Biochemical Pharmacology, Vol. 51, pp. 919–929, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02401-8

Reactivity of Lipophilic diSchiff-Base Coordinated Copper in Rat Hepatocytes

*Lars-Oliver Klotz,[1] Jörg Müller,[1] Martina Fausel,[2] Rolf Gebhardt[2] and Ulrich Weser[1]**

[1]ANORGANISCHE BIOCHEMIE UND [2]PHYSIOLOGISCH-CHEMISCHES INSTITUT DER EBERHARD-KARLS-UNIVERSITÄT, HOPPE-SEYLER STRASSE 4, D-72076 TÜBINGEN, GERMANY

ABSTRACT. The membrane permeability and intracellular fate of {[N,N′-bis(2-pyridyl-phenyl-methylene)-1,4-butanediamine](N,N′,N″,N‴)-copper(II)}-diperchlorate (CuPuPhePy), a copper-diSchiff-base complex of superoxide dismutase (SOD)-mimetic activity surviving biochelation, were examined using rat hepatocytes. Lipophilicity was quantified by determining the octanol/water partition coefficients (K_p) employing PBS as the aqueous phase. K_p (octanol/water) was close to 1 (0.7 ± 0.31) for Cu-PuPhePy. The complex associates with phosphatidylcholine liposomes, as deduced from ultracentrifugation and gel filtration experiments. The ability of the complex to permeate cellular membranes was proven by correlating copper release and viability of rat hepatocytes preincubated with CuPuPhePy and treated with digitonin and diethylmaleate (DEM), respectively. The toxicity and reactivity of CuPuPhePy ($LD_{50} \approx 10$ μM for rat hepatocytes under the given conditions) were higher than those of $CuSO_4$ ($LD_{50} \approx 16$ μM) and CuZn-SOD (no toxicity in the tested range of concentration). Unlike $CuSO_4$ and CuZn-SOD, the toxicity and reactivity of the diSchiff-base complex were increased ($LD_{50} \approx 5$ μM) when the concentration of intracellular glutathione was reduced to 16% of the initial content, by preincubating the cells with DEM. The toxicity of Cu-PuPhePy paralleled lipid peroxidation. This phenomenon was strongly enhanced when Cu-PuPhePy and cumene hydroperoxide (CumOOH) were simultaneously allowed to react with rat hepatocytes. This effect was intensified following preincubation with DEM. A decline in Cu(II)-EPR signals was indicative of the reduction of CuPuPhePy by GSH and liver extract, respectively. The concomitant formation of the Cu(I)-GSH complex during this reduction was monitored by the formation of luminescent Cu(I)-thiolate chromophores. BIOCHEM PHARMACOL 51;7:919–929, 1996.

KEY WORDS. copper metabolism; superoxide dismutase analogues; partition coefficients; membrane permeability; cumene hydroperoxide toxicity; glutathione

Copper plays a prominent role in the growth and development of eucaryotic cells, with those cells exposed to an oxygen-containing environment being particularly susceptible. Respiration, as well as the control of transiently formed reactive byproducts of oxygen-consuming processes, are highly dependent on the body's copper content.

Among the many different proposals for the physiological role of copper, CuZnSOD† (E.C. 1.15.1.1), the catalysis of superoxide dismutation [1] and copper stress [2, 3] are favourably considered although still intensively debated [4, 5]. The 32 kDa protein consists of 2 identical subunits, each containing one tetra-histidyl-coordinated copper and one histidine-bridged zinc ion [6].

The half-life of the cytosolic enzyme in blood is limited by proteolytic degradation. The fairly large molecular size does not allow simple diffusion across biomembranes. Thus, many substances that exhibit SOD activity, most of which—in analogy to the active centre of CuZnSOD—contain copper, have been synthesized to transfer the beneficial effects of external CuZnSOD (such as antiinflammatory activity [7–9]) into the cell. Contrary to the intact enzyme, the use of these mimics allows a systemic application, thereby avoiding any undesired immunological response.

Early SOD mimics belonged to a number of copper complexes of the carboxylate- or biuret-type [10–13] that did not survive chelators, including bovine serum albumin (BSA) and EDTA [14]. In regard to the copper complexes of putrescine- and pyridine carbonyl-based diSchiff-base ligands called Cu-PuPy and Cu-PuPhePy, two important criteria should be emphasized [14, 15]. First, they do not merely mimic SOD activity, as the Cu(II)-binding centre in CuZn-SOD is also imitated by the coordination to four soft

* Corresponding author: Prof. Dr. U. Weser, Anorganische Biochemie, Physiologisch-Chemisches Institut der Universität, Hoppe-Seyler Strasse 4, D-72076 Tübingen. Tel., Fax +49-7071-296391; e-mail: ulrich.weser@uni-tuebingen.de

† *Abbreviations:* AAS, atomic absorption spectrometry; CumOOH, cumene hydroperoxide; Cu-PuPy, {[N,N′-bis(2-pyridyl-methylene)-1,4-butanediamine] (N,N′,N″,N‴)-copper(II)}-diperchlorate; Cu-PuPhePy, {[N,N′-bis(2-pyridyl-phenyl-me-thylene)-1,4-butanediamine](N,N′,N″,N‴)-copper(II)}-diperchlorate; CuZnSOD, Cu_2, Zn_2-superoxide dismutase (E.C. 1.15.1.1); DEM, diethylmaleate; GSH, reduced glutathione; GSSG, glutathione disulfide; HBS, HEPES-buffered saline; LDH, lactate dehydrogenase (E.C. 1.1.1.27); MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium-bromide; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

Received 15 June 1995; accepted 17 November 1995.

nitrogens in a way very similar to that found in the intact enzyme. Second, Cu-PuPy and especially Cu-PuPhePy demonstrate pronounced stability against biochelators: the stability of Cu-PuPhePy (log K = 18.33; K = [Cu-ligand]/([Cu^{2+}] × [ligand])) [14] is approximately 2 orders of magnitude greater than that of Cu-BSA (log K = 16.2) [15], whereas the stability of Cu-PuPy (log K = 16.4) [15] is equivalent to that of Cu-BSA.

It was intriguing to see that Cu-PuPy does not exhibit intracellular SOD activity [16, 17]. Attributable to the generation of reactive oxygen species, it proved to be more toxic to erythroleukemia cells than to human lymphocytes and undergoes intracellular redox cycling depending on GSH concentration. During this reaction, Cu(I)-PuPy can be controlled *via* Cu(I)-GSH in the presence of normal GSH levels in the cell, whereas a reduced GSH concentration allows intracellular Fenton chemistry of Cu-PuPy. From this phenomenon, it was concluded that Cu-PuPy might have a medical application. Unlike normal cells, many degenerated cells are known to contain substantially diminished levels of reduced glutathione and should, thus, be much more sensitive to Cu-PuPy. Erythroleukemia cells were employed in these earlier studies.

It was of interest to examine whether or not the thermodynamically much more stable Cu-PuPhePy is metabolized in an analogous way using cultured primary hepatocytes. This was done by monitoring its GSH-dependent reactivity on rat hepatocytes and its influence on lipid peroxidation induced in the presence of CumOOH.

Total Cu(II) was monitored by electron paramagnetic resonance. Similarly, the formation of Cu(I) was paralleled by the decline of the characteristic Cu(II)-EPR signal.

The lipophilicity of Cu-PuPhePy was examined by the determination of octanol/water partition coefficients. The degree to which Cu-PuPhePy is able to migrate through hepatocyte membranes was measured using cells preincubated with Cu-PuPhePy. The resulting viability was correlated with the release of this Cu complex into the incubation medium.

MATERIALS AND METHODS

Chemicals

Cu-PuPhePy was synthesized as previously described [14] and kept in an 8-mM stock solution in DMSO. Cu-PuPy was the same as published earlier [18]. HEPES and CuZnSOD from bovine erythrocytes (E.C. 1.15.1.1) were purchased from Serva (Heidelberg, Germany) and CumOOH and Octyl-β-D-glucopyranoside were from Fluka (Buchs, Switzerland). Glutathione reductase from baker's yeast (E.C. 1.6.4.2.), 1,1,3,3-tetraethoxypropane and phosphatidylcholine from egg yolk were supplied by Sigma (Munich, Germany).

