Metabolic Effects of Copper in Intact Cells: Comparative Activity of Cupric Chloride and the Cupric Chelate of Kethoxal Bis(thiosemicarbazone)

BARBARA A. BOOTH AND ALAN C. SARTORELLI

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

(Received January 30, 1967)

SUMMARY

The exposure of sarcoma 180 ascites cells to the cupric chelate [Cu(II)KTS] of kethoxal bis(thiosemicarbazone) (KTS) resulted in the death of a large proportion of the cell population; both the ligand portion of the molecule, KTS, and cupric chloride were less toxic. Cells isolated from mice treated with Cu(II)KTS contained considerably greater amounts of copper than did those cells exposed to the same number of gram-atoms of copper presented as either copper chloride or copper stearate. The results suggested that the relatively lipid-soluble chelate-form of copper was more readily assimilated. Dissociation of Cu(II)KTS occurred within neoplastic cells, and this resulted in a relatively rapid loss from the cells of the ligand portion, KTS. In contrast, the copper derived from Cu(II)KTS persisted for a much longer period of time. The relationship of nucleic acid and protein synthesis to the phenomenon of cell death induced by these agents was assessed by measuring the effects of the compounds on these metabolic processes. The formation of DNA was more sensitive to the inhibitory action of Cu(II)KTS, $CuCl_2$, and KTS, than were either the syntheses of RNA or protein. In agreement with the cellular toxicity, Cu(II)KTS caused more pronounced depression of the incorporation of isotopic precursors into DNA than did either $CuCl_2$ or KTS. The copper present in cells treated with Cu(II)KTS induced at least three metabolic blocks on the pathways of DNA biosynthesis. The most sensitive site was measured by the incorporation of thymidine-^{3}H into DNA and presumably was the result of the loss of activity of thymidine kinase. The findings obtained using a variety of isotopic precursors of DNA as biochemical probes suggested that the intracellular localization of copper derived from Cu(II)KTS differed from that of $CuCl_2$.

INTRODUCTION

2-Keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) [kethoxal bis(thiosemicarbazone); KTS] is an agent that chelates metals of the first, second, and third transition series; in this regard, one molecule of KTS is coordinated to one metal ion to yield a tetradentate chelate (1–3). In mammalian cell systems the compound is capable of retarding growth; thus, the development of a variety of neoplastic cell types (4–9) as well as the growth of normal cells (10) is inhibited by this agent. The antineoplastic potency of KTS is enhanced by the presence of either cupric or zinc ions (11, 12). Furthermore, of several different metal chelates of KTS, the preformed cupric chelate has been found to be the most potent (1, 11).

The metabolic process that is most sensitive to mixtures of cupric ions and KTS in neoplastic cells is the biosynthesis of DNA; inhibition of the formation of RNA and protein also occurs but is less pronounced. Moreover, the presence of KTS under these conditions markedly enhances the uptake of cupric ions by neoplastic cells (12). Preliminary communications have reported that the biochemical potency of combinations of

KTS and cupric ions is essentially duplicated by the preformed cupric chelate of KTS [Cu(II)KTS] (13, 14) and that within neoplastic cells dissociation of Cu(II)KTS occurs; this phenomenon is followed by the cellular elimination of the KTS portion and deposition of copper (15). A role for thiol-containing compounds in the mechanism by which KTS causes growth inhibition has been shown in *Saccharomyces carlsbergensis*. In this system, toxicity of KTS is prevented competitively by glutathione, whereas the inhibition of this organism produced by Cu(II)KTS is not antagonized by the thiol (16).

The data in this report describe the comparative effects of Cu(II)KTS, $CuCl_2$, and KTS on the formation of DNA, RNA, and protein in intact sarcoma 180 ascites cells, and provide evidence to support the concept that the carcinolytic and biochemical actions of Cu(II)KTS are predominantly the result of the effective transport of this compound and selective deposition of copper into areas of sensitive metabolic processes.

MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female CD-1 mice (Charles River Breeding Laboratories, North Wilmington, Massachusetts) bearing sarcoma 180 ascites cells. Transplantation of the tumor was carried out by withdrawing peritoneal fluid from a donor mouse bearing a 7-day growth. The suspension was centrifuged for 2 min (1600 *g*), the supernatant peritoneal fluid was decanted, a 15-fold dilution with isotonic saline was made, and 0.1 ml of the resulting cell suspension was injected intraperitoneally into each animal (17).

In those experiments in which the mice were pretreated with the drugs, cupric chloride was made up in isotonic saline, while Cu(II)KTS, KTS, and copper stearate were homogenized in absolute ethanol (adjusted so that the final concentration of the drug solution was 5% with respect to ethanol) and 2–3 drops of 20% aqueous Tween 80, and then made up to volume with isotonic saline.

Metabolic effects were measured in animals bearing 6-day growths of sarcoma 180. A single intraperitoneal injection of drug was administered at selected times before each mouse received either 200 μg of thymidine-methyl-^{3}H (5.7×10^3 cpm/μg), 100 μg of orotic-6-^{14}C acid hydrate (2.1×10^4 cpm/μg), 125 μg of DL-leucine-1-^{14}C (1.4×10^4 cpm/μg), 100 μg of L-glycine-2-^{14}C (1.8×10^4 cpm/μg), 200 μg of uridine-5,6-^{3}H (7.1×10^3 cpm/μg), 90 μg of sodium formate-^{14}C (1.2×10^5 cpm/μg), 50 μg of adenine-8-^{14}C (2.9×10^4 cpm/μg), or 660 μg of deoxycytidine-^{3}H (1.1×10^3 cpm/μg); 1 hr was allowed for metabolic utilization of isotope.

In experiments involving the incorporation of either thymidine-^{3}H, orotic-6-^{14}C acid, formate-^{14}C, or deoxycytidine-^{3}H into nucleic acids, sodium nucleates were isolated by the method of Tyner *et al.* (18) and hydrolyzed for 1.5 hr with 70% perchloric acid (19). Extracts were desalted on charcoal columns, and the desired pyrimidine bases were purified and analyzed as described by Danneberg *et al.* (20). In the case of adenine-8-^{14}C and uridine-^{3}H, the dried sodium nucleates were subjected to alkaline hydrolysis to separate deoxyribonucleotides from ribonucleotides, the DNA was precipitated by acidification and separated from the RNA by centrifugation. With adenine-^{14}C, the purine nucleotides of both fractions were hydrolyzed to their free bases with acid, and the bases were then purified and analyzed (21). With uridine-^{3}H, aliquots of acid-hydrolyzed DNA and RNA were analyzed both for radioactivity and for deoxyribose and ribose content, using deoxyadenosine and adenosine, respectively, as the standards (22).

