Oxidative Stress Induced by a Copper-Thiosemicarbazone Complex†

Robert W. Byrnes,‡ Madan Mohan,‡ William E. Antholine,‡,§ Robert X. Xu,‡,∥ and David H. Petering*,‡
Department of Chemistry, University of Wisconsin—Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201, and National Biomedical ESR Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Wauwatosa, Wisconsin 53226
Received December 19, 1989; Revised Manuscript Received April 2, 1990

ABSTRACT: Copper thiosemicarbazones cause considerable oxidative stress. This effect may be related to their cytotoxicity. In the present work, the chemical and cellular properties of a new ligand, pyridoxal thiosemicarbazone (H_2T), and its copper(II) chelate (CuT) are assessed. CuT is toxic to cultured Ehrlich ascites tumor cells, producing nearly complete cell kill at drug/cell ratios of 2.5–4 nmol/10^5 cells in a monolayer culture over a 48-h treatment period. This concentration is at least 1 order of magnitude lower than those required for a similar degree of cytotoxicity by H_2T or $CuCl_2$. The following observations support the view that activated oxygen species are generated by interaction of CuT with Ehrlich cells: (1) Room-temperature electron spin resonance spectroscopy and atomic absorption measurements show rapid cellular uptake and CuT–thiol adduct formation. (2) Cellular thiol content is reduced. (3) High levels of DNA strand scission result from 1-h treatments of cells by concentrations of CuT that cause growth inhibition and toxicity. (4) The extent of strand scission can be increased by addition of superoxide dismutase and decreased by catalase or DMSO in the treatment medium. Catalase and DMSO do not inhibit the toxic effect of CuT. This suggests that DNA damage is not responsible for inhibition of cell proliferation by CuT.

The lower oxidation states of several copper and iron complexes that bind to DNA are known to cause cell-free degradation of the macromolecule. This is achieved through redox reactions that activate molecular oxygen to forms that directly attack and cleave the DNA backbone. These complexes include copper(I) and iron(II) bleomycin and the 1,10-phenanthroline complex of Cu(I) (Sausville et al., 1976; Ehrenfeld et al., 1985, 1987; Sigman, 1986). The metallobleomycins may exert cytotoxic effects (Lyman et al., 1986) by causing DNA degradation in cells (Suzuki et al., 1969).

It has been proposed that thiosemicarbazonato metal complexes induce toxic effects through generation of active oxygen species (Saryan et al., 1981; Antholine et al., 1985). This mechanism is supported by work demonstrating that copper and iron complexes are much more toxic to cultured cells than metal-free ligands (Petering & van Giessen, 1966; Saryan et al., 1981) and by studies showing an extensive similarity in oxidation–reduction activities of copper 2-formylpyridine thiosemicarbazone (CuL)[1] in chemical reactions and in cells. In particular, binding of CuL to thiols and their oxidation together with stimulation of oxygen consumption have been measured in both chemical and cellular model systems. Production of hydroxyl radicals by CuL and glutathione in a cell-free system has also been demonstrated by electron spin resonance spectroscopy (Saryan et al., 1981). DNA binding by some copper thiosemicarbazones (Mikelens et al., 1976, 1978; Rohde et al., 1979) suggests the possibility that the cellular oxidative chemistry of copper thiosemicarbazones may produce DNA damage by reactions similar to those of bleomycin or phenanthroline complexes.

The increasing interest in mechanisms of metal-catalyzed oxidative damage to DNA (Stubbe & Kozarich, 1987) and parallel interest in metal-dependent oxidative damage to cells (Aust et al., 1985) have stimulated the present study. Even though it is commonly assumed that oxidative cleavage of DNA by agents such as bleomycin is responsible for cytotoxicity (Petering et al., 1990), the causal relationship between DNA damage and cell toxicity remains unresolved and relatively unexplored. Experiments were undertaken to examine the redox chemistry and DNA binding of (pyridoxal thiosemicarbazone)copper(II) (CuT), possessing a nitrogen, oxygen, and sulfur (NOS) set of ligand donor atoms for metal chelation (Figure 1), the effects of its presence in tumor cells on the integrity of DNA, and the relationship between cellular oxidative damage to DNA and cell viability.

Experimental Procedures

All solutions were prepared in deionized, distilled water with acid- or NaOH/EDTA-washed glassware. All reagents were of laboratory grade. Catalase (from bovine liver, 11 000 units/mg) superoxide dismutase (from bovine erythrocytes, 3000 units/mg) from Sigma were prepared in PBS prior to use. Glutathione (Sigma) was prepared daily in water and kept on ice until use to minimize oxidation. Radiolabeled DNA precursors were from RPI Products.

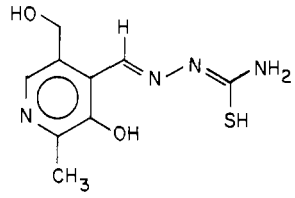
Preparation of Pyridoxal Thiosemicarbazone. Pyridoxal thiosemicarbazone (H_2T ligand) was prepared from pyridoxal and thiosemicarbazide as described previously (Mohan et al., 1989).

Synthesis of Copper Monoaquomono(pyridoxal thiosemicarbazone) Chloride Monohydrate [$[Cu(C_9H_{11}N_4O_2S)(OH_2)]Cl\cdot H_2O$]. Pyridoxal thiosemicarbazone (1 mM, 0.240 g) was dissolved in 15 mL of methanol and added with constant stirring to a solution of $CuCl_2\cdot 2H_2O$ (1 mM, 0.17

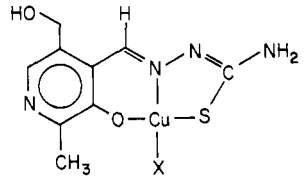
† Supported by NIH CA-22184, ES-04184, RR-01008, and a grant to M.M. from the International Union Against Cancer (UICC), Switzerland.
* Author to whom correspondence should be addressed.
‡ University of Wisconsin—Milwaukee.
§ Medical College of Wisconsin.
∥ Present address: Department of Biochemistry, University of Washington, Seattle, WA 98195.

[1] Abbreviations: CuL, the copper complex of 2-formylpyridine thiosemicarbazone; H_2T, 3-hydroxy-5-(hydroxymethyl)-2-methyl-4-formylpyridine thiosemicarbazone or pyridoxal thiosemicarbazone; CuT, the copper complex of pyridoxal thiosemicarbazone; NBT, nitroblue tetrazolium; GS or GSH, glutathione; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; BSA, bovine serum albumin.