Liposomes

Liposomes were produced by controlled dialysis of mixed micelles of lipid and detergent [19] from egg yolk phosphatidylcholine and octyl-β-D-glucopyranoside. On average, the resulting liposomes were homogeneous in size and 200 nm in diameter, as determined by laser autocorrelation spectroscopy (Nanosizer,™ Coulter Electronics Ltd., Harpenden, U.K.). The phospholipid content of liposome solutions was evaluated according to Stewart [20]. The association processes of Cu-PuPhePy to phosphatidylcholine vesicles were examined by gel filtration in micro columns (Microspin-S400,™ Pharmacia, Freiburg, Germany). Liposomes (7.8 mg phosphatidylcholine) in HEPES-buffered saline (HBS; 150 mM NaCl, 10 mM HEPES, pH 7.35) and Cu-PuPhePy (final concentration 5 μM) were mixed and incubated at 38°C for up to 190 min. In the subsequent gel filtration, liposomes and associated Cu-PuPhePy migrated through the columns, whereas the nonassociated Cu-PuPhePy was retained. Liposomes mixed with Cu-PuPhePy and incubated for 190 min were also centrifuged at 140,000 *g* for 3.5 hr at 10°C. To calculate the amount of copper complex accompanying the sedimented liposomes, copper in the mixture before and in the supernatant after ultracentrifugation was determined by atomic absorption spectrometry (AAS).

Partition Coefficients

Octanol/water and butanol/water partition coefficients ($K_{p(\text{octanol/water})}$ and $K_{p(\text{butanol/water})}$) were determined by the vigorous mixing of Cu-PuPhePy or Cu-PuPy solutions (approx. 2 to 4 μM) in PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) with the same volume of either 1-octanol or 1-butanol and by maintaining the mixture at 24°C for more than 5 hr to ensure that the partition equilibrium was reached. The copper concentration of the aqueous phases was measured and partition coefficients calculated from the respective aqueous and nonaqueous Cu-concentrations (with $[Cu]_{(\text{nonaqueous phase})} = [Cu]_{(\text{aqueous phase})}$ before mixing minus $[Cu]_{(\text{aqueous phase})}$ after mixing) according to $K_p = [Cu]_{(\text{nonaqueous phase})}/[Cu]_{(\text{aqueous phase})}$.

Rat Liver Extract

The liver of a male Sprague Dawley rat (620 g) was cut into portions on solid ice and homogenized in an Elvejhem potter suspended in approximately 5 times the liver volume of ice-cold PBS. The final mixture had an approximate volume of 50–60 mL and was centrifuged for 1 hr at 16,000 *g* at 4°C. The supernatant was centrifuged again at 22,000 *g* at 4°C for 2 hr in a Beckman JA-20 rotor. The extract was decanted from the pellet while the upper white fat layer remained closely attached to the centrifuge tube wall. The extract was stored in aliquots at −20°C. Protein was assayed according to Bradford [21] and Whitaker and Granum [22] and ranged near 26 mg/mL. The specific activity of lactate dehydrogenase was 6 U/mg. The protein was precipitated with 5% (w/v; final concentration) of trichloroacetic acid and the thiol content assessed in the protein-free superna-

tant according to Grassetti and Murray [23] using GSH as a standard. It was found to be in the order of 1.7 mM.

Cell Culture

Liver parenchymal cells were isolated from male Sprague-Dawley rats by the two-step collagenase perfusion technique of Seglen [24], according to modifications previously published [25]. Freshly prepared hepatocytes were resuspended in Williams Medium E (Sigma, Munich, Germany) containing additional glutamine (2 mM), streptomycin (0.05 g/L), penicillin G (0.03 g/L), dexamethasone (100 nM) and fetal calf serum (10%). The suspension was spread over culture plates precoated with collagen and the plates were incubated at 37°C for a minimum of 2 hr. All later incubations were in Williams Medium E in the presence of additional glutamine (2 mM), streptomycin (0.05 g/L), penicillin G (0.03 g/L) and dexamethasone (100 nM). The reactivity and toxicity of Cu-PuPhePy were assessed by incubating hepatocytes on 24-well plates with 250,000 cells and 0.2 mL of incubation medium containing Cu-PuPhePy in the tested concentration per well for 24 hr at 37°C and 5% CO_2. Cell viability was measured either by evaluating the activity of lactate dehydrogenase released into the incubation medium or by means of the Cell Proliferation Kit I (MTT) from Boehringer (Mannheim, Germany), thereby determining the activity of a mitochondrial dehydrogenase that occurs in living cells only *via* the production of the blue-colored formazan using the tetrazolium salt MTT. LDH activity was measured by mixing 10 to 50 μL of the sample and 1 mL of an assay mixture containing 0.62 mM sodium pyruvate and 0.24 mM NADH in 50 mM potassium phosphate buffer, pH 7.5. The time-dependent decrease in absorbance at 340 nm was observed and converted into activity values (ϵ_{340} = 6220 $M^{-1}cm^{-1}$ for NADH [26]). GSH and GSSG were determined using the glutathione reductase enzymatic cycling assay [27] after 2.5 × 10^6 hepatocytes had been collected, sonified, and separated from protein precipitated by 4.5% (w/v; final concentration) 5-sulfosalicylic acid.

Lipid Peroxidation

Hepatocytes (7.5 × 10^6) were placed on a 90-mm incubation plate and incubated at 37°C and 5% CO_2 for 40 min with a substance likely inducing lipid peroxidation, such as CumOOH or Cu-PuPhePy, or both. This was carried out either with or without prior incubation with 7 mM DGM in medium for 2 hr and subsequent rapid washing with 0.9% NaCl. After two rapid washings with 0.9% NaCl, cells were collected in 1 mL of 50 mM potassium phosphate buffer (pH 7.5) and sonified on ice for 20 sec with a Branson sonifier rod. 0.5 mL of the resulting cell homogenate and 1 mL of TBA reagent (15% (w/v) trichloroacetic acid, 0.375% (w/v) TBA, 0.05% (w/v) butylated hydroxytoluene (BHT) in 0.25 N HCl) were mixed, placed in a boiling water bath for 15 min, and centrifuged for 15 min at 3300 *g* and 10°C. The absorbance of the supernatant at 532 nm was measured and compared with that of a standard curve obtained from hydrolysed 1,1,3,3,-tetraethoxypropane. Protein was determined according to Lowry *et al.* [28].

Spectrometry

Copper was analysed with a Perkin-Elmer AAS 400. Fluorescence spectra of Cu(I)-GSH were recorded in the 500 to 700 nm range after excitation at 336 nm on a Perkin-Elmer LS 50 luminescence spectrometer. EPR measurements were done in liquid nitrogen (77 K) in a quartz finger dewar on a Bruker ESP 300 E spectrometer with the following parameters: modulation frequency 100 × 10^3 s^{-1}, modulation amplitude 0.1 mT and microwave power 20 mW.

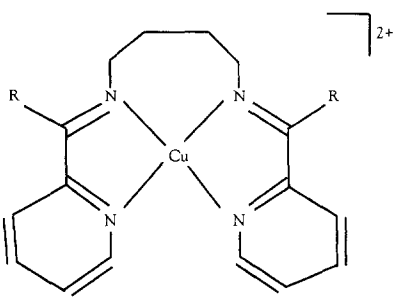
RESULTS

Partition Coefficients and Membrane Permeability

To evaluate the lipophilicity of the Cu-diSchiff-base complex, the different solubilities in water and octanol were compared. The octanol/water partition coefficients of Cu-PuPhePy and, thus, its solubility in octanol strongly depend on the pH and chloride content of the aqueous phase which, for this reason, must be buffered and contain a definite concentration of chloride. Partition coefficients were determined in systems using PBS as the aqueous phase (Table 1). The ability of Cu-PuPhePy to associate with phosphatidylcholine vesicles was verified using ultracentrifugation and gel filtration studies. The liposomes retained some 40% of the initial Cu-PuPhePy concentration during sedimentation or in the course of passing through gel filtration columns, respectively. The association of Cu-PuPhePy to phosphatidyl choline vesicles was complete after 10–40 min. Thus, washing procedures limited to less than 2 min

TABLE 1. Partition coefficients (K_p) of Cu-PuPhePy and Cu-PuPy for octanol/water and butanol/water systems using PBS as the aqueous phase. The values are means of 3 independent measurements ± standard deviations.