After exposure of cells to either leucine-1-^{14}C or glycine-2-^{14}C, residual protein was isolated and analyzed as previously described (23). Radioactivity in residual protein was measured with a Nuclear-Chicago gas flow counter equipped with a "Micromil" window, and in all other instances radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer. The phosphor solution contained 100 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene and 8 g of

2,5-diphenyloxazole dissolved in a mixture of 2 liters of toluene and 1 liter of absolute ethanol.

The cellular concentration of Cu(II)KTS was determined by measuring the radioactivity associated with cells exposed to Cu(II)KTS-^{14}C; the radioactive carbon was present in the thiosemicarbazide portions of the molecule. A suspension of Cu(II)KTS-^{14}C (32.4×10^4 cpm/μmole) was administered to animals by intraperitoneal injection in a dose of 5 mg/kg; at selected time intervals thereafter, ascites fluid was removed from each mouse and the cells were collected by centrifugation for 2 minutes (1600 *g*). The fluid was decanted and the packed cells were diluted with 4 volumes of distilled water; of this suspension, 1 ml was solubilized by the addition of a concentrated solution of NaOH (final concentration, 0.2 N NaOH). An aliquot was taken for measurement of radioactivity; the results are expressed as mμmoles of drug per gram of packed cells.

The copper content of sarcoma 180 cells was determined after removal from mice subsequent to appropriate drug treatment; the cells were collected by centrifugation, and 4 volumes of distilled water were added. A 1-ml portion of this suspension was wet-ashed using concentrated nitric acid followed by a 1:1 perchloric acid–nitric acid mixture, and the copper content was then measured colorimetrically according to the method described by Stoner and Dasler (24) using copper sulfare as the standard.

Total cells in the peritoneal cavity were determined by quantitatively washing the cells out of the cavity and determining the cell number using a Coulter particle counter, model A. The cell volumes were measured in hematocrit tubes by determining the volume occupied by a known number of cells.

Enzyme extracts were prepared from 6-day growths of ascites cells and the activities of thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21), thymidine monophosphate kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9), and thymidine monophosphate phosphohydrolase were assessed by methods previously described (25). The activity of DNA nucleotidyltransferase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) was assayed by the method of Mantsavinos and Canellakis (26), except that radioactivity was determined by scintillation spectrometry.

RESULTS

Sarcoma 180 ascites cells exposed to Cu(II)KTS over a range of concentrations exhibit a marked inhibition of the incorporation of thymidine-^{3}H into the thymine of DNA. This is shown under conditions in which the isotopic tracer was administered 12 hr after injection of the drug (Fig. 1).

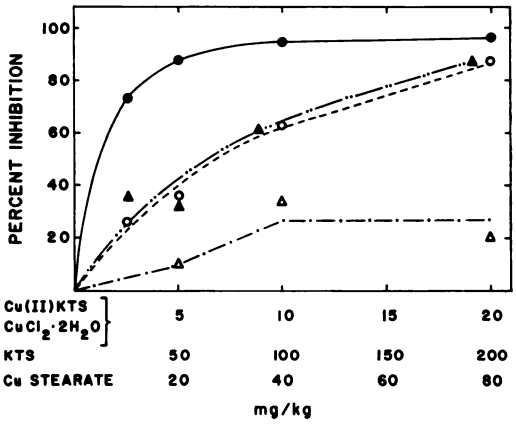

FIG. 1. *Comparative effects of Cu(II)KTS, $CuCl_2$, copper stearate, and KTS on the incorporation of thymidine-^{3}H into the thymine of DNA*

Twelve hours before administration of thymidine-^{3}H, various doses of either Cu(II)KTS, $CuCl_2 \cdot 2\,H_2O$, copper stearate, or KTS were given by intraperitoneal injection to each mouse. Each value represents the mean obtained from the separate analyses of ascites cells from 4 to 37 animals. Cu(II)KTS, ●——●; copper stearate, ▲—·—▲; $CuCl_2$, ○- - - -○; KTS, △—·—△.

Under these conditions, KTS, the chelating portion of the molecule, caused relatively little retardation of the synthesis of DNA as measured by the incorporation of thymidine into these molecules, whereas comparable molar quantities of cupric chloride ($CuCl_2$) and copper stearate (Cu stearate) produced considerably less inhibition of the utilization of thymidine-^{3}H for the formation of DNA than did Cu(II)KTS. Molar concentrations

of cupric acetate, cupric sulfate, and cuprous chloride equivalent to 10 mg of $CuCl_2 \cdot 2\ H_2O$ per kg caused similar magnitudes of inhibition of the incorporation of isotopic thymidine into DNA. The duration of inhibition of the biosynthesis of DNA afforded by a single dose of each of the various agents was measured to ascertain continuance of this metabolic stress. Figure 2 presents the time course of this effect at a dose of 5.0 mg of Cu(II)KTS per kg; the level of $CuCl_2$ selected for comparison was such that the dose contained four times the number of copper atoms present in 5 mg of Cu(II)KTS per kg. KTS was administered at 200 mg/kg of body weight, a level containing fifty equivalents of ligand as compared to the selected level of the cupric chelate (5 mg/kg). Although $CuCl_2$ caused a greater degree of inhibition of the incorporation of thymidine into DNA when the isotope was administered 2 hr after the drug, cells exposed to $CuCl_2$ recovered their ability to synthesize DNA more rapidly than did those cells exposed to Cu(II)KTS. KTS was relatively noninhibitory.