0006-2960/90/0429-7046$02.50/0



3-HYDROXY-5-HYDROXYMETHYL-2-METHYL-4-FORMYLPYRIDINE THIOSEMICARBAZONE (H_2T)



3-HYDROXY-5-HYDROXYMETHYL-2-METHYL-4-FORMYLPYRIDINE THIOSEMICARBAZONATO Cu^{2+} (CuT)

FIGURE 1: Structures of pyridoxal thiosemicarbazone (H_2T) and its copper complex (CuT).

g) in 20 mL of methanol. The solution was refluxed for 1 h and allowed to cool at −25 °C overnight. The dark green crystalline solid was filtered, washed with ethanol and diethyl ether, and dried in vacuo over P_2O_5. Analyses for C, H, and N were carried out on a Perkin-Elmer Model 240C elemental analyzer. Anal. Calcd for $CuC_9H_{15}N_4O_4SCl$: C, 28.87; H, 4.01; N, 14.97. Found: C, 28.72; H, 3.97; N, 14.90. The compound decomposed above 225 °C. ^{63}CuT was prepared with ^{63}CuO from Oak Ridge National Laboratory, Oak Ridge, TN.

CuT stock solutions were prepared in either DMSO (Aldrich) or water. Aqueous stock solutions stored at 4 °C were noted to gradually decrease in cellular strand scission effectiveness over a 3-month period. Consequently, aqueous stocks were either used immediately or frozen at −25 °C and used within 2–3 weeks, with no apparent loss of optical absorption or strand scission properties. No time-dependent alterations in DMSO stock properties were noted.

Growth Inhibition of Cultured Ehrlich Cells. Effects of CuT, H_2T, and $CuCl_2$ on growth of Ehrlich cells during 48-h exposure periods were studied as previously described (Lyman et al., 1986). In one set of experiments, CuT, H_2T, or $CuCl_2$ was added to 1-mL wells in a 24-well culture plate immediately prior to addition of 2×10^5 cells in 1 mL of complete fresh Eagles' minimal essential medium plus Earle's salts with 2.5% fetal bovine serum. After 48 h at 37 °C, cells were trypsinized and counted. Counts were normalized relative to H_2O ($CuCl_2$) or DMSO (CuT, H_2T) controls.

In studies examining the effects of DMSO and catalase on the toxicity of CuT, CuT (in DMSO or H_2O, respectively) was added immediately prior to addition of cells. Catalase activity in treatment media was measured by the decrease in absorbance of H_2O_2 in phosphate buffer at 240 nm (Lück, 1963). Aliquots of media were added to a cuvette containing 2 mL of assay buffer plus H_2O_2 and the time for the decrease of H_2O_2 absorbance from 0.45 to 0.40 was measured. The absorbance span was altered appropriately to correct for drug absorbance when solutions containing copper complex were assayed. The absence of an inhibitory effect of CuT on catalase was examined by using 20 μM CuT plus 0.5 units of catalase/mL of assay buffer; no effect on activity was found.

ESR Spectroscopy. ESR spectra were obtained either at room temperature in a flat cell or at 77 K. Samples were placed in standard quartz ESR tubes prior to freezing. The sides of the tubes were then warmed slightly to allow the frozen samples to slide into a finger Dewar filled with liquid nitrogen for acquisition of spectra.

Spin trapping spectra were obtained at ambient temperature by using a flat cell, a Varian dual cavity, and a Varian E-line Century Series spectrometer. DMPO was supplied by Aldrich Chemical Co., Inc. Aqueous stock solutions were treated with charcoal to remove interfering impurities (Buettner & Oberley, 1978). Samples were mixed and pipeted into the flat cell with scanning initiated within 60 s.

For cellular uptake experiments, ascites tumor cells freshly obtained from mice were used. Cell suspensions at concentrations of $(1–1.5) \times 10^8$/mL in PBS were treated with CuT and either placed in an ESR flat cell for ambient temperature measurements or frozen in liquid nitrogen for measurements at 77 K.

Oxygen Consumption. Consumption of oxygen in air-saturated solutions was followed by using a Yellow Springs Instrument Co. Model 53 oxygen monitor. $CuCl_2$ and CuT concentrations were checked by atomic absorption spectroscopy. The buffer contained 50 mM HEPES buffer, 0.1 M NaCl, and 2% DMSO at pH 7.4. Reactions were started by addition of GSH.

Absorption Spectroscopy. Absorption spectra were taken on a Hewlett-Packard 8451A diode array spectrophotometer. Digitized spectra were taken at 60-s time intervals and stored for later retrieval and analysis.

Acid Solubilization of Labeled DNA. Radiolabeled DNA from Ehrlich cells was prepared as follows (Blin & Stafford, 1976; Maniatis et al., 1982). Suspension cultures (8×10^5/mL, 250 mL) were incubated 24 h in complete growth media containing 0.02 μCi/mL [2-^{14}C]thymidine (specific activity 50 mCi/mmol). Cells were washed with fresh medium without serum, resuspended in 10 mM Tris–1 mM EDTA (pH 7.4), and lysed by addition of 0.5% Sarcosyl. Following digestion with 500 μg/mL pronase, the digest was extracted successively with phenol, 1:1 phenol/chloroform, and chloroform and dialyzed against Tris/EDTA/NaCl. The sample was then further digested with RNase, extracted with 1:1 phenol/chloroform, and dialyzed against 50 mM sodium phosphate buffer, pH 7.4. The DNA was concentrated by ethanol precipitation and resuspended in phosphate buffer (Maniatis et al., 1982). The product possessed an A_{260}/A_{280} ratio of 1.77 and specific activity 0.27 μCi/μmol DNA bases.

Reactions were carried out in 0.4 mL of 20 mM phosphate buffer (15 mM Na_2HPO_4 and 5 mM NaH_2PO_4) at pH 7.4 and 25 °C, using a concentration of 0.32 mM DNA bases. Components were added in the order buffer, DNA, CuT, and finally reducing agent. Typical reaction mixtures contained 4 mM GSH and 0.2 mM metal. Reactions were incubated for 15 minutes, followed by addition of 0.05 M EDTA, 5 mg/mL BSA, and 0.5 M perchloric acid. After a 15 minute precipitation at 0°, the precipitate was removed by centrifugation, and radioactivity in supernatant and precipitate determined separately by liquid scintillation counting (Rao et al., 1980).

Uptake of CuT by Ehrlich Cells. Ehrlich cells maintained in spinner culture were suspended in fresh, prewarmed complete medium at 37 °C at 10^7 cells/mL. CuT was added after 20 min. Beginning immediately after addition, 1–2.5-mL samples were taken at intervals and cells rapidly removed by centrifugation. Supernatants were analyzed for total copper by atomic absorption spectroscopy. The fraction of CuT remaining in the medium was determined after first subtracting out the background contribution of Cu in the medium. Measurement of uptake of CuT into cells incubated in PBS

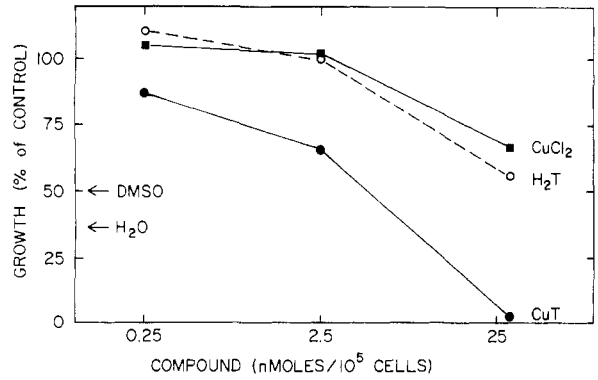


FIGURE 2: Growth inhibition and cell killing of Ehrlich cells by CuT, Cu, and H_2T. Arrows on the Y axis indicate starting cell numbers relative to final cell counts in H_2O and DMSO control treatments.

was carried out at room temperature.