Cu-complex	R=	K_p (octanol/water)	K_p (butanol/water)
Cu-PuPhePy	C_6H_5	0.7 ± 0.31	8.8 ± 4.85
Cu-PuPy	H	0.14 ± 0.06	0.22 ± 0.21



ensure that Cu-PuPhePy remains bound to the hepatic cells employed.

The permeability of membranes to Cu-PuPhePy was proven by destroying rat hepatocytes previously incubated with Cu-PuPhePy that was shown to be released from the ruptured cells. Membrane breakage was achieved using 1. the nonionic detergent digitonin or 2. DEM:

1. Hepatocytes were preincubated with different concentrations of Cu-PuPhePy for 4 hr and, subsequently, treated with 50 μM digitonin for 1 hr (control: 1 hr of incubation with medium). Concentration of copper and activity of LDH were measured in the incubation medium (Fig. 1). This result cannot be explained by mere adhesion to the cellular membranes; it is much more likely that Cu-PuPhePy has penetrated the hepatocyte membrane in the course of the preincubation.

   The curves A and B in Fig. 1 (curves A + B, left and right panel) run essentially parallel—a fact that points to membrane permeation by the copper complex. Curves A (left and right panel) show that approximately 90% of the offered Cu-concentration that the cells were incubated with was accumulated by these cells. In the presence of the highest Cu concentration, a decline to 50% was observed. This is attributable to the destruction of the cells with a concomitant release of the previously accumulated complex (curve A, LDH, right panel and the corresponding curve B). In the presence of 27 μM Cu-PuPhePy, the cells were fully destroyed and all LDH completely released, allowing no further release of the enzyme after digitonin treatment. The digitonin treatment released approximately half of the Cu-PuPhePy concentration theoretically expected (sum of the 26 μM values of curves A and B, left panel). The partial association of Cu-PuPhePy to membrane-containing fractions of the destroyed cells was considered to be the cause. This was paralleled by the release of Cu-complex at the 26 μM point in curve B (left panel), almost certainly the result of the simple diffusion of adsorbed Cu-PuPhePy from cell remnants into the altered medium, according to the concentration gradient. Curve C (left panel) shows a release of Cu-PuPhePy using the cells at the highest Cu-PuPhePy concentration. The release of the Cu-complex was incomplete following the destruction of the hepatocytes by preincubation with 26 μM complex. Some portions remained bound to cellular fragments. When the medium was changed, a new state of equilibrium was reached by Cu-PuPhePy diffusing into the medium.
2. Preincubation of hepatocytes with DEM resulted in the lowering of the concentration of cellular GSH (see below), thereby increasing the cell's susceptibility to lipid peroxidation caused by metabolic processes influencing membrane integrity and, ultimately, leading to membrane perforation. Hepatocytes were either preincubated with different concentrations of DEM for 2.5 hr and treated with 9.8 μM Cu-PuPhePy (Fig. 2) or preincubated with 1 mM DEM for 2 hr and treated with different concentrations of Cu-PuPhePy (Fig. 3). Following this incubation, the copper concentration in the incu-

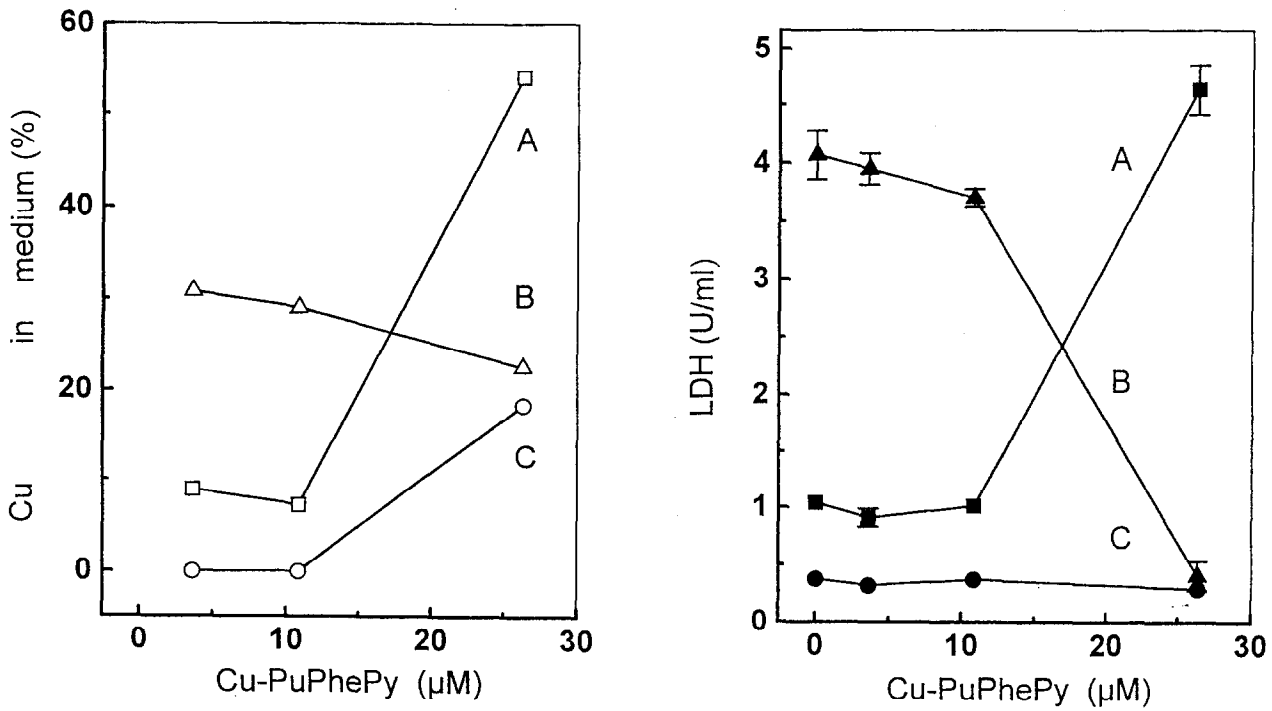


FIG. 1. Release of copper (left) and lactate dehydrogenase (LDH, right) from rat hepatocytes preincubated with varying concentrations of Cu-PuPhePy by digitonin. The cells were incubated with Cu-PuPhePy for 4 hr at 37°C and 5% CO_2 and treated with medium (control) and 50 μM digitonin in medium, respectively, for 1 additional hr. (A) Cu-PuPhePy left in the medium and LDH released from the cells during preincubation; (B) treatment with digitonin after preincubation; and (C) control. The values are means of 3 independent measurements; error bars smaller than the symbols are omitted.

