A. C. Sartorelli and W. A. Creasey, *Cancer Res.* **32**, 1455 (1972).
15. I. H. Krakoff, E. Etcubanas, C. Tan, K. Mayer, V. Bethune and J. H. Burchenal, *Cancer Chemother. Rep.* (Pt. 1) **58**, 207 (1974).
16. W. E. Antholine, J. M. Knight and D. H. Petering, *Inorg. Chem.* **16**, 569 (1977).
17. D. T. Minkel and D. H. Petering, *Cancer Res.* **38**, 117 (1978).
18. D. T. Minkel, L. A. Saryan and D. H. Petering, *Cancer Res.* **38**, 124 (1978).
19. R. F. W. Thorne and F. L. Bygrave, *Cancer Res.* **33**, 2562 (1973).
20. C. H. Chan-Stier, D. Minkel and D. H. Petering, *Bioinorg. Chem.* **6**, 203 (1976).
22. G. C. Ellman, *Archs. Biochem. Biophys.* **82**, 70 (1959).
23. G. R. Buettner and L. W. Oberley, *Biochem. biophys. Res. Commun.* **83**, 69 (1978).
24. J. Peisach and W. E. Blumberg, *Molec. Pharmac.* **5**, 200 (1969).
25. J. Peisach and W. E. Blumberg, *Archs. Biochem. Biophys.* **165**, 691 (1974).
26. D. Solaiman, E. A. Rao, D. H. Petering, R. C. Sealy and W. E. Antholine, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1519 (1979).

27. E. A. Sausville, R. W. Stern, J. Peisach and S. B. Horwitz, *Biochemistry* **17**, 2746 (1978).
28. K. Downey, B. G. Que and A. G. So, *Biochem. biophys. Res. Commun.* **93**, 264 (1980).
29. R. Cone, S. K. Hasan, J. W. Lown and A. R. Morgan, *Can. J. Biochem.* **54**, 219 (1976).
30. W. E. Antholine, P. Gunn and L. E. Hopwood *Int. J. Radiat. Oncol. Biol. Phys.* in press (1981).
31. K. C. Agrawal and A. C. Sartorelli, in *Antineoplastic and Immunosuppressive Agents* Part II (Eds. A. C. Sartorelli and D. G. Johns), pp. 793–807. Springer, Berlin (1975).
32. A. C. Sartorelli, K. C. Agrawal, A. S. Tseftsoglou and E. C. Moore, *Adv. Enzyme Regulat.* **15**, 117 (1976).