Depletion of Cellular Thiols by CuT. CuT was added to a suspension of cultured Ehrlich cells (10^7 cells/mL) in complete media at 37 °C. At intervals, 1-mL aliquots were taken and washed by addition to 14 mL of cold PBS followed by centrifugation. Cell pellets were suspended in 2 mL of Ellman's reagent (10 mM DTNB in PBS) and sonicated immediately as described previously (Saryan et al., 1981). Cellular debris was removed by centrifugation on a tabletop centrifuge at maximum speed for 10 min and the absorbance at 412 nm determined (Saryan et al., 1981). Thiol content was determined colorimetrically using ϵ_{412} = 13 600 M^{-1} cm^{-1} (Ellman, 1959). Total cellular protein was calculated assuming a factor of 2.5 mg/10^7 cells as previously determined (Kraker et al., 1985).

Alkaline Elution. Alkaline elution experiments for single-strand breaks in cellular DNA were performed by procedure B of Kohn et al. (1981). Cultured Ehrlich cells suspended in fresh, complete MEM were used. [^{14}C]TdR-labeled cells (8×10^5/mL) were treated with $CuCl_2$ (Aldrich), H_2T, or CuT at 37 °C for 1 h, followed by addition to cold PBS prior to subsequent filter elution. [^{3}H]TdR-labeled internal standard cells irradiated with 300-rad ^{137}Cs γ-radiation were used to provide a corrected time scale of elution. Forty-minute (1.4 mL) fractions were collected.

RESULTS

Effect of CuT on Cell Proliferation. Cultured Ehrlich ascites tumor cells have been extensively utilized as a model system in studies of inhibition of cell growth by metal complexes (Lyman et al., 1986; Antholine et al., 1985). Inhibition of Ehrlich cell growth by CuT, the free ligand (H_2T), and by $CuCl_2$ are shown in Figure 2. Essentially complete inhibition by CuT is achieved at concentration ratios in the range 2.5–25 nmol/(10^5 cells)/mL. This effect is achieved by concentrations at least one order of magnitude lower than required for the same level of inhibition by H_2T or by $CuCl_2$.

DMSO exhibits a concentration-dependent toxicity to Ehrlich cells. At 2%, cell growth is inhibited by 33% relative to non-DMSO-treated cells. CuT displays no significant differences in growth inhibition relative to water or DMSO-treated controls when added in the form of aqueous or DMSO stock solutions at 2% final DMSO concentration (not shown).

Described below are results of experiments directed toward understanding how CuT reacts in Ehrlich cells.

Properties of CuT in Solution. The ESR spectrum of CuT in DMSO at 77 K is typical of a square-planar cupric complex with $g_\parallel$ = 2.22 and $A_\parallel$ = 177 G (supplementary Figure 1 and Table I). At room temperature, the ESR spectrum of CuT in DMSO (Figure 3A) is attributed to a low molecular weight

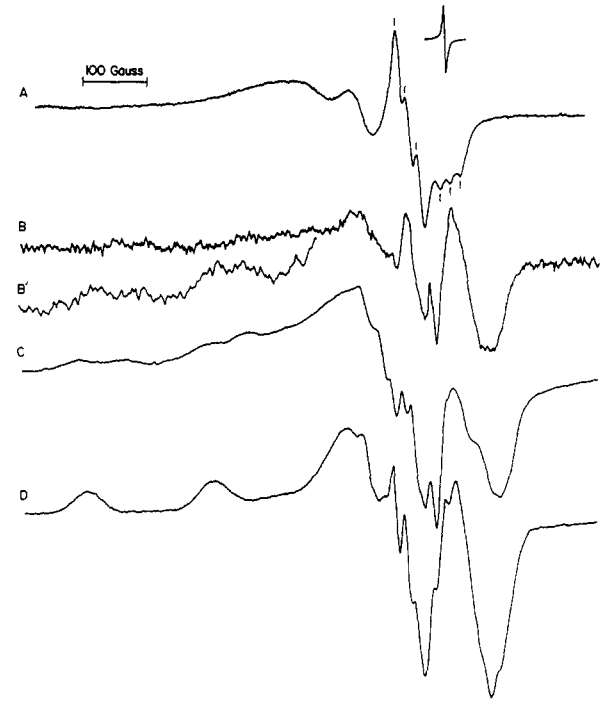


FIGURE 3: ESR spectra for (A) CuT (5 mM) in DMSO at room temperature; (B) CuT (0.5 mM) plus Ehrlich cells (about 1×10^7 cells/mL) in PBS (95%) and DMSO (5%) at room temperature; (B′) scan of solution as in (B) at increased gain; (C) solution as in (B) frozen in liquid nitrogen; and (D) 2 mM CuT frozen in 60% ascites fluid/40% DMSO. Spectrometer conditions. Gain: (A) 4×10^3; (B) 2×10^4; (B′) 10^5; (C) 4×10^3; (D) 4×10^3. Microwave frequency: (A) 9.188 GHz; (B and B′) 9.393 GHz; (C) 9.183 GHz; (D) 9.238 GHz. Microwave power, 100 mW; modulation amplitude, 5 G; modulation frequency, 100 KHz. Splitting of the high-field line into a triplet feature by a single nitrogen atom is indicated in (A). Spectral parameters are given in the text.

complex, g_{iso} = 2.11 and A_{iso} about 72 G. The high-field line is split into a triplet due to a single nitrogen donor atom (A^N = 17 G), consistent with coordination of copper by nitrogen, oxygen, and sulfur ligand atoms. A second set of three lines (A^N about 15 G) suggests the presence of a minor species, one-tenth the concentration of the dominant species. Presumably the tridentate complex, CuT, completes its coordination sphere to form square-planar complexes with either DMSO or trace amounts of water (see supplementary material Figure 1 and Table I).

CuT is stable in the presence of competing ligands. For example, the addition of CuT (75 μM) to ascites fluid, a model biological medium (Petering, 1974), shifts the absorbance bands of the complex as seen in phosphate buffer from 328 and 402 nm to 331 and 410 nm, while preserving the relative intensities of these bands (1.5:1). This indicates that CuT forms adduct species in this medium but is not dissociated to yield H_2T (maxima at 314 and 386 nm with equal band intensity) (Table II of supplementary material). The absorption spectrum does not change for at least 60 min. The perturbation of 77 K ESR parameters, $g_\parallel$ = 2.175 and A^N = 195 G (Figure 3D), also supports the view that CuT forms adducts with Lewis bases in ascites fluid without undergoing ligand substitution.