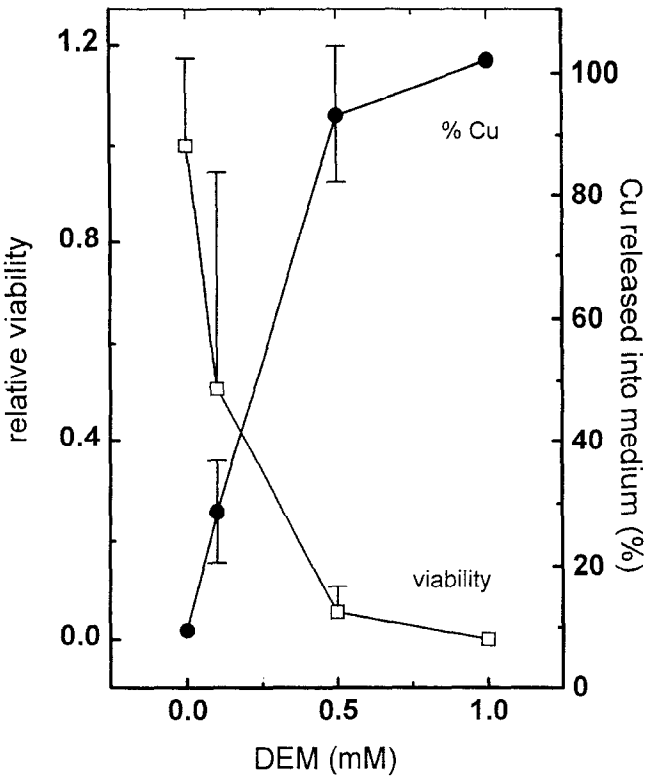


FIG. 2. Relative viability (measured as formazan blue production (MTT)) of rat hepatocyte cultures treated with 9.8 μM Cu-PuPhePy for 23.5 hr after preincubation with varying concentrations of DEM for 2.5 hr. Viability and percentage of copper released into the medium are inversely correlated. The values shown are means of 2 (% Cu) and 3 (viability) independent measurements. Error bars are omitted, if they are smaller than the symbols.

bation medium was measured and correlated with the respective viability of the employed cells (Figs. 2, 3). Again, the results support the conclusion that permeability of Cu-PuPhePy through the membrane may very well have taken place. This is consistent with the observation that the lower viability of the cells paralleled a higher copper release into the medium.

Glutathione Levels in Rat Hepatocytes after Treatment with DEM

Intracellular concentration of reduced glutathione can be lowered by incubating rat hepatocytes with DEM, an electrophilic agent coupled to GSH by glutathione-S-transferases [29]; the minimum level of GSH + GSSG measured—approximately 16% of the initial concentration—was noted after 2 hr of incubation (Table 2). The recovery of the glutathione state in the later course of incubation may be explained by an induction of systems providing the import of glutathione precursors [29].

The LD_{50} value for DEM for rat hepatocytes in a 24-hr incubation was determined to be approximately 2.3 mM. No toxicity to the cells was observed for DEM in concentrations up to 1.4 mM after 5 hr of incubation.

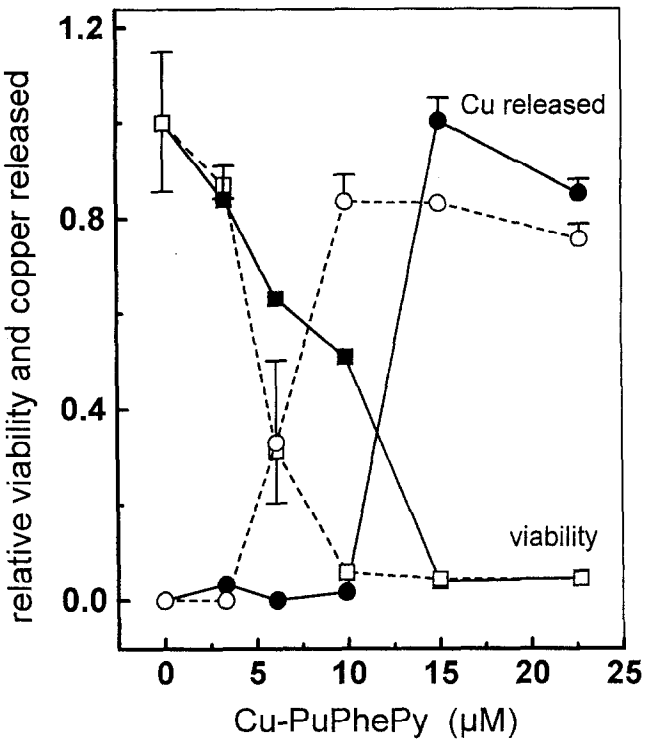


FIG. 3. Viability of rat hepatocytes preincubated with Cu-PuPhePy and copper released into the medium after 24 hr of incubation. Cells were incubated with varying concentrations of Cu-PuPhePy for 24 hr with (dashed lines) and without (solid lines) preincubation with 1 mM diethylmaleate for 2 hr. Viability of the cells (curves with square symbols) was measured as formazan dye production (MTT), and the portion of copper set in for incubation as Cu-PuPhePy that could be found in the incubation medium (curves with circles as symbols) was determined by atomic absorption spectrometry. The values are means of 2 (copper release) and 3 (viability) independent measurements, respectively. Error bars smaller than the symbols are omitted.

Reactivity and Toxicity of Cu-PuPhePy on Rat Hepatocytes

The toxicity of Cu-PuPhePy and detectable lipid peroxidation appear to correlate with each other (Fig. 4). The reactivity of Cu-PuPhePy was higher than that of either $CuSO_4$ or CuZnSOD examined for reasons of comparison: under the given conditions, the LD_{50} value for Cu-PuPhePy (tested on rat hepatocytes) was approximately 10 μM (Figs. 3–5) and, thus, lower than that of $CuSO_4$ ($LD_{50} \approx 16$ μM,

TABLE 2. Glutathione (GSH + GSSG) levels of rat hepatocytes after 2 and 25 hours of incubation with diethylmaleate (means of 2 independent measurements each ± maximum deviations). The physiological conditions with regard to glutathione content agree with the data of Tonda and Hirata [30]

Diethylmaleate (mM)	Glutathione (nmol/mg protein)	
0	163.9 ± 9.4	135.6 ± 1.0
0.5	27.6 ± 4.5	81.2 ± 1.0
1	23.2 ± 1.1	188.7 ± 34.8
Incubation (hours)	2	25

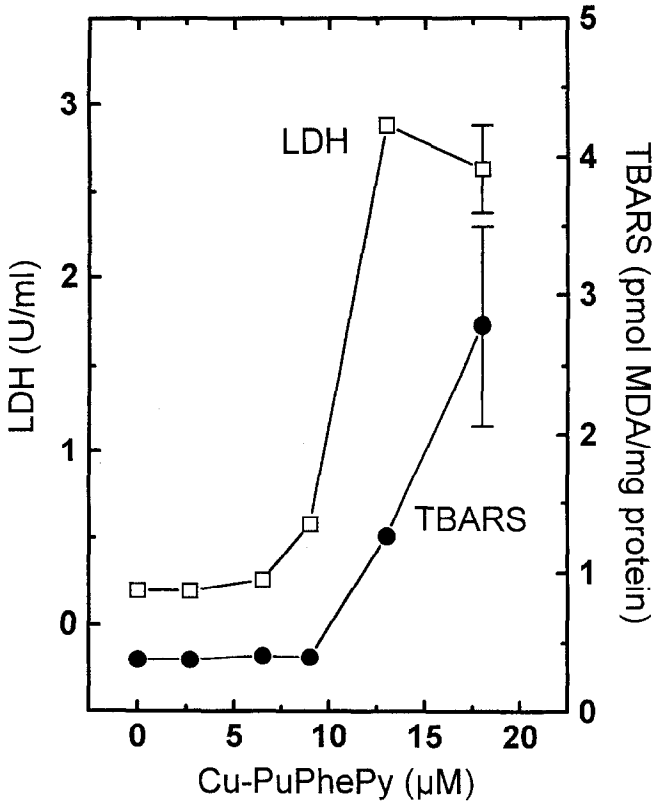


FIG. 4. Toxicity of Cu-PuPhePy and lipid peroxidation. 7.5 × 10^6 rat hepatocytes were incubated with 6 mL of medium containing Cu-PuPhePy for 22.5 hr at 37°C/5% CO_2. Toxicity was assessed by determining lactate dehydrogenase (LDH) released and lipid peroxidation by measuring the amount of TBA-reactive substances (TBARS) produced. The values are means of 3 independent measurements; error bars smaller than the symbols are omitted.