The effects of these agents on the synthesis of macromolecules other than DNA were investigated to determine whether drug-induced lesions occurred in other metabolic areas. Table 1 presents data obtained using three different isotopic tracers as indicators of the synthesis of RNA. The administration of $CuCl_2$ caused 57% inhibition of the incorporation of orotic-6-^{14}C acid into RNA uracil, whereas, under these conditions, 39 and 35% inhibition were effected by Cu(II)KTS and KTS, respectively. This lesser sensitivity of

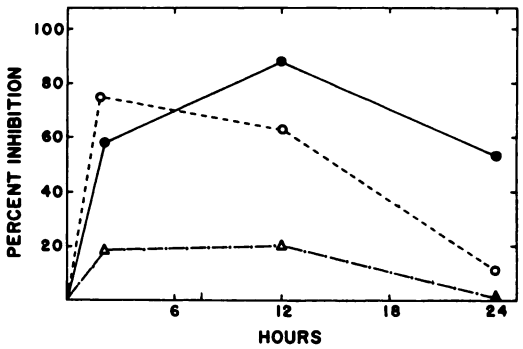

FIG. 2. *Duration of inhibition of the synthesis of DNA by Cu(II)KTS, $CuCl_2$, and KTS*

At various times before administration of thymidine-^{3}H, either 5.0 mg of Cu(II)KTS/kg, 9.9 mg of $CuCl_2 \cdot 2\ H_2O$/kg, or 200 mg of KTS/kg were given by intraperitoneal injection to each mouse. Each value represents the mean obtained from the separate analyses of ascites cells from 4 to 37 animals. Cu(II)KTS, ●——●; $CuCl_2$, O----O; KTS △—·—△.

RNA synthesis to the ligand KTS, its cupric chelate, and $CuCl_2$ as compared with the formation of DNA was corroborated by monitoring the synthesis of RNA with adenine-8-^{14}C and uridine-^{3}H.

The biosynthesis of protein in sarcoma 180 ascites cells was measured by the incorpora-

TABLE 1

Incorporation of radioactive precursors into RNA of sarcoma 180 ascites cells treated with either Cu(II)KTS, $CuCl_2$, or KTS

Twelve hours before the administration of an isotopic precursor, either 5.0 mg of Cu(II)KTS/kg, 9.9 mg of $CuCl_2 \cdot 2\ H_2O$/kg, or 200 mg of KTS/kg were given by intraperitoneal injection to each mouse. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 6 to 20 animals.

Treatment	Orotic acid-6-^{14}C into RNA uracil[a]	Adenine-8-^{14}C into RNA adenine[a]	Uridine-5,6-^{3}H into RNA[b]
None	28.2 ± 5.8 (9)[c]	350.8 ± 28.0 (19)	13.8 ± 1.3 (19)
Cu(II)KTS	17.0 ± 2.7 (11)	232.9 ± 50.0 (8)	8.7 ± 0.8 (19)
$CuCl_2$	12.5 ± 4.8 (6)	175.4 ± 23.2 (11)	11.2 ± 1.3 (19)
KTS	18.0 ± 2.4 (9)	237.0 ± 42.6 (8)	11.0 ± 1.2 (20)

[a] Expressed as cpm/μmole × 10^{-3}.

[b] Expressed as cpm/mμmole RNA pentose by the orcinol reaction.

[c] Number in parentheses indicates total number of animals per group.

tion of either leucine-^{14}C or glycine-^{14}C into residual protein (Table 2). The formation of these molecules appeared to be relatively insensitive to these agents and little or no effect was evidenced 12 hours after administration of the drugs.

TABLE 2

Incorporation of radioactive precursors into residual protein of sarcoma 180 ascites cells treated with either Cu(II)KTS, $CuCl_2$, or KTS

Experiments were carried out as described in Table 1. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 8 to 16 animals.

Treatment	DL-Leucine-1-^{14}C into residual protein[a]	L-Glycine-2-^{14}C into residual protein[a]
None	42.0 ± 4.2 (12)[b]	36.7 ± 5.4 (8)
Cu(II)KTS	31.3 ± 1.9 (16)	32.7 ± 6.2 (8)
$CuCl_2$	29.6 ± 4.0 (8)	44.0 ± 6.8 (8)
KTS	33.7 ± 3.8 (11)	35.1 ± 4.0 (8)

[a] Values are given as cpm/mg × 10^{-2}.

[b] Number in parentheses indicates total number of animals per group.

Of the metabolic processes measured, the formation of DNA appeared to be the most sensitive to the inhibitory properties of Cu(II)KTS; hence, it was of interest to probe further for the sites of metabolic lesions induced by Cu(II)KTS on the DNA biosynthetic pathways. Accordingly, other isotopic substrates that trace the formation of DNA through both the purine and pyrimidine synthetic routes were employed; the results obtained are shown in Table 3. The incorporation of thymidine into the thymine of DNA was the pathway most sensitive to the action of Cu(II)KTS, and 88% inhibition of the utilization of this isotopic substrate was produced. Less, but nevertheless marked, inhibition by Cu(II)KTS of the incorporation of formate-^{14}C, deoxycytidine-^{3}H, adenine-^{14}C and uridine-^{3}H into DNA was induced; under the conditions employed, the degree of inhibition was similar for all these isotopic tracers. $CuCl_2$ caused less inhibition of the formation of DNA as monitored by these isotopic precursors than did Cu(II)KTS; in this regard, the utilization of thymidine-^{3}H, formate-^{14}C, and adenine-^{14}C for the fabrication of DNA was depressed by $CuCl_2$ to a greater extent than was the incorporation of deoxycytidine-^{3}H and uridine-^{3}H. In contrast to both Cu(II)KTS and $CuCl_2$, the incorporation of thymidine-^{3}H into DNA was much less sensitive to the action of KTS than was the utilization of the other isotopic substrates.

The relatively greater sensitivity to Cu(II)KTS of the incorporation of thymidine into DNA as compared with that of the other isotopic precursors employed suggested the presence of a lesion on an enzymic step(s) involved in the formation of thymine nucleotides. To test this possibility, cell-free extracts were prepared from ascites cells isolated from either untreated mice or from those treated 12 hr previously with one of

TABLE 3

Incorporation of radioactive precursors into DNA of sarcoma 180 ascites cells treated with either Cu(II)KTS, $CuCl_2$, or KTS

Experiments were carried out as described in Table 1. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 8 to 37 animals.