Reaction of CuT with GSH. The effects of GSH on the absorption spectrum of CuT in aqueous phosphate solution are shown in Figure 4. Immediately upon mixing thiol and complex a new spectrum is evident, which is thought to be that of a GS–Cu(II)T adduct. This is consistent with ESR results (Figure 1 of supplementary material), which indicate that a new species of Cu(II) with $g_\parallel$ = 2.15 is formed upon intro-

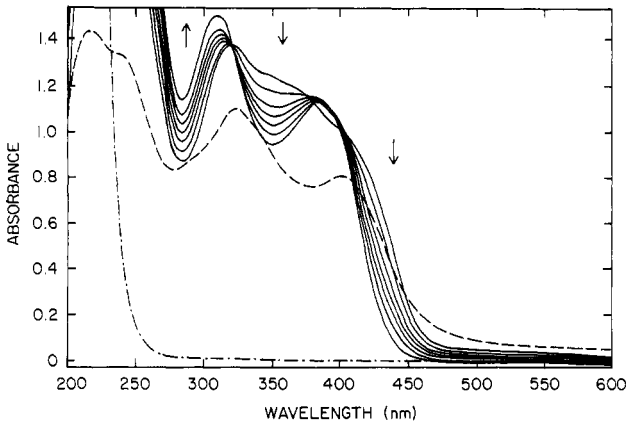


FIGURE 4: Absorption spectra of CuT before and after addition of glutathione. CuT concentration was 70 μM and the buffer was 20 mM phosphate, pH 7.4. Final glutathione concentration was 4 mM. Spectra were taken at 1-min intervals; selected spectra are shown. Arrows indicate direction of change from initial (t = 0) spectrum recorded immediately after addition of GSH. (--) CuT in the absence of GSH; (—) spectra recorded at 0, 1, 3, 7, 13, 19, and 40 min after addition of GSH; (-·-), spectrum of GSH in the absence of CuT.

duction of GSH. Isosbestic points at 318 nm for all spectra of the reaction mixture and at 386 nm after the initial 3 min support the view that the GSH adduct proceeds on to products. The direction of change throughout the time course of reaction is toward the production of free ligand and copper, presumably bound to GSH. This follows from examination of the 280–350-nm region. At the end of 24 h in air the spectrum of CuT is quantitatively restored as GSH is completely oxidized and Cu(II) rebinds to the thiosemicarbazone ligand.

Absorption spectral changes qualitatively similar to those seen in the aerobic reaction are observed when GSH is added to CuT under anerobic conditions. However, they occur more rapidly. This suggests that oxygen is competing for Cu(I)T and reoxidizing it to Cu(II)T, setting up a redox cycle in which electrons from GSH reduce O_2. To investigate this possibility consumption of oxygen by CuT and GSH was measured by means of an oxygen electrode (Antholine & Petering, 1979). Addition of an excess of glutathione (4 mM) to a solution of CuT (0.1 mM) stimulates reduction of oxygen (approximately 0.2 mM in air-saturated solution). Measurements of reaction of oxygen was made during a 10-min observation period beginning 6 min after addition of GSH, in which the rate of decrease of oxygen concentration is constant. The rate of loss of oxygen occurring in the absence of added drug, 0.01 mM/h, is enhanced 30-fold, to 0.3 mM/h. This rate does not change in the presence of 20% DMSO. In contrast, $CuCl_2$ at an equivalent concentration enhances consumption to a lesser degree (0.1 mM/h in the absence of DMSO, 0.05 mM/h in the presence of DMSO). An enhanced rate of consumption by CuT continues until 100% of the oxygen is consumed.

In results supplied in the supplementary material it is shown that CuT catalyzes the dismutation of O_2^- to H_2O_2 and O_2 with efficiency comparable to that of $CuCl_2$. In spin trapping experiments, the reaction of CuT with GSH in DMSO produces a DMPO spin adduct (supplementary material, Figure 2) with proton splittings (A^H = 22.5 G) and nitrogen splittings (A^N = 16 G) that are consistent with the trapping of either a carbon-centered radical or H$^\bullet$ (Buettner, 1987). A carbon-centered radical results when DMPO reacts with a DMSO radical, generated by prior reaction of DMSO with hydroxyl radicals (Lagercrantz & Forshult, 1969).

In Vitro DNA Strand Scission. The activity of various forms of copper in supporting DNA degradation by reduced forms of oxygen was examined. Acid solubilization of

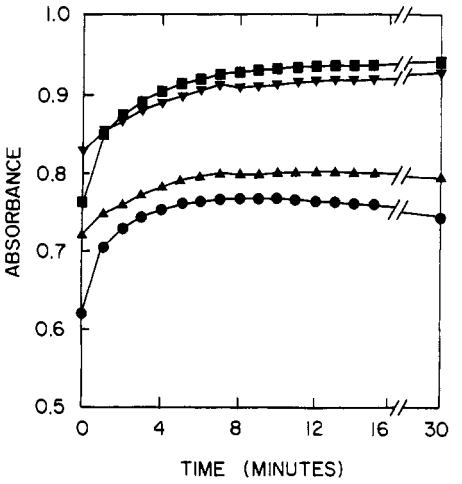


FIGURE 5: Comparison of absorbance changes to CuT following GSH addition in the presence and absence of DNA. Reactions were carried out in the presence or absence of calf thymus DNA (0.5 mM base pairs) in 20 mM phosphate buffer. The CuT concentration was 70 μM. Measurements were initiated immediately after addition of GSH at t = 0. Symbols represent absorbance at (▼) 310 nm in the absence of DNA, (■) 310 nm in the presence of DNA, (▲) 386 nm in the absence of DNA, and (●) 386 nm in the presence of DNA.

[^{14}C]thymidine-labeled DNA obtained from Ehrlich cells was used as an assay of degradation, based upon previous applications of this method (Sausville et al., 1976; Sigman et al., 1979; Rao et al., 1980). Release of radioactivity was not observed, even when conditions were altered to include 60 min of incubation, addition of 5 mM H_2O_2, or addition of GSH prior to CuT. Varying the CuT concentration (between 20 μM and 0.5 mM), addition of 5 mM H_2O_2, or use of CuL in place of CuT was also ineffective. Similarly, 40 μM $CuCl_2$ plus 4 mM GSH solubilized less than 1%. The result with $CuCl_2$ is similar to that of controls in all experiments (<1%). However, a positive control, 40 μM $CuCl_2$ plus 80 μM phenanthroline (Sigman et al., 1979) in the presence of 4 mM GSH, achieved solubilization of 49% of the radioactivity in 15 min. Ferrous bleomycin was also effective in acid solubilization using the same DNA preparation (not shown).