Fig. 5) and of CuZnSOD (no toxicity in the experimental range, Fig. 5). When $CuSO_4$ was dissolved in the incubation medium, it was shown by EPR that Cu(II) was present as an uncontrolled mixture of Cu-amino acid- and -protein complexes. The EPR spectrum of Cu-PuPhePy remained unchanged, indicating the survival of this diSchiff-base complex. In other words, the reactivity of $CuSO_4$ can be seen as the sum of many uncontrolled reactions. By way of contrast, Cu-PuPhePy reacted in a much more pronounced and specific manner with regard to the observed toxicity. Although the LD_{50}-values of Cu-PuPhePy and $CuSO_4$ were rather similar, the slope of the toxicity curve of Cu-PuPhePy was much steeper (Fig. 5). Whereas preincubation of hepatocytes with 1 mM DEM for 2 hr lowered the LD_{50}-value of Cu-PuPhePy from 10 μM to 5 μM (approximately), no influence on the relative toxicity of $CuSO_4$ and CuZnSOD was seen (Fig. 5). Hence, the toxicity of Cu-PuPhePy was enhanced when intracellular GSH was diminished, as opposed to CuZnSOD, to which membranes are impermeable.

Modulation of Cumene Hydroperoxide-induced Lipid Peroxidation by Cu-PuPhePy

Incubation of rat hepatocytes with CumOOH induces lipid peroxidation that can be measured as formation of TBA-reactive substances. This CumOOH-induced lipid peroxidation was progressively enhanced by Cu-PuPhePy (Fig. 6); similar rates of TBARS formation were found when hepatocytes were preincubated with DEM and treated with Cu-PuPhePy (Fig. 7). Preincubation with DEM strongly intensifies TBARS production through Cu-PuPhePy + CumOOH (Fig. 7). By way of contrast, Cu-PuPhePy alone induced TBARS formation in an insignificant manner under the given conditions (Figs. 6, 7); $CuSO_4$ and CuZnSOD were used as control substances and did not produce a substantial amount of TBARS either with or without CumOOH (not shown).

Reducibility of Cu-PuPhePy and Cu(I)-GSH Formation

The possible *in vivo* reduction of Cu(II)-PuPhePy by glutathione was examined in separate experiments *ex vivo*. Cu-PuPhePy 80 μM was reduced in the presence of 3.3 mM GSH and rat liver extract containing 1.7 mM of nonprotein thiols, respectively. This reduction, controlled by the decline in the Cu(II)-EPR signal (Fig. 8), was accompanied by the concomitant formation of Cu(I)-GSH. The Cu(I)-thi-

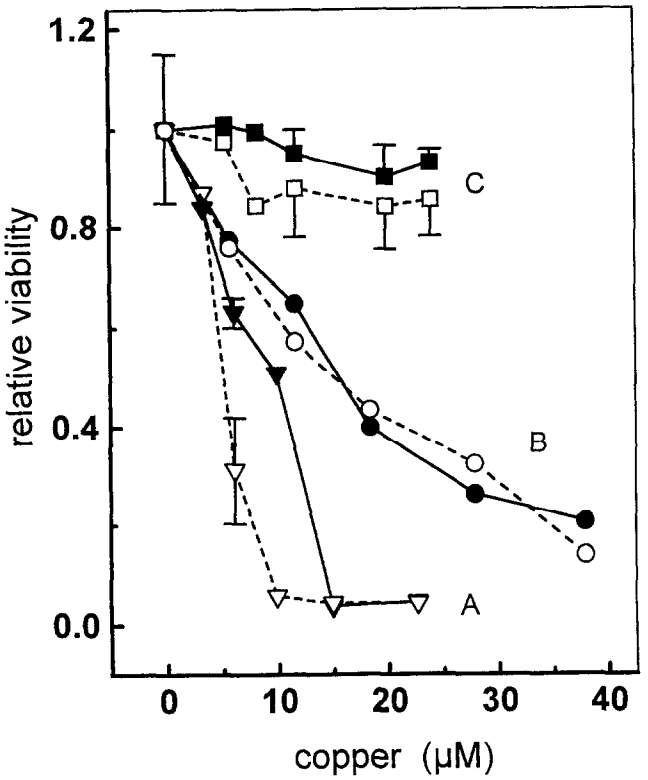


FIG. 5. Reactivity and toxicity of Cu-PuPhePy (A); $CuSO_4$ (B); and CuZnSOD (C). The viabilities were measured via formazan blue production (MTT) after 24-hr incubation of rat hepatocytes with different concentrations of the substances (solid lines) and a 24-hr incubation after 2 hr of preincubation with diethylmaleate (DEM, dashed lines). The viabilities are shown as values relative to the respective control values. Only the toxicity of Cu-PuPhePy is strongly influenced by DEM treatment of the cells (A). The values are means of 3 independent measurements. For reasons of clarity, error bars are shown only at important points.

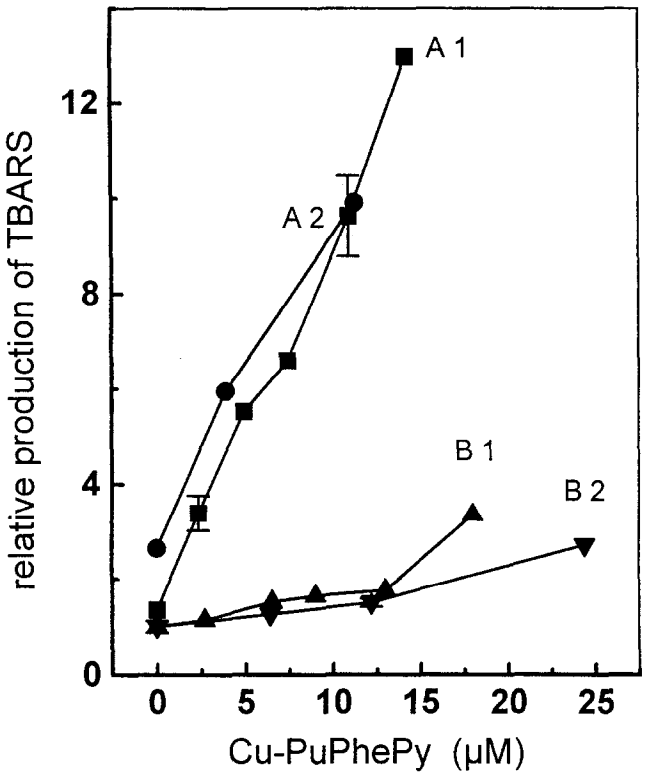


FIG. 6. Lipid peroxidation (measured as the relative production of TBA-reactive substances, TBARS) in 7.5 × 10^6 rat hepatocytes incubated with 6 mL of incubation medium containing Cu-PuPhePy (B1 and B2) and Cu-PuPhePy + 0.4 mM cumene hydroperoxide (A1 and A2), respectively, for 40 min on 90-mm incubation dishes. The four curves are results of 4 independent experiments; B2 is identical with "D" in Fig. 8. The values shown are means of 2 independent measurements each; error bars smaller than the symbols are omitted.

olate coordination was successfully demonstrated by fluorescence spectroscopy (Fig. 9). No reduction was seen when 2 × 10^6 rat liver cells were incubated together with 500 μL of 0.1 mM Cu-PuPhePy. The magnitude of the Cu(II)-EPR signals remained essentially unchanged, attributable to the minute concentration of reducing equivalents. However, some transient changes in the hyperfine splittings were seen, but disappeared in the dead cells (Fig. 10). The observed slight changes seen in Fig. 8 between spectrum A and B or E, respectively, may be assigned to changes in the axial positions of the coordination sphere of Cu-PuPhePy, due to changes in the composition of the solution containing the copper complex. The ligands in these positions are easily changed in the presence of different coordinating compounds, replacing water molecules. This influences the respective EPR spectra. The identity of the axial ligands remains speculative.