Treatment	Thymidine-methyl-^{3}H into DNA thymine[a]	Formate-^{14}C into DNA thymine[a]	Deoxycytidine-^{3}H into DNA thymine[a]	Adenine-8-^{14}C into DNA adenine[a]	Uridine-5,6-^{3}H into DNA[b]
None	149.8 ± 5.8 (37)[c]	105.5 ± 6.8 (18)	13.1 ± 1.3 (11)	58.8 ± 5.2 (20)	8.6 ± 0.8 (8)
Cu(II)KTS	17.9 ± 3.0 (16)	43.0 ± 2.2 (8)	7.0 ± 1.5 (10)	18.6 ± 2.2 (12)	4.7 ± 0.6 (8)
$CuCl_2$	58.1 ± 10.1 (15)	53.0 ± 3.9 (8)	11.9 ± 2.0 (11)	32.0 ± 2.9 (11)	6.2 ± 0.7 (8)
KTS	119.3 ± 16.5 (12)	50.1 ± 7.2 (8)	5.7 ± 1.1 (11)	32.0 ± 4.8 (12)	3.4 ± 0.2 (8)

[a] Expressed as cpm/μmole × 10^{-2}.

[b] Expressed as cpm/μmole DNA deoxypentose × 10^{-2} by the diphenylamine reaction.

[c] Number in parentheses indicates total number of animals per group.

the various agents employed in the concentrations described in Table 1; the extracts were then assayed for thymidine kinase, thymidine monophosphate kinase, and DNA nucleotidyltransferase activities (Table 4).

TABLE 4

Thymidine kinase, thymidine monophosphate kinase, and DNA nucleotidyltransferase activities of sarcoma 180 ascites cells treated with either Cu(II)KTS, $CuCl_2$, or KTS[a]

Cell-free extract	Thymidine kinase	Thymidine monophosphate kinase	DNA nucleotidyltransferase
Untreated	16.4	25.0	0.13
Cu(II)KTS	3.2	33.6	0.11
$CuCl_2$	10.2	33.7	0.08
KTS	15.4	50.8	0.16

[a] Each value represents the mean of 2 to 6 determinations expressed as mμmoles/hr/mg.

In confirmation of the isotopic data obtained with intact cells, no inhibition of the conversion of thymidine-^3H into either phosphorylated intermediates or DNA was detected in enzymic extracts prepared from cells exposed to KTS. When extracts were prepared from cells that were pretreated with either Cu(II)KTS or $CuCl_2$, there was a pronounced decrease in the activity of thymidine kinase. Dialysis for 12 hr at 4° against 0.05 M Tris buffer, pH 7.5, containing either thymidine (5×10^{-5} M) or thymidine and EDTA (1×10^{-4} M) resulted in a partial loss in enzymic activity in both treated and non-drug-treated extracts; however, no relief of inhibition was afforded by dialysis. No decrease in the activities of thymidine monophosphate kinase was found in extracts prepared from cells following pretreatment with either $CuCl_2$ or Cu(II)KTS. Neither Cu(II)KTS nor KTS pretreatment caused a decrease in the cellular activities of DNA nucleotidyltransferase, while $CuCl_2$ gave about 38% inhibition.

To determine whether these agents caused direct inhibition of enzyme activity, the drugs were added to enzymic extracts prepared from untreated sarcoma 180 ascites cells. A summary of these data is given in Table 5. Addition of Cu(II)KTS at a concentration of 1×10^{-4} M did not inhibit the activities of either thymidine kinase or thymidine monophosphate kinase. DNA nucleotidyltransferase activity was insensitive to Cu(II)KTS at 1×10^{-5} M; addition of higher concentrations of this inhibitor was limited by the sensitivity of the enzyme to the ethanol necessary to dissolve the agent. Thymidine monophosphate phosphohydro-

TABLE 5

Effect of Cu(II)KTS, $CuCl_2$, and KTS on the activities of thymidine kinase, thymidine monophosphate kinase, thymidine monophosphate phosphohydrolase and DNA nucleotidyltransferase

Cu(II)KTS and KTS were dissolved with the aid of ethanol; the final concentration of ethanol in the DNA nucleotidyltransferase reaction was 0.2% and in the other enzymic assays 1.1%; these concentrations of ethanol did not inhibit the activities of these enzymes. Each value represents the mean of 3–10 determinations, expressed as percent inhibition.

Inhibitor	Concentration (mM)	Thymidine kinase	Thymidine monophosphate kinase	Thymidine monophosphate phosphohydrolase	DNA nucleotidyltransferase
Cu(II)KTS	0.1	0	0	65	—[a]
	0.01	—	—	—	0
$CuCl_2$	0.1	77	65	99	—
	0.05	33	56	100	—
	0.01	15	0	82	50
	0.001	0	0	53	—
KTS	0.1	21	0	48	—
	0.01	—	—	—	0

[a] Not determined at indicated concentrations.

lase, however, was sensitive to Cu(II)KTS. $CuCl_2$ was an effective inhibitor of thymidine kinase at concentrations of 0.5 to 1×10^{-4} M. This inhibition was reversible because addition of 1×10^{-4} M $CuCl_2$ to the enzymic extract followed by dialysis for 18 hr against 100 volumes of 0.05 M Tris buffer, pH 7.5, containing 0.05 μmole of thymidine/ml resulted in complete reversal of the copper ion-induced inhibition. In contrast to results obtained with preparations made from $CuCl_2$-treated cells, thymidine monophosphate kinase activity also was inhibited by $CuCl_2$. DNA nucleotidyltransferase also was markedly sensitive to the addition of cupric ions and 50% inhibition was produced by 1×10^{-5} M $CuCl_2$. Thymidine monophosphate phosphohydrolase was the enzyme most sensitive to the addition of $CuCl_2$ and pronounced inhibition was produced by concentrations from 0.01 to 1×10^{-4} M. KTS (1×10^{-4} M) produced only slight inhibition of the activity of thymidine kinase (21%); however, this drug caused a more marked depression (48%) of the activity of thymidine monophosphate phosphohydrolase.