Absorption spectroscopy was used to study the interaction of CuT and the free ligand with DNA. Aliquots of calf thymus DNA, prepared by stirring at 4 °C in phosphate buffer overnight, were added to aqueous solutions of CuT or H_2T (70 μM) in 20 mM phosphate buffer. The absorption spectrum of CuT decreases in intensity in the presence of DNA (Figure 3 of supplementary material), indicative of binding interaction between CuT and DNA. This hypochromic effect is uniform throughout the entire spectrum, including 260 nm, the maximum for DNA. Maximum hypochromicity (15%) occurs at a ratio of CuT to DNA base pairs (r) of 2 to 3. H_2T also associates with DNA as shown by a decrease in intensity of the absorbance band at 386 nm (supplementary material, Figure 3).

To gain insight into the inactivity of CuT in the DNA strand scission assay, the reaction of DNA-bound CuT with GSH was studied by absorption spectroscopy (Figure 5). Upon addition of GSH, similar absorption bands and an isosbestic point at 322 nm are observed as in the absence of DNA. After an initial rapid increase in absorbance indicative of thiol adduct formation, the changes at 310 nm for the reaction in the presence of DNA parallel those of the DNA-free system. The 386-nm band remains hypochromic relative to the reaction in the absence of DNA, indicating that binding of this chromophore persists. The similar reaction of CuT with GSH in the presence and absence of DNA shows that GSH reduces

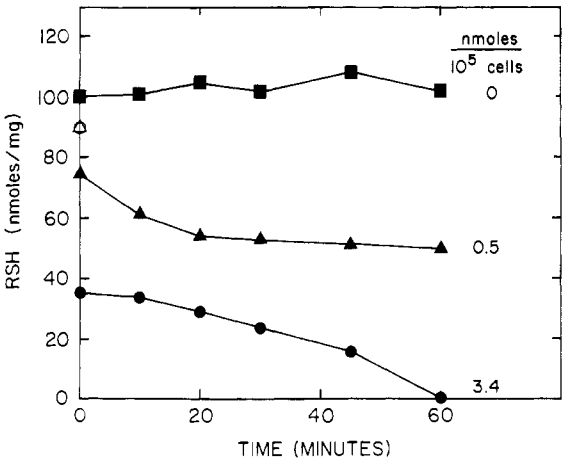


FIGURE 6: Depletion of thiols in Ehrlich cells by CuT (37 °C). The open symbols on the ordinate indicate the quantity of reduced thiol in Ehrlich cells immediately prior to addition of CuT to cells at (Δ) 0.5 nmol/10^5 cells and (O) 3.4 nmol/10^5 cells. The filled symbols at zero time points indicate thiol levels measured immediately after CuT addition. The cell concentrations were 10^7 cells/mL.

and competes for Cu(I) under both conditions. Nevertheless, the capacity to bind to DNA is not sufficient to guarantee that reactive forms of oxygen, generated in proximity to DNA, will be effective in causing damage measurable by the acid solubilization assay.

Cellular Uptake and Reactions of CuT. Cultured cells (10^7/mL) were suspended in complete medium (2.5% serum) or PBS, and CuT was added at ratios of 0.25 or 2.5 nmol/10^5 cells. Rapid distribution of drug between cells and medium occurs, with cells taking up 11% (at 0.25 nmol/10^5 cells) or 21% (at 2.5 nmol/10^5 cells) of the drug within the time of separation of medium and cells. At the lower concentration in medium, a slow additional uptake occurs, for a total uptake of about 27% of the total drug after 60 min. No additional uptake occurs at the higher concentration within this time period. In PBS at 2.5 nmol/10^5 cells, 75% uptake occurs immediately, with no additional uptake after 60 min. It is likely that Lewis base adducts of CuT that form in complete medium partition less well into cells than the uncharged CuT molecule.

Interaction between Cu complexes and cells can be observed if ESR spectra acquired at room temperature show changes in magnetic parameters or in mobility of the complex (Saryan et al., 1981; Antholine et al., 1984). Upon addition of CuT to cells in saline solution (Figure 3B), an ESR spectrum resembling the rigid limit spectrum in the frozen state (Figure 3C) is immediately obtained. In the room temperature spectrum with cells, lines on both the high-field and low-field side of the rapidly tumbling spectrum for CuT in 100% DMSO (Figure 3A) are observed. ESR parameters for two species revealed in the room temperature spectrum are $g_{\|}$ = 2.15 and 2.18 and A^N = 160 and 180 G. This spectrum does not change in intensity for at least 60 min after mixing, indicating that a steady-state concentration of $Cu^{II}T$ exists in the presence of cells.

Lines in the $g_{\|}$ region, which help to identify the nature of the adduct species, are more evident after freezing the sample (Figure 3C). Again, two sets of lines are observed. One set ($g_{\|}$ = 2.15, A^N = 185 G) possesses a g value that is identical with the value found for CuT in the presence of GSH. By *analogy with* CuL, the second species ($g_{\|}$ = 2.19, A^N = 190 G) may be a histidine adduct (Saryan et al., 1981).

CuT depletes cellular thiols rapidly (Figure 6). Between the time of mixing drug and cells and initiation of the cellular thiol assay, 20% and 60% of the thiols are oxidized at ratios of 0.5 and 3.4 nmol/10^5 cells, respectively. Further depletion occurs as time progresses, leading to oxidation of 45% (0.5 nmol of CuT/10^5 cells) or 100% (3.4 nmol of CuT/10^5 cells) of the total sulfhydryl groups at 60 min. Thus, although ESR spectra show the presence of Cu(II)T, the loss of cellular thiols shows that redox cycling of Cu(II)T occurs within cells.

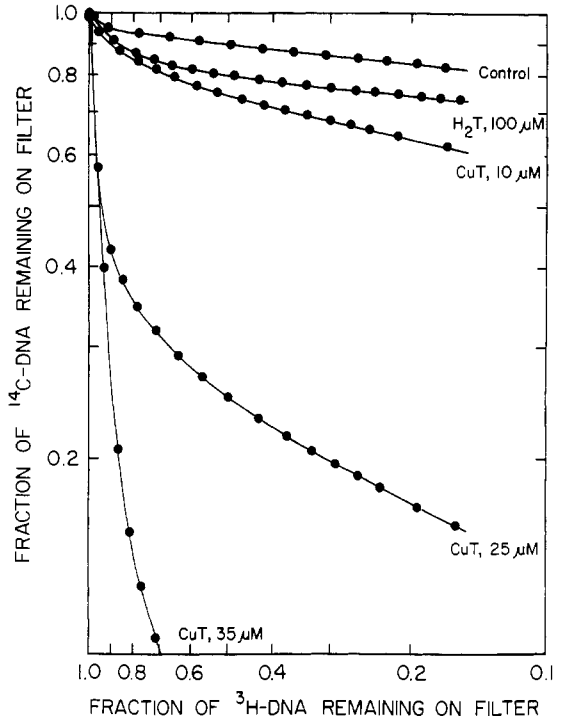


FIGURE 7: Alkaline elution of DNA from cells treated with H_2T or CuT for 1 h.