DISCUSSION

We attempted to quantify the lipophilicity of CuPuPhePy by determining octanol/water and butanol/water partition coefficients. The resulting values were compared with those determined for the membrane permeant [16] CuPuPy; the values for CuPuPhePy exceed those for CuPuPy, which means that the former (due to its higher lipophilicity) should be even more soluble in membrane systems. In general, conclusions regarding membrane permeabilities drawn on the basis of these experiments must be made with caution. Schubert [31] emphasizes significant differences between octanol/water and membrane/water interfaces. In contrast to octanol/water interfaces, membrane/water interfaces are distinctively larger, thereby offering access to almost any molecule in the aqueous phase. Unlike octanol/water interfaces, which can be permeated only by uncharged substances, membranes are charged and may strongly bind charged molecules. Furthermore, the asymmetry of membranes cannot be simulated by octanol/water systems. Nevertheless, the permeability of membranes to CuPuPhePy was, in fact, demonstrated and is consistent with the partition coefficients of the complex and its association to liposomes. Hence, partition coefficients seem to be quite appropriate for estimating both lipophilicity and membrane permeability to copper complexes, provided the pH and ionic strength of the aqueous phases used are unchanged. In this context, octanol/water partition coeffi-

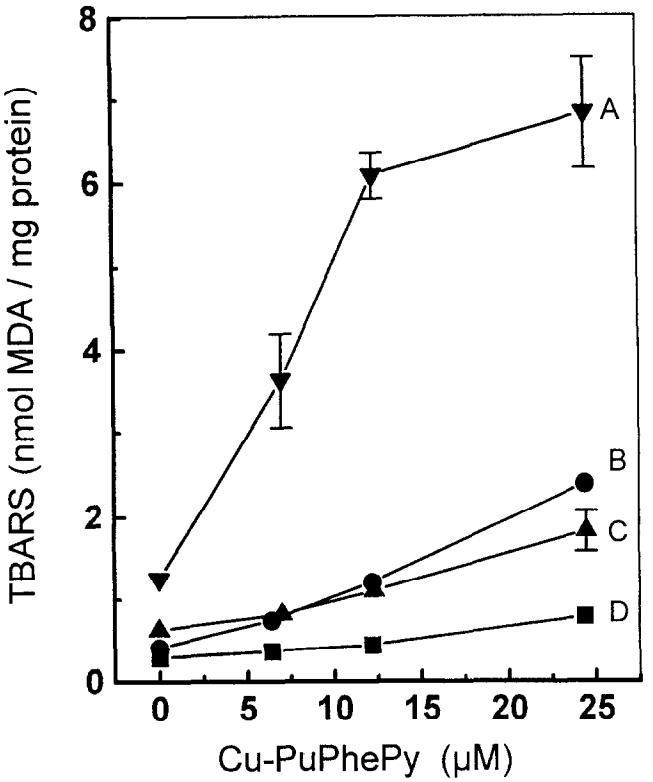


FIG. 7. Lipid peroxidation (measured as the production of TBA-reactive substances, TBARS) in rat hepatocytes incubated for 40 min with varying concentrations of Cu-PuPhePy (D); Cu-PuPhePy + cumene hydroperoxide (C); Cu-PuPhePy with a 2-hr preincubation with 1 mM diethylmaleate (B); and Cu-PuPhePy + cumene hydroperoxide with 2-hr preincubation with 1 mM diethylmaleate (A). The values are means of 2 independent measurements; error bars smaller than the symbols are left out. Control values are denoted at the respective zero concentrations of Cu-PuPhePy.

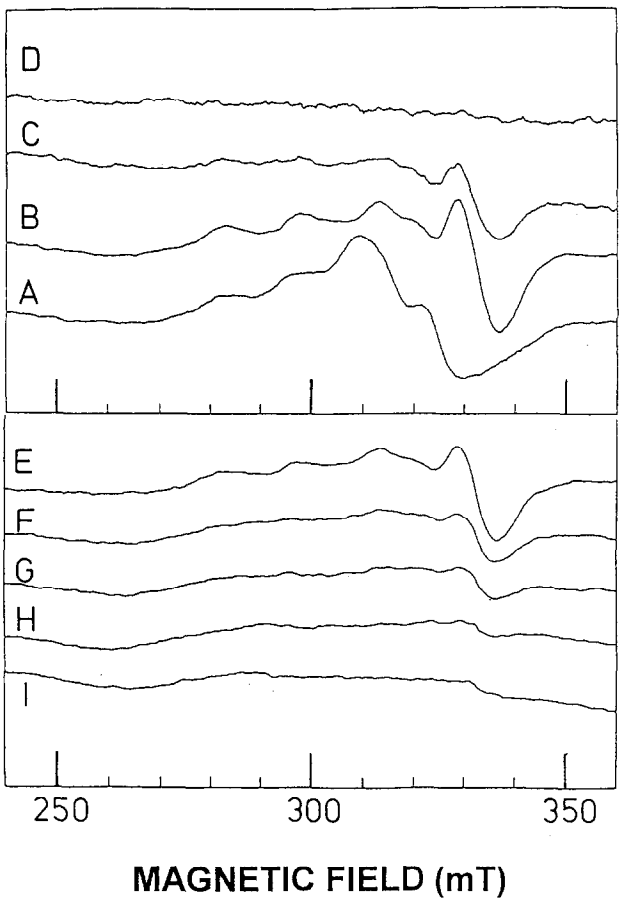


FIG. 8. EPR spectra (77 K) demonstrating the reduction of Cu-PuPhePy by GSH (A–D) and by rat liver extract (E–I). Cu-PuPhePy 80 μM was incubated with 3.3 mM GSH and extract containing 1.7 mM of free thiol groups, respectively. (A) 80 μM Cu-PuPhePy in PBS; (B) 80 μM Cu-PuPhePy + 3.3 mM GSH after 1 min of incubation (24°C); (C) 16 min; (D) 76 min of incubation; (E) 80 μM Cu-PuPhePy + rat liver extract after 1 min of incubation (24°C); (F) after 10 min; (G) after 21 min; and (H) after 60 min of incubation; (I) pure rat liver extract.

cients close to 1 (as is the case for CuPuPhePy) should be the optimum condition for a real partition between lipid and aqueous phases, given that the substances are to migrate through membranes and not accumulate in the membranes or other lipoid compartments.

It appears that membrane permeability of CuPuPhePy is one of the main reasons why the reactivity and toxicity of CuPuPhePy are much more pronounced than those observed after the addition of $CuSO_4$ and CuZnSOD. Likewise, the GSH dependences of the reactivities are extremely distinct. Lipid peroxidation and CuPuPhePy-induced hepatotoxicity can be correlated (Fig. 4). Cu(II)-PuPhePy must either *per se* or in a modified form be able to induce peroxidation of membrane systems that results in a decrease in the cell membrane's integrity and a concomitant increase in its permeability to cell contents.

The reducibility of Cu(II)-PuPhePy by rat liver extracts and GSH solutions (each with an approximate molar ratio of thiol groups to CuPuPhePy of approximately 10:1 to 30:1) points out a possible intracellular reduction of Cu(II)-PuPhePy to Cu(I)-PuPhePy, the latter being a substance with Fenton-like activity. This would mean that CuPuPhePy lowers the concentration of intracellular GSH and thus that of cellular reduction equivalents, with the consequence that glutathione peroxidase, for example, will be unable to degrade lipid peroxides. The formation of Cu(I)-GSH in the course of reduction of CuPuPhePy by GSH (Fig. 9) shows that Cu(I)-PuPhePy has a limited stability in the presence of Cu(I) chelators.