Cu(II)KTS is a relatively lipid-soluble form of copper (3); therefore, it should penetrate cellular membranes with greater ease than do more water-soluble forms of copper (e.g., $CuCl_2$). To gain evidence in support of such a premise, the levels of copper associated with sarcoma 180 ascites cells were measured 12 hr after administration of either Cu(II)KTS, $CuCl_2$, or copper stearate to tumor-bearing mice; the results obtained are shown in Fig. 3. The neoplastic cells present in the peritoneal cavities of mice exposed to 5.0 mg of Cu(II)KTS per kilogram of body weight contained approximately 175 mμmoles of copper per gram of cells; this quantity of copper is a measure of the amount that is both assimilated and retained. The achievement of cellular copper concentrations comparable to those obtained with Cu(II)KTS required exposure of sarcoma 180 to approximately four times the gram-equivalents of copper in the form of either $CuCl_2$ or copper stearate. Thus, presentation to the cells of copper in chelate form with KTS appeared either to be a more effective method of transporting copper into these neoplastic cells or to cause greater retention of copper after the uptake of Cu(II)KTS. No increase in the amount of copper associated with the neoplastic cells was seen after exposure to KTS. These results suggest that in this system the biological effects produced by KTS are not the result of sequestration of copper from other tissues, formation of Cu(II)KTS, and subsequent transport of this copper into the tumor cells.

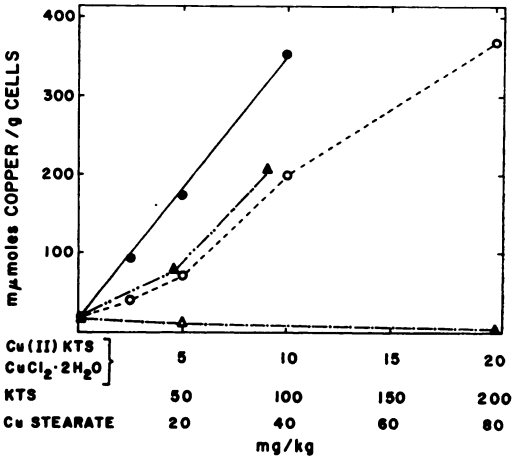

FIG. 3. *The copper content of sarcoma 180 ascites cells after exposure to either Cu(II)KTS, $CuCl_2$, copper stearate, or KTS*

Twelve hours after various doses of either Cu(II)KTS, $CuCl_2 \cdot 2\,H_2O$, copper stearate, or KTS, tumor cells were collected and the copper content was determined. Each value represents the mean obtained from the separate analyses of ascites cells from 2 to 20 animals. Cu(II)KTS, ●——●; $CuCl_2$, O- - - -O; Cu stearate, ▲—·—▲; KTS, △—·—△.

A comparison was made of the retention of copper by cells treated with a dose of either 9.9 mg of $CuCl_2$ per kg or 5 mg of Cu(II)KTS per kg, as well as of the disposition of the KTS portion of the Cu(II)KTS molecule by measurement of the ^{14}C content of cells exposed to Cu(II)KTS-^{14}C (Fig. 4). Essentially equal amounts of copper were associated with cells exposed to these concentrations of Cu(II)KTS and $CuCl_2$ at 2 hr after exposure. Copper was lost progressively regardless of the form to which the cells were exposed, and by 24 hr only slightly more copper was found in the cells treated

with Cu(II)KTS. These data support the concept that higher concentrations of copper were achieved in Cu(II)KTS-treated cells because this agent was transported more effectively than was $CuCl_2$, rather than the alternative proposal that differential retention of copper occurred. Measurement of the ^{14}C content of cells exposed to Cu(II)KTS-^{14}C (labeled in each of the groups derived from thiosemicarbazide) is indicative of the disposition of the KTS portion of the molecule; at 2 hr after administration of the

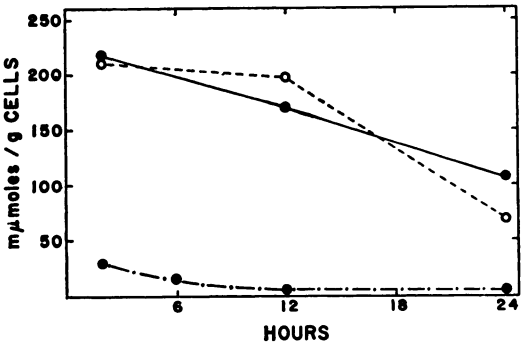

FIG. 4. *The cellular retention of Cu(II)KTS and $CuCl_2$*

At various times after administration of either 9.9 mg of $CuCl_2 \cdot 2\ H_2O$/kg or 5.0 mg of Cu(II)KTS-^{14}C/kg, cells were collected and either the copper content or the radioactivity was determined. Each value represents the mean obtained from the separate analyses of ascites cells from 4 to 8 animals. Copper from Cu(II)KTS, ●——●; copper from $CuCl_2$, O- - - -O; radioactivity from Cu(II)KTS-^{14}C, ●—·—●.

cupric chelate only about 14% of the copper present in the neoplastic cells was accounted for as Cu(II)KTS, and by 12 hr only a fraction of the KTS portion remained [<4% of the copper concentration could be derived from Cu(II)KTS]. Thus, dissociation of the cupric chelate occurred, presumably because of greater avidity of copper for some of the cellular constituents, and copper was retained by the cells while KTS was relatively rapidly expelled.

That the amount of copper associated with cells treated with either Cu(II)KTS or $CuCl_2$ bore no relationship to the degree of inhibition of thymidine-^3H incorporation into the thymine of DNA afforded by these agents is indicated by comparison of the results shown in Fig. 2 and Fig. 4. Thus, although similar quantities of copper were associated with cells after exposure to either Cu(II)KTS or $CuCl_2$ at both 12 and 24 hr, the percentage inhibition of the incorporation of thymidine into DNA was significantly greater in those cells treated with Cu(II)KTS. Since the metabolic lesions induced in sarcoma 180 at 12 and 24 hr after Cu(II)KTS should result primarily from copper, the finding of differential inhibition