Intracellular DNA Strand Scission. The cell-free redox chemistry, ready cellular uptake, and cellular thiol consumption by copper thiosemicarbazones indicate that generation of reactive, reduced forms of oxygen may occur upon addition of the complexes to cells. To assess some of their effects, the capacity of CuT and H_2T to cause strand breakage of DNA was studied by the sensitive alkaline elution technique (Kohn et al., 1981). Results are shown in Figure 7. High levels of strand breaks are generated by 1-h treatments with CuT as the concentration is raised from 10 μM (1.2 nmol/10^5 cells) to 35 μM (4.3 nmol/10^5 cells). This closely parallels the range over which CuT exerts its growth inhibitory and toxic effect over a 48- treatment period (Figure 2 and below). In contrast, 100 μM H_2T is less than 10% as effective as 100 μM CuT. No effect is observed for concentrations of $CuCl_2$ up to 50 μM (data not shown).

Scavengers of activated oxygen species were utilized to detect the possible intermediacy of these species in cellular strand scission (Figure 8). Superoxide dismutase at 10 μg/mL did not inhibit strand scission, and instead significantly enhanced the degree of strand scission. Catalase at 10 μg/mL nearly completely inhibited DNA damage. Addition of 2.5% DMSO also strongly inhibited strand scission. Treatment of cells with SOD, catalase, or DMSO alone showed no strand scission as compared with untreated controls (not shown).

To determine whether similar reactions might be important in the toxic effect of CuT, experiments were conducted to determine whether catalase could reverse the inhibition of cellular growth by the complex. The results, shown in Table I, reveal no inhibition of toxicity by catalase. To ensure that no alteration in enzyme activity occurred during these treat-

Table I: Absence of Reversal of CuT Growth Inhibition by Catalase

treatment	[CuT] (nmol/10^5 cells)	catalase[a]	% growth[b]
1	2	−	50
2	2	+	40
3	4	−	1
4	4	+	2
5	10	−	3
6	10	+	1

[a] Presence or absence of catalase at 100 μg/mL. [b] Percent live cells remaining after a 48-h treatment relative to PBS control. Catalase had no effect on growth (growth relative to PBS control 99.6%). Initial cell count was 42% of final control cell count.

ments, in a separate experiment, aliquots of media were centrifuged to remove suspended cells and the supernatants analyzed for catalase activity at the beginning of treatment and after 48 h. The activity of the enzyme in all of the treatments was unaffected over the treatment period. Neither CuT (at 20 μM) nor complete growth media (1% in assay buffer) in the absence of added catalase was found to have any catalase activity.

Discussion

The present study characterizes features of the interaction of (pyridoxal thiosemicarbazone)copper(II) with Ehrlich cells with the objective of determining whether, and by what mechanism, this complex causes oxidative damage in cells. Like (2-formylpyridine thiosemicarbazonato)copper(II) (CuL) the complex is stable to ligand substitution in the presence of competing biological ligands in a model biological medium, ascites fluid (Saryan et al., 1981). It also forms adducts readily to fill out its tridentate, in-plane coordination structure.

These properties are seen as well in the reactions of CuT with Ehrlich cells. As expected from its relatively nonpolar character and zero charge, the complex is rapidly accumulated by cells. This association is accompanied by adduct formation between CuT and thiol donors, as shown by shifts in the ESR spectrum of CuT at 77 K (Figure 3C), which can be modeled by the interaction of GSH with CuT. Also evident from room temperature spectra is the immobilization of CuT in cells (Figure 3B). Model studies show that CuL can bind to hemoglobin, in part because of adduct formation with a sulfhydryl group (Antholine & Taketa, 1982). Thus, in the current study, the combination of loss of motion and evidence of adduct formation by CuT suggests that it is immobilized through binding to macromolecular structures.

Because of the equilibrium stability of CuT in the presence of competing ligands, such as histidine (data not shown), it can undergo oxidation–reduction reactions in cells as an intact structure. The nature of reactions of CuT with the cellular reducing agent glutathione has been studied by absorbance and ESR spectroscopy (Figures 3–5). The following pathway of reaction is consistent with the spectral data:

$$\mathrm{Cu(II)T + GSH \xrightarrow{fast} GS\text{–}Cu(II)T^- + H^+} \quad (1)$$

$$\mathrm{GS\text{–}Cu(II)T^- \rightarrow GS^\bullet + Cu(I)T^-} \quad (2)$$

$$\mathrm{Cu(I)T^- + O_2 \rightarrow O_2^- + Cu(II)T} \quad (3)$$

$$\mathrm{Cu(I)T^- + GSH + H^+ \rightarrow GS^-\text{–}Cu(I) + H_2T} \quad (4)$$

$$\mathrm{GS^-\text{–}Cu(I) + GS^\bullet + H^+ \rightarrow GSSG + Cu(I)} \quad (5)$$

Both absorbance and ESR (77 K and ambient temperature) results indicate that a glutathione adduct of CuT forms immediately upon addition of GSH. The adduct undergoes oxidation–reduction (reaction 2) to a $Cu(I)T^-$ species and an oxidized form of GSH, which ultimately forms GSSG (Saryan et al., 1981). The absorbance experiments also suggest displacement of copper from the ligand. In contact with air, thiols will eventually be completely oxidized by copper and Cu(II)T can reform as seen in these experiments.

Like many copper complexes, CuT catalyzes the dismutation of superoxide ion (Goldstein & Czapski, 1986):

$$\mathrm{Cu(II)T + O_2^- \rightarrow Cu(I)T^- + O_2} \quad (6)$$

$$\mathrm{Cu(I)T^- + O_2^- + 2H^+ \rightarrow Cu(II)T + H_2O_2} \quad (7)$$

Once hydrogen peroxide is formed, Fenton chemistry can convert it to hydroxyl radical

$$\mathrm{Cu(I)T + H_2O_2 \rightarrow {}^\bullet OH + OH^- + Cu(II)T} \quad (8)$$

The generation of $^\bullet OH$ is inferred from the primary spin trapping of DMSO$^\bullet$, formed as $^\bullet OH$ reacts with DMSO present in the reaction mixture. The spin adduct between hydroxyl radical and DMPO was detected directly in the aerobic reaction of CuL^+ and glutathione (Saryan et al., 1981).

That redox chemistry involving CuT occurs in Ehrlich cells is shown in several ways. First, the presence of an ESR signal modeled by a GS–Cu(II)T ESR signal suggests interaction with cellular thiols. This interpretation is supported by the CuT-dependent loss of sulfhydryl groups from Ehrlich cells over a 60-min incubation period (Figure 6). The maintenance of cellular CuT during redox cycling is interesting in light of the rapid reduction/dissociation of the complex during its reaction with GSH. Apparently, in cells reaction 3 is favored over reaction 4. As described below, the effects of CuT on cellular DNA and their modification by superoxide dismutase and catalase are also consistent with the participation of CuT in reactions such as reactions 1–8 in cells. That CuT and not other forms of Cu is probably the major catalytic species for the oxidation of thiols and reduction of oxygen in cells is demonstrated by the presence of a steady-state concentration of CuT (Figure 3b), at least during a 1-h treatment.