To understand why CuPuPhePy enhances CumOOH-induced lipid peroxidation, one has to look at the ways CumOOH can be metabolized in a normal cell. Due to its lipophilicity, the cell's interior is easily accessible to CumOOH. It can be degraded mainly in 2 ways: first, it can undergo enzymic reductions at the expense of GSH (glutathione peroxidase) and NADPH (glutathione reductase) [32] or cytochrome P-450-dependent reactions using different electron donors, for example, NADPH [33, 34]. Second, CumOOH can actively participate in Fenton-like re-

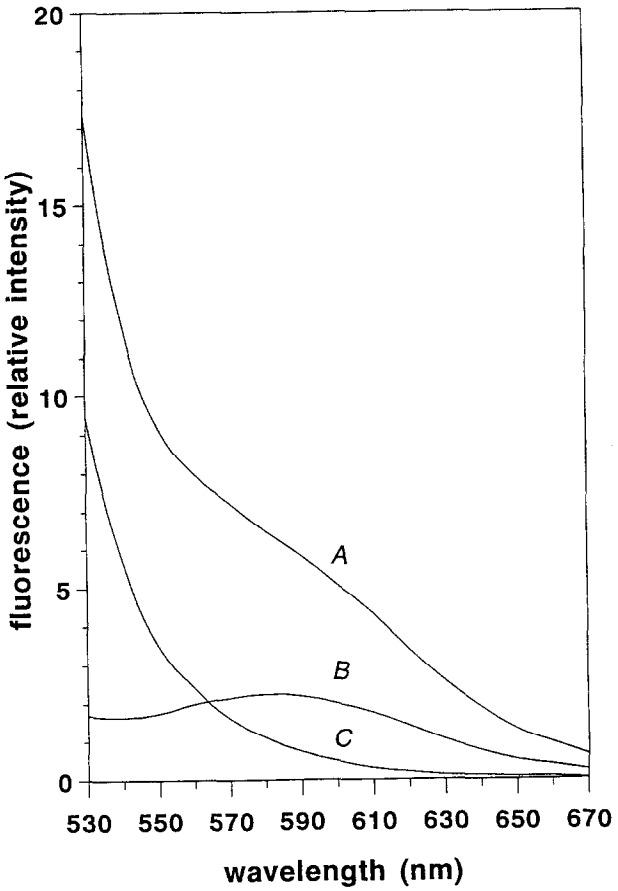


FIG. 9. Generation of Cu(I)-GSH. Fluorescence spectra of 1 mM Cu-PuPhePy + 10 mM GSH (A); 1 mM $CuSO_4$ + 10 mM GSH (B); and 1 mM Cu-PuPhePy (C). All measurements were done in PBS. Excitation was at 336 nm. All mixtures were treated with the same volume of n-butanol to extract insoluble PuPhePy-ligand, in the case of (A), and for reasons of comparison; Cu(I)-GSH remains in the aqueous phase. No fluorescence could be seen in solutions of 1 mM $CuSO_4$ and 10 mM GSH, respectively.

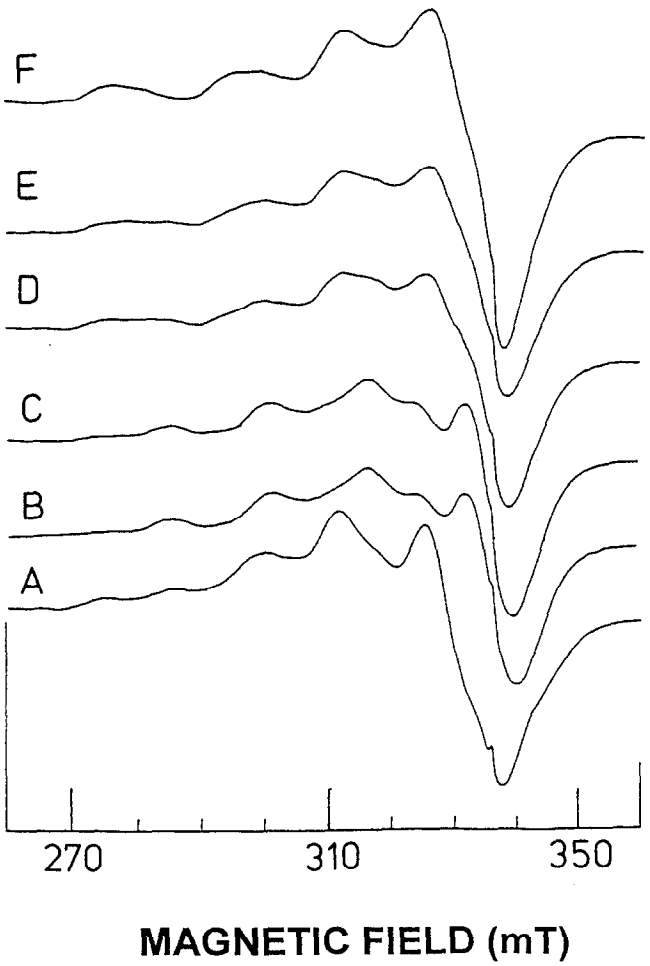


FIG. 10. EPR spectra (77 K) of Cu-PuPhePy incubated with rat hepatocytes (500 µL of 0.1 mM Cu-PuPhePy + 2 × 10^6 cells). The complex previously suspended in medium was added to the cells. The resulting spectra seem to be shifted, and a peak at approximately 335 mT transiently appears, disappearing again when the cells die. (A) 0.1 mM Cu-PuPhePy in medium; (B) 1 min after adding Cu-PuPhePy to the cells (cells alive); (C) 10 min of incubation (24°C; cells alive); (D) 1 hr of incubation (37°C; cells beginning to die); (E) 2 hr (37°C; cells dead); (F) 7 hr (2 hr 37°C, 5 hr 24°C, no change of medium; cells dead).

actions, in the course of which chelated transition metals (e.g., Fe^{2+}-porphyrin [35, 36] and Fe^{2+}-ATP [37]) reduce CumOOH to the cumoxyl radical CumO· and OH^-, leaving the metal in the oxidized state. CumO· can cause many uncontrolled reactions producing a variety of radicals (such as the cumene peroxyl radical, CumOO· or, in a beta-scission reaction, the methyl radical [38, 39]), all of which are possible initiators of lipid peroxidation in that they are able to abstract hydrogen atoms from methylene groups.

An intracellular reduction of Cu(II)-PuPhePy produces Cu(I)-PuPhePy, a very powerful Fenton-active complex that can reduce CumOOH to OH^- and CumO·, thus, supporting CumOOH-dependent lipid peroxidation. Glutathione peroxidase diminishes the concentration of intracellular GSH by reducing CumOOH. Furthermore, the reduction of Cu(II)-PuPhePy requires additional GSH. Both GSH-diminishing reactions are aggravated by treatment of the cells with DEM (Fig. 7).

Why do the preincubation with DEM and consecutive treatment with CuPuPhePy result in a degree of TBARS formation similar to that of CuPuPhePy + CumOOH? And how can the toxicity of CuPuPhePy to hepatocytes rise when the cellular GSH level is decreased, although a higher concentration of GSH provides for the reduction of more CuPuPhePy to the Fenton-active Cu(I)-PuPhePy and should, hence, induce lipid peroxidation?

Steinkühler *et al.* [16, 17] proposed an intracellular reduction-oxidation cycling of the copper complex CuPuPy and GSH, the latter reducing the former to Cu(I)-PuPy that *per se* can reduce O_2 to H_2O_2, which can be decomposed by Cu(I)-PuPy in a reaction of the Fenton-type with formation of hydroxyl radicals capable of attacking (e.g., membrane lipids). By analogy, this proposal strongly supports the same reactivity of CuPuPhePy.

Another point of great importance is that the more GSH is present, the more Cu(I)-PuPhePy can be detoxified *via* Cu(I)-GSH (Figs. 9, 11), a redox-inert complex capable of transporting copper to apo-copper-proteins [40–42] such as metallothionein. Preincubation of the cells with DEM diminishes the concentration of GSH capable of preventing Cu(I)-PuPhePy-induced Fenton reactions by transferring Cu(I) to cellular copper sinks.

There are many known nonFenton reactions of copper transported into cells that support peroxidative effects. Cu(I)-PuPhePy has limited stability in the presence of competing Cu(I)-chelators; proteins containing sulfhydryl groups are potent ligands that compete with PuPhePy for the copper ion. In this context, glutathione reductase should be mentioned, which contains a disulfhydryl group in the active centre [43]. This enzyme is strongly inhibited by copper ions [44] and, in this form is unable to restore cellular GSH levels by reducing GSSG at the expense of NADPH.

CuPuPhePy remains stable in cellular systems; it is stable in lipophilic solvents and in the presence of chelating substances under nonreducing conditions. Time-dependent changes in the EPR spectrum of CuPuPhePy incubated with hepatocytes (Fig. 10) suggest some transient modifications in the ligand resulting in changes in the complex geometry. This assumption sounds reasonable, because the phenyl residues of CuPuPhePy render it a very good substrate for cytochrome-P450-dependent reactions. These modifications could alter the stability of CuPuPhePy in both its cupric and cuprous state.