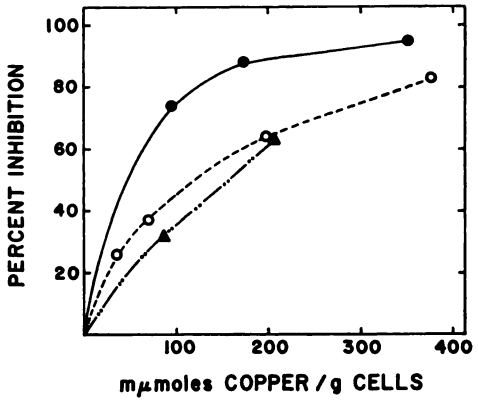

FIG. 5. *Inhibition of the incorporation of thymidine-^3H into the thymine of DNA by Cu(II)KTS, $CuCl_2$, and copper stearate*

Twelve hours before administration of thymidine-^3H, various doses of either Cu(II)KTS, $CuCl_2 \cdot 2\ H_2O$, or copper stearate were given to each mouse. Each value represents the mean obtained from the separate analyses of ascites cells from 4 to 37 animals. Cu(II)KTS, ●——●; $CuCl_2$, O- - - -O; Cu stearate, ▲—·—▲.

of the utilization of isotopic thymidine suggests that the intracellular localization of copper derived from Cu(II)KTS differs from that of $CuCl_2$. Further evidence that differences in the compartmentalization of copper exist in cells exposed to either $CuCl_2$, Cu stearate, or Cu(II)KTS was obtained from a more extensive comparison of the amount of inhibition of the utilization of thymidine for the formation of DNA caused by different cellular levels of copper (Fig. 5). Similar results were obtained when adenine-8-^{14}C incorporation into the adenine of DNA was used as the biochemical probe to delineate differences in the intracellular localization of copper.

To gain further evidence that differences in the metabolic effects produced by copper are dependent upon the form in which the cation was administered, a comparison was made of the amount of blockade of the utilization of several different isotopic precursors of DNA 12 hr after the intraperitoneal treatment of tumor-bearing mice with various doses of either Cu(II)KTS or $CuCl_2$. The results obtained are shown in Fig. 6. With Cu(II)KTS, incorporation of

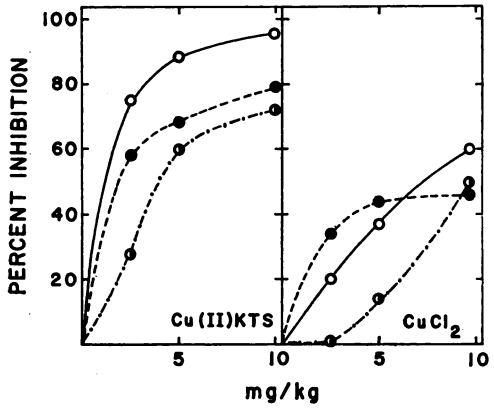

FIG. 6. *The comparative effects of Cu(II)KTS and $CuCl_2$ on the incorporation of thymidine-^3H, adenine-8-^{14}C, and formate-^{14}C into DNA*

Twelve hours before administration of isotopes, various doses of either Cu(II)KTS or $CuCl_2 \cdot 2\ H_2O$ were given. Each value represents the mean obtained from the separate analyses of ascites cells from 3 to 37 animals. Thymidine-methyl-^3H, O——O; adenine-8-^{14}C, ●----●; formate-^{14}C, ◑——◑.

thymidine-^3H into DNA was more sensitive to the drug then was the utilization of adenine-8-^{14}C for the formation of these macromolecules, whereas the reverse was true at lower dose levels of $CuCl_2$ (i.e., 2.5–5 mg/kg). The incorporation of formate-^{14}C into the thymine of DNA is the system that was the least inhibited by both agents. If it be assumed that the drug treatments produced no alterations either in permeability to the radioactive precursors of the nucleic acids or in the size of metabolic pools through which the isotopic tracers must pass, then the incorporation of formate-^{14}C into the thymine of DNA is an expression of ,the maximum amount of inhibition of the formation of these polymeric molecules that can occur with each drug, since the least amount of blockade was obtained with this isotopic tracer. With Cu(II)KTS, the increased amount of inhibition observed using adenine-8-^{14}C to measure the rate of synthesis of DNA suggests the presence of an additional site(s) of blockade in the metabolic interconversions of purine nucleotides, and the results obtained with thymidine-^3H suggest a still more sensitive third site. The latter site presumably reflects a decrease in the activity of thymidine kinase in Cu(II)KTS-treated cells. In support of the hypothesis of a biochemical lesion on the purine nucleotide biosynthetic pathways, isotopic studies utilizing several different radioactive precursors which monitor the formation of purine nucleotides by alternate routes have suggested the presence of several Cu(II)KTS-sensitive enzymes (B. A. Booth and A. C. Sartorelli, unpublished observations).

To determine the degree of cell death under conditions that produced the observed metabolic alterations, the effects of Cu(II)KTS, $CuCl_2$, KTS, and Cu stearate on the total number of cells present in the abdominal cavities of mice at 12 and 24 hr after a single dose of drug were measured. The data obtained are shown in Table 6. Twelve hours after exposure to Cu(II)KTS, there was a 55% decrease in the total number of cells present in the peritoneal cavities; this loss in cells progressed, and by 24 hr there was a 71% decrease. An increase in the average volume of the cells accompanied the loss in cell number, and at 12 and 24 hr after Cu(II)KTS the cells had increased their average size. Treatment of cells with $CuCl_2$ caused a marked cell enlargement at 12 hr, but no further increase occurred at 24 hr after drug; under these conditions a 43% loss in cell number was induced by $CuCl_2$ (12 hr) and no further significant decrease occurred at 24 hr. Exposure of the cells to KTS caused some cell death; however, this was a transient phenomenon and by 24 hr the cell number had returned to the control level. Thus, the effect of a single dose of KTS is a relatively short-lived event. Moreover, the initial increase in the average cell volume induced by KTS recovered within 24 hr to

TABLE 6

Toxicity of Cu(II)KTS, $CuCl_2$, KTS, and copper stearate for sarcoma 180 ascites cells

Twelve and 24 hr before collection of cells, mice were each given either 5.0 mg of Cu(II)KTS/kg, 9.9 mg of $CuCl_2 \cdot 2\ H_2O$/kg, 200 mg of KTS/kg, or 36.6 mg of copper stearate/kg by intraperitoneal injection. Ascites cells were harvested quantitatively from the abdominal cavities, and the numbers were determined. Each value represents the mean (± the standard error) obtained from 4–24 animals.