Alkaline elution provides a sensitive indication of strand breakage of cellular DNA. In experiments with CuT, increased rates of elution of DNA were seen only after denaturation at high pH (12.1). Other treatments, such as bleomycin, high X-ray doses, or elevated H_2O_2 concentrations (Iqbal et al., 1976; Bradley & Kohn, 1979), lead to the elution of treated cell DNA under relatively neutral conditions, such as found in detergent lysis solution (pH 10) (Kohn et al., 1981). Elution under these conditions is taken as an indication of double-strand breaks (Bradley & Kohn, 1979). Since no such elution was seen after treatment with CuT up to 35 μM, formation of single-strand breaks seems to occur without appreciable double-strand breakage in these experiments.

The need for copper in the DNA strand cleavage reaction of CuT is shown by greatly increased levels of strand scission by CuT above those of H_2T (Figure 7). The involvement of an intracellular pathway of reaction including reactions analogous to reactions 1–8 is supported by the finding that DMSO partially inhibits strand cleavage (Figure 8). In vitro studies have demonstrated that systems that generate hydroxyl radicals introduce single-strand breaks into DNA. Such DNA damage can be inhibited by scavengers of hydroxyl radicals, including DMSO (Repine et al., 1981; Brawn & Fridovich, 1981). Because of the highly reactive character of $^\bullet OH$, which can initiate the strand scission reaction through abstraction of hydrogen from nearest-neighbor C–H bonds or perhaps through other reactions (von Sonntag et al., 1981; Deeble & von Sonntag, 1984), only $^\bullet OH$ formed in close proximity to DNA will cause cleavage and only DMSO molecules in the

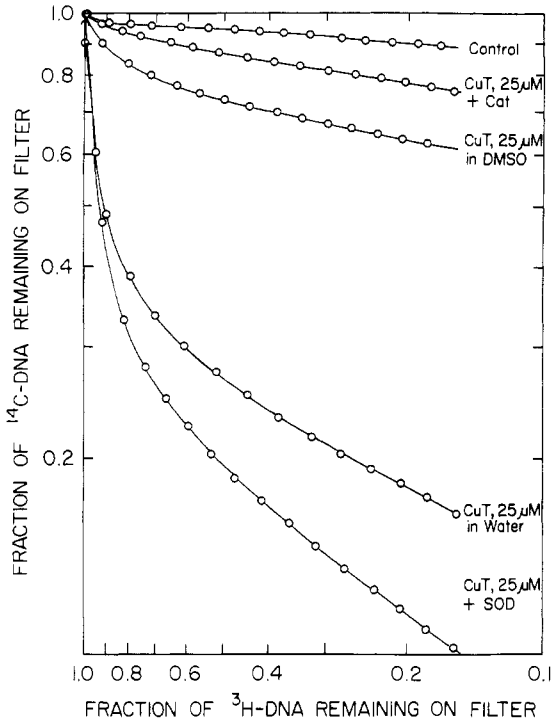


FIGURE 8: Effect of activated oxygen scavengers on alkaline elution of DNA from cells treated with CuT for 1 h. Stock solutions of CuT in either water or DMSO (final DMSO concentration, 2.5%) were added to 8×10^5 cells/mL Ehrlich cells as indicated. Parallel treatments contained catalase (Cat) or superoxide dismutase (SOD) at 10 μg/mL, plus 25 μM CuT added from an aqueous stock solution. Separate addition of aqueous CuT stock solution and DMSO to achieve the same final CuT and DMSO concentrations showed a closely similar degree of inhibition as when DMSO containing CuT was used in a single addition.

same vicinity can compete for hydroxyl radicals.

Hydrogen peroxide added to cells causes single-strand breaks in DNA. The effects of this reagent can also be inhibited by DMSO, implying that H_2O_2 is converted to hydroxyl radical (Bradley & Erickson, 1981). In the present study, nearly complete elimination of DNA damage by catalase, which converts H_2O_2 to O_2 and H_2O, also argues strongly that production of H_2O_2 is required for DNA damage. Indeed, the stimulation of strand breakage by superoxide dismutase, which catalyzes the formation of H_2O_2 by reactions analogous to reactions 6 and 7, supports this view and indicates that O_2^- is formed in cellular reactions of CuT.

Alterations to levels of cellular DNA damage by addition of catalase and superoxide dismutase to the treatment media have been seen in other models of oxidative stress (Kaneko et al., 1984; Kaneko & Leadon, 1986; Leadon et al., 1988). It is conceptually difficult to understand how extracellular enzymes can have decisive effects on the extent of intracellular reactions leading to formation of hydroxyl radical. Still, superoxide ion and hydrogen peroxide can traverse large distances relative to cellular dimensions and can partition through biological membranes (Lynch & Fridovich, 1978; Hassan & Fridovich, 1979). Thus, it seems that the reactions producing O_2^-, H_2O_2, and $^{\bullet}OH$ need not be closely coupled in space or time. This being the case, once the reduction cascade of molecular oxygen begins, it may possibly be catalyzed by normal intracellular components such as superoxide dismutase and iron or copper as well as by CuT.

Chemical results show that GSH-dependent breakage of DNA does not occur in the presence of CuT or the related complex CuL, although CuT is fully reactive with GSH in the presence of DNA. Clearly, the capacity of CuT to associate with DNA does not ensure that damage to the polymer will result from this redox chemistry. Thus, CuT does not resemble copper phenanthroline or iron bleomycin in producing acid solubilization of DNA. Both copper phenanthroline and iron bleomycin achieve DNA degradation through close association of reduced metal with DNA (reviewed in Stubbe & Kozarich, 1987).

Neither catalase nor DMSO inhibit the cytotoxic effect of CuT, though they depress DNA strand breakage. Other work has shown that H_2O_2 treatment of cells at concentrations far below those required for cell death produces high levels of single-strand breaks (Ward et al., 1985). Since the damage to DNA caused by CuT seems to be mediated by H_2O_2, the apparent lack of correlation of CuT toxicity with single-strand breaks agrees well with these data. In the case of H_2O_2, there is evidence that DNA damage in the form of double-strand breaks, detected after treatment at higher, toxic concentrations, may be lethal (Prise et al., 1989).

Alkaline elution provides a sensitive measure of overall single- and double-strand DNA breaks. The inability of scavengers to affect toxicity over the same range of CuT-to-cell ratios used to detect DNA breakage suggests that one must look elsewhere than DNA to find a key molecular site of reaction of CuT. For the same reason, a mechanism requiring activation of cellular nucleases (McConkey et al., 1989) also appears dissociated from CuT toxicity. Thiol oxidation, itself, rather than generation of reactive intermediates, may prove to be the source of copper–thiosemicarbazone toxicity (Petering & Antholine, 1988).

ACKNOWLEDGMENTS

We thank Dr. Ralph Grunewald, Department of Biology, UW—Milwaukee, for use of a ^{137}Cs irradiator.