Treatment	Time after drug (hr)	Total cells/mouse × 10^{-6}	Cell volume (μ^3/cell × 10^{-3})
None	—	481 ± 33 (24)[a]	3.12 ± 0.17 (24)
Cu(II)KTS	12	214 ± 40 (11)	4.30 ± 0.66 (11)
	24	139 ± 12 (13)	6.14 ± 0.76 (13)
$CuCl_2$	12	273 ± 47 (11)	7.51 ± 1.05 (11)
	24	261 ± 57 (11)	5.64 ± 0.53 (12)
KTS	12	272 ± 21 (8)	4.61 ± 0.41 (8)
	24	429 ± 50 (11)	3.68 ± 1.39 (11)
Cu stearate	12	379 ± 60 (4)	5.64 ± 0.98 (4)

[a] Number in parentheses indicates total number of animals per group.

approach the size of the untreated population. A single injection of Cu stearate resulted in a slight (21%) decrease in the number of cells present in the peritoneal cavity at 12 hr and an increase in the average cell volume.

DISCUSSION

Exposure of sarcoma 180 ascites cells to Cu(II)KTS results in a degree of cell death and a consequential prolongation of survival time of mice bearing this neoplasm that are greater than those achieved by similar or even greater concentrations of $CuCl_2$. These findings appear to be the result of several factors. The first is that the assimilation of copper by neoplastic cells treated with Cu(II)KTS is considerably greater than by those populations of tumor cells exposed to similar doses of $CuCl_2$, a result that is presumably an expression of the relatively greater lipid solubility of the cupric chelate (3). Secondly, copper is transferred from Cu(II)KTS to the cellular constituents; preferential retention of copper occurs, with most of the KTS portion of the molecule expelled by 12 hr after dosage with the drug. In systems in which KTS may be retained in the cellular environment for a relatively long period of time, a "shuttle" mechanism may be envisioned, wherein a KTS molecule binds an atom of copper in the extracellular milieu to form Cu(II)KTS, thereby serving as a carrier to transport copper into a cell. After dissociation of the cupric chelate and deposition of copper within the cell, the KTS is expelled into the medium, and this process may be repeated. Such a mechanism is compatible with the results of Petering and Van Giessen (3), who showed that the addition of noncytotoxic levels of cupric ions in combination with Cu(II)KTS to Walker 256 tumor cells in culture resulted in considerably greater cytotoxicity than could be achieved with Cu(II)KTS alone. To obtain such a result in whole animal systems, however, requires the simultaneous availability of a large pool of copper ions with KTS. Since the treatment of mice bearing sarcoma 180 ascites cells with KTS alone did not increase the copper content of the cells, this presumably reflects either the unavailability of tissue stores of copper or the greater avidity of tissues for this metal as compared to KTS.

The metabolic lesions induced by Cu(II)KTS and $CuCl_2$ were measured predominantly 12 hr after tumor-bearing animals were treated with these agents; under these conditions, less than 4% of the copper associated with the cells after exposure to Cu(II)KTS could be accounted for in a chelate form with KTS. Thus, the biochemical changes resulting from such treatment would appear to be due primarily to

copper. The data from these experiments clearly suggest that intracellular copper derived from Cu(II)KTS modifies biochemical processes differently than does the cation obtained from $CuCl_2$. In this regard, the copper derived from Cu(II)KTS appears to be considerably more toxic to the biochemical systems studied than was copper obtained from $CuCl_2$. These results may be the expression either of differences in intracellular localization of the metal or of the binding of a larger percentage of copper from $CuCl_2$ to external cellular membranes; in the latter alternative, the total amount of copper associated with the cell pack after exposure to Cu(II)KTS or $CuCl_2$ may be similar; however, the actual intracellular concentrations achieved differ. Both alternatives can be visualized as expressions of the greater lipid solubility of Cu(II)KTS.

The results obtained with the different isotopic precursors of DNA suggest the presence of at least three distinctly different copper-induced biochemical lesions. The most sensitive process to Cu(II)KTS, that of the incorporation of thymidine-^{3}H into the thymine of DNA, is accounted for by a decrease in the cellular activity of thymidine kinase. The decrease in the activity of this enzyme in cells treated with either Cu(II)KTS or $CuCl_2$ seems to be either an indirect effect which results in a loss in the number of enzymic molecules present in the cells or a copper-induced alteration in catalytic efficiency. Direct inhibition of thymidine kinase by either Cu(II)KTS or KTS would appear to be eliminated. Although thymidine kinase is directly sensitive to copper ions, direct inhibition of this enzyme by copper in whole cells requires the subsequent denaturation of copper-bound enzyme molecules, since the Cu(II)KTS-induced lowering of the activity of thymidine kinase in drug-pretreated cells cannot be restored by dialysis under conditions in which inhibitory levels of $CuCl_2$ can be removed after its addition to the enzyme. Furthermore, since both thymidine kinase and thymidine monophosphate kinase are inhibited to essentially the same degree by the addition of copper ions to cell-free extracts, the differential sensitivity that occurs in cells pretreated with either Cu(II)KTS or $CuCl_2$ requires either a differential distribution of copper ions within cells, with the achievement of a higher concentration in the vicinity of thymidine kinase than of thymidine monophosphate kinase or a differential sensitivity of thymidine monophosphate kinase in its natural cellular environment from that found in cell-free extracts. Alternatively, since the cellular levels of thymidine kinase activity decrease in nongrowing cells, this enzyme is exquisitely sensitive to agents that retard cellular growth (21); it is conceivable, therefore, that the decrease in growth rate induced by Cu(II)KTS and $CuCl_2$ creates a situation that results in the cellular loss of catalytically active kinase molecules. DNA nucleotidyltransferase activity was also sensitive to the presence of cupric ions both under conditions in which the enzyme was isolated from cells that were exposed to $CuCl_2$ and when this salt was added directly to cell-free extracts. The finding that DNA nucleotidyltransferase activity isolated from copper-pretreated cells is lower than normal suggests a direct inhibitory effect of copper on the DNA nucleotidyltransferase enzyme; however, the decreased efficiency may also be the result of an inability of residual copper-bound DNA to serve as an effective primer because of resultant metal ion-induced changes in structure (27, 28). This latter alternative assumes that residual sarcoma 180 DNA and added calf-thymus DNA both contribute as primers in the formation of DNA in this system.