SUPPLEMENTARY MATERIAL AVAILABLE

A description of ESR spectral results and a summary table of CuT ESR parameters obtained in various experiments, a description of experiments demonstrating dismutation of superoxide by CuT, a table of optical absorption features of H_2T and CuT in different buffers, a table of conditions utilized to examine whether CuT achieved acid solubilization of radiolabeled DNA, a figure containing ESR spectra of CuT in various buffers at 77 K, a figure containing spin adduct signals generated by CuT and GSH in the presence of DMPO, and a figure containing absorption spectra of H_2T and CuT in the absence or presence of calf thymus DNA (6 pages). Ordering information is given on any current masthead page.

REFERENCES

Antholine, W. E., & Petering, D. H. (1979) *Biochem. Biophys. Res. Commun. 90*, 384–389.

Antholine, W. E., & Taketa, F. (1982) *J. Inorg. Biochem. 16*, 145–154.

Antholine, W. E., Basosi, R., Hyde, J. S., Lyman, S., & Petering, D. H. (1984) *Inorg. Chem. 23*, 3543–3548.

Antholine, W. E., Kalyanaraman, B., & Petering, D. H. (1985) *Environ. Health Perspect. 64*, 19–35.

Auclair, C., & Voisin, E. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., Ed.) pp 123–132, CRC Press, Boca Raton, FL.

Aust, S. D., Morehouse, L. A., & Thomas, C. E. (1985) *J. Free Radicals Biol. Med. 1*, 3–25.

Beauchamp, C., & Fridovich, I. (1971) *Anal. Biochem. 44*, 276–287.

Blin, N., & Stafford, E. W. (1976) *Nucleic Acids Res. 3*, 2303–2308.

Bradley, M. O., & Kohn, K. W. (1979) *Nucleic Acids Res. 7*, 793–804.
Bradley, M. O., & Erickson, L. C. (1981) *Biochim. Biophys. Acta 654*, 135–141.
Brawn, K., & Fridovich, I. (1981) *Arch. Biochem. Biophys. 106*, 414–419.
Buettner, G. R. (1987) *Free Radical Biol. Med. 3*, 259–303.
Buettner, G. R., & Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun. 89*, 69–74.
Deeble, D. J., & von Sonntag, C. (1984) *Int. J. Radiat. Biol. 46*, 247–260.
Ehrenfeld, G. M., Rodriguez, L. O., Hecht, S. M., Chang, C., Basus, V. J., & Oppenheimer, N. (1985) *Biochemistry 24*, 81–92.
Ehrenfeld, G. M., Shipley, J. B., Heimbrook, D. C., Sugiyama, H., Long, E. C., van Boom, J. H., van der Marel, G. A., Oppenheimer, N. J., & Hecht, S. M. (1987) *Biochemistry 26*, 931–942.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Fridovich, I. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., Ed.) pp 51–53, CRC Press, Boca Raton, FL.
Goldstein, S., & Czapski, G. (1986) *J. Free Radicals Biol. Med. 2*, 3–11.
Hassan, H., & Fridovich, I. (1979) *Arch. Biochem. Biophys. 196*, 385–395.
Iqbal, Z. M., Kohn, K. W., Ewig, R. A. G., & Fornace, A. J., Jr. (1976) *Cancer Res. 36*, 3834–3838.
Kaneko, M., & Leadon, S. A. (1986) *Cancer Res. 46*, 71–75.
Kaneko, M.; Nakayama, T., Kodama, M., & Nagata, C. (1984) *Gann 75*, 349–354.
Kohn, K. W., Ewig, R. A. G., Erickson, L. C., & Zwelling, L. A. (1981) in *DNA Repair: A Laboratory Manual of Research Procedures* (Friedberg, E. C., & Hanawalt, P. C., Eds.) Vol. 1, Part B, pp 379–401, Marcel Dekker, New York.
Kraker, A., Krezoski, S., Schneider, J., Minkel, D., & Petering, D. H. (1985) *J. Biol. Chem. 260*, 13710–13718.
Lagercrantz, C., & Forshult, S. (1969) *Acta Chem. Scand. 23*, 811–817.
Leadon, S. A., Stampfer, M. R., & Bartley, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4365–4368.
Lück, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., Ed.) pp 885–888, Academic Press, New York.
Lyman, S., Ujjani, B., Renner, K., Antholine, W., Petering, D. H., Whetstone, J. W., & Knight, J. M. (1986) *Cancer Res. 46*, 4472–4478.
Lynch, R. E., & Fridovich, I. (1978) *J. Biol. Chem. 253*, 1838–1845.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 280–281, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McConkey, D. J., Hartzell, P., Nicotera, P., & Orrenius, S. (1989) *FASEB J. 3*, 1843–1849.
Mikelens, P. E., Woodson, B. A., & Levinson, W. E. (1976) *Biochem. Pharmacol. 25*, 821–827.
Mikelens, P. E., Woodson, B. A., & Levinson, W. E. (1978) *Bioinorg. Chem. 9*, 469–478.
Mohan, M., Mudhuranath, P. H., Kumar, A., Kumar, M., & Jha, N. (1989) *Inorg. Chem. 28*, 96–99.
Petering, D. H. (1974) *Biochem. Pharmacol. 23*, 567–576.
Petering, D. H., & Antholine, W. E. (1988) *Rev. Biol. Toxicol. 9*, 225–270.
Petering, H. G., & van Giessen, G. J. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P., & Blumberg, W. E., Eds.) pp 197–209, Academic Press, New York.
Petering, D. H., Byrnes, R. W., & Antholine, W. A. (1990) *Chem.-Biol. Interact. 73*, 133–182.
Prise, K. M., Davies, S., & Michael, B. D. (1989) *Int. J. Radiat. Biol. 55*, 583–592.
Rao, E. A., Saryan, L. A., Antholine, W. E., & Petering, D. H. (1980) *J. Med. Chem. 23*, 1310–1318.
Repine, J. E., Pfenninger, O. W., Talmage, D. W., Berger, E. M., & Pettijohn, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1001–1003.
Rohde, W., Shafer, R., Idriss, J., & Levinson, W. (1979) *J. Inorg. Biochem. 10*, 193–194.
Saryan, L. A., Mailer, K., Krishnamurti, C., Antholine, W., & Petering, D. H. (1981) *Biochem. Pharmacol. 12*, 1595–1604.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814–822.
Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180–186.
Sigman, D. S., Graham, D. R., D'Aurora, V., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269–12272.
Stubbe, J., & Kozarich, J. W. (1987) *Chem. Rev. 87*, 1107–1136.
Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1969) *J. Antibiot. 22*, 446–448.
von Sonntag, C., Hagen, U., Schön-Bopp, A., & Schulte-Frohlinde, D. (1981) *Adv. Radiat. Biol. 9*, 109–142.
Ward, J. F., Blakely, W. F., & Jones, E. I. (1985) *Radiat. Res. 103*, 383–392.