Biochemical alterations in the synthetic routes leading to the formation of DNA also are produced by KTS. The pattern of inhibition differs from that obtained with Cu(II)KTS, for although the data indicate that the metabolic pathway from thymidine to DNA is relatively insensitive to KTS, fairly good blockade of the incorporation of formate-^{14}C, deoxycytidine-^{3}H, uridine-^{3}H, and adenine-^{14}C into DNA was observed. The decrease in the conversion of formate, deoxycytidine, and uridine into thymine of DNA caused by exposure of the cells to KTS, under conditions in which the utilization of isotopic thymidine for the biosyn-

thesis of thymine of DNA is normal, implies the presence of a metabolic lesion(s) prior to the formation of deoxythymidine 5′-phosphate.

That some biochemical specificity to the toxic effects of these agents exists was shown by the fact that the formation of macromolecules other than DNA (i.e., RNA and protein) was much less susceptible to the inhibitory effects of Cu(II)KTS, $CuCl_2$, and KTS. Noteworthy in this regard was the finding that with $CuCl_2$-treated cells the conversion of orotic-^{14}C acid to the uracil of RNA was inhibited more than was the incorporation of either uridine or adenine into the uracil and adenine moieties, respectively, of RNA. Thus, it may be supposed either that a $CuCl_2$-sensitive reaction exists on the metabolic sequence between orotic acid and uridine 5′-phosphate, or that the labeled orotic-^{14}C acid is being preferentially bound by copper ions, resulting in a decreased availability of the labeled substrate.

The relative importance of the various biochemical lesions in the process of cell death is unknown; however, the data indicate that the magnitude and duration of inhibition of the synthesis of the precursors of DNA, which is a product of several sites of blockade, correlate with the loss of cells from the peritoneal cavity, a process indicative of the disintegration of cells. The utilization of Cu(II)KTS as an antineoplastic agent for the treatment of malignancy in man, however, would appear to be limited by the relatively greater avidity of copper for cellular constituents as compared to KTS. Cu(II)KTS may, however, find utility in the treatment of neoplastic growths located in body cavities into which the Cu(II)KTS may be introduced directly. Under these conditions, selective deposition of copper in neoplastic cells could occur, thereby resulting in therapeutic advantage to the host.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Harold G. Petering, formerly of the Upjohn Company, for generous supplies of kethoxal bis(thiosemicarbazone) and its cupric chelate and Miss Sheila J. Feld, Miss Florence C. Dunmore, and Miss Lynn A. Bon Tempo for capable assistance. This investigation was supported by U.S. Public Health Service Grant CA-02817 from the National Cancer Institute.

REFERENCES

1. G. J. Van Giessen and H. G. Petering, *Abstr. Am. Chem. Soc. Meeting,* 13 N (1965).
2. M. R. Taylor, E. J. Gabe, J. P. Glusker, J. A. Minkin and A. L. Patterson, *J. Am. Chem. Soc.* **88**, 1845 (1966).
3. H. G. Petering and G. J. Van Giessen, *in* "The Biochemistry of Copper" (J. Peisach, P. Aisen and W. E. Blumberg, eds.), p. 197. Academic Press, New York, 1966.
4. F. A. French and B. L. Freedlander, *Cancer Res.* **20**, Part 2, 505 (1960).
5. H. G. Petering and H. H. Buskirk, *Federation Proc.* **21**, 163 (1962).
6. E. Mihich and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.* **4**, 44 (1963).
7. H. G. Petering, H. H. Buskirk and G. E. Underwood, *Cancer Res.* **24**, 367 (1964).
8. E. Mihich and A. I. Mulhern, *Federation Proc.* **24**, 454 (1965).
9. E. Mihich and C. A. Nichol, *Cancer Res.* **25**, 1410 (1965).
10. E. Mihich, C. L. Simpson and A. I. Mulhern, *Cancer Res.* **25**, 1417 (1965).
11. H. G. Petering, H. H. Buskirk, J. A. Crim and G. J. Van Giessen, *Pharmacologist* **5**, 271 (1963).
12. B. A. Booth and A. C. Sartorelli, *Nature* **210**, 104 (1966).
13. H. G. Petering, H. H. Buskirk and F. P. Kupiecki, *Federation Proc.* **24**, 454 (1965).
14. A. C. Sartorelli, A. D. Welch and B. A. Booth, *Federation Proc.* **24**, 454 (1965).
15. A. C. Sartorelli and B. A. Booth, *Proc. Am. Assoc. Cancer Res.* **7**, 62 (1966).
16. E. Mihich and L. Jassy, *Federation Proc.* **25**, 453 (1966).
17. G. A. Fischer and A. C. Sartorelli, *in* "Methods in Medical Research" (H. Eisen, ed.), Vol. 10, p. 247. Yearbook Publishers, Chicago, Illinois, 1964.
18. E. P. Tyner, C. Heidelberger and G. A. LePage, *Cancer Res.* **13**, 186 (1953).
19. A. Marshak and J. Vogel, *J. Biol. Chem.* **189**, 597 (1951).
20. P. B. Danneberg, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 329 (1958).
21. A. C. Sartorelli and B. A. Booth, *Mol. Pharmacol.* **3**, 71 (1967).
22. W. C. Schneider, *in* "Methods in Enzymology" (S. P. Colowick and N. D. Kaplan, eds.), Vol. 3, p. 447. Academic Press, New York, 1957.

23. B. A. Booth and A. C. Sartorelli, *J. Biol. Chem.* **236**, 203 (1961).
24. R. E. Stoner and W. Dasler, *Clin. Chem.* **10**, 845 (1964).
25. A. C. Sartorelli and B. A. Booth, *Cancer Res.* **25**, 1393 (1965).
26. R. Mantsavinos and E. S. Canellakis, *Cancer Res.* **19**, 1239 (1959).
27. G. L. Eichhorn and P. Clark, *Proc. Natl. Acad. Sci. US.* **53**, 586 (1965).
28. G. L. Eichhorn, P. Clark and E. D. Becker, *Biochemistry* **5**, 245 (1966).