A scheme describing the intracellular metabolism of Cu-PuPhePy may be suggested (Fig. 11).

Cu-PuPhePy could also be regarded as a channelling system for copper into cells. It appears to present advantages over simple copper salts when copper is administered to cells in culture. Copper sulphate and other simple copper salts would merely result in the uncontrolled complexation with many potent chelators richly abundant in the employed incubation medium. By way of contrast, Cu-

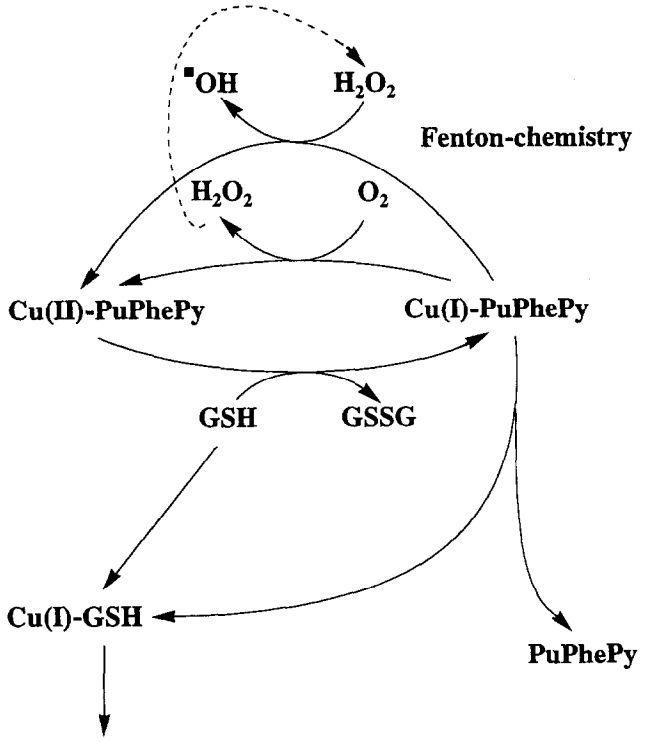


FIG. 11. Proposed scheme for the intracellular fate of Cu-PuPhePy. Cu-PuPhePy is reduced by GSM's ability to exert Fenton chemistry, and may produce hydroxyl radicals, HO·. The reduction of O_2 to H_2O_2 by Cu(I)PuPhePy depicted in the upper part of the figure is the sum of 2 separate reactions according to:

$$2\ GSH + 2\ Cu(II)\text{-}PuPhePy \rightarrow 2\ Cu(I)\text{-}PuPhePy + 2H^+ + GSSG$$

$$2\ Cu(I)\text{-}PuPhePy + O_2 + 2\ H^+ \rightarrow 2\ Cu(II)\text{-}PuPhePy + H_2O_2$$

$$net:\ 2\ GSH + O_2 \rightarrow GSSG + H_2O_2$$

A ligand exchange occurs in the case of Cu(I)-PuPhePy and Cu(I)-GSH is formed, which can act as a transporter of Cu(I) into apo-copper-proteins such as metallothionein or superoxide dismutase [40–42] (see text for further details).

PuPhePy—due to both its stability against biochelation and its membrane permeability—should permit a controlled copper application and might be, for example, rather useful for research on pathological copper disorders in cell culture.

This study was supported by the Deutsche Forschungsgemeinschaft (We 401/) and the Fonds der chemischen Industrie. We are indebted to Dr. H.-J. Hartmann for stimulating discussions. We also thank Sabine Lutz, Chirurgische Klinik, Tübingen, for expert help in preparing the liposomes. Further thanks go to Michael Milchsack, University of Giessen, for discussions on glutathione and the glutathione assay.

References

1. McCord JM and Fridovich I, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244:** 6049–6055, 1969.
2. Steinkühler C, Sapora O, Carri MT, Nagel W, Marcocci L, Ciriolo MR, Weser U and Rotilio G, Increase of Cu, Zn-superoxide dismutase activity during differentiation of human K562 cells involves activation by copper of a constantly expressed copper-deficient protein. *J Biol Chem* **266:** 24580–24587, 1991.
3. Steinkühler C, Carri MT, Micheli G, Knoepfel L, Weser U and Rotilio G, Copper-dependent metabolism of Cu, Zn-superoxide dismutase in human K562 cells. *Biochem J* **302:** 687–694, 1994.
4. Weser U, Paschen W and Younes M, Singlet oxygen and superoxide dismutase (cuprein). *Biochem Biophys Res Comm* **66:** 769–777, 1975.
5. Khan AK and Kasha M, Singlet molecular oxygen in the Haber-Weiss reaction. *Proc Natl Acad Sci USA* **91:** 12365–12367, 1994.
6. Tainer JA, Getzoff ED, Beem KM, Richardson JS and Richardson DC, Determination and analysis of the 2 Å-structure of copper, zinc superoxide dismutase. *J Mol Biol* **160:** 181–217, 1982.
7. Flohé L, Superoxide dismutase for therapeutic use: clinical experience, dead ends and hopes. *Mol Cell Biochem* **84:** 123–131, 1988.
8. Vaille A, Jadot G and Elizagaray A, Anti-inflammatory activity of various superoxide dismutases on polyarthritis in the lewis rat. *Biochem Pharmacol* **39:** 247–255, 1990.
9. Deby C and Goutier R, New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. *Biochem Pharmacol* **39:** 399–405, 1990.
10. Brigelius R, Spöttl R, Bors W, Lengfelder E, Saran M and Weser U, Superoxide dismutase activity of low molecular weight Cu^{2+}-chelates studied by pulse radiolysis. *FEBS Lett* **47:** 72–75, 1974.
11. Brigelius R, Harmann HJ, Bors W, Saran W, Lengfelder E and Weser U, Superoxide dismutase activity of $Cu(Tyr)_2$ and Cu,Co-erythrocuprein. *Hoppe Seyler's Z Physiol Chem* **356:** 739–745, 1975.
12. Younes M, Lengfelder E, Zienau S and Weser U, Pulse radiolytically generated superoxide and Cu(II)-salicylates. *Biochem Biophys Res Comm* **81:** 576–580, 1978.
13. Weser U, Sellinger KH, Lengfelder E, Werner W and Strähle J, Structure of Cu_2(Indomethacin)$_4$ and the reaction with superoxide in aprotic systems. *Biochim Biophys Acta* **631:**232–245, 1980.
14. Müller J, Felix K, Maichle C, Lengfelder E, Strähle J and Weser U, Phenyl substituted Cu-di-Schiff-base, a potent Cu_2Zn_2 superoxide dismutase mimic surviving biochelation. *Inorg Chim Acta* **233:** 11–19, 1995.
15. Linss M and Weser U, Redox behaviour and stability of active centre analogues of Cu_2Zn_2 superoxide dismutase. *Inorg Chim Acta* **138:** 163–166, 1987.
16. Steinkühler C, Mavelli I, Rossi L, Pedersen JZ, Melino G, Weser U and Rotilio G, Cytotoxicity of a low molecular weight Cu_2Zn_2 superoxide dismutase active center analog in human erythroleukemia cells. *Biochem Pharmacol* **39:** 1473–1479, 1990.
17. Steinkühler C, Pedersen JZ, Weser U and Rotilio G, Oxidative stress induced by a di-Schiff-base copper complex is both mediated and modulated by glutathione. *Biochem Pharmacol* **42:** 1821–1827, 1991.
18. Felix K, Lengfelder E, Deters D and Weser U, Pulse radiolytically determined superoxide dismutase mimicking activity of copper-putrescine-pyridine, a diSchiff base coordinated copper complex. *BioMetals* **6:** 11–15, 1993.
19. Milsmann MHW, Schwendener RA and Weder HG, The preparation of large single bilayer liposomes by a fast and controlled dialysis. *Biochim Biophys Acta* **512:** 147–155, 1978.
20. Stewart JCM, Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem* **104:** 10–14, 1980.

21. Bradford MM, A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* **72:** 248–254, 1976.
22. Whitaker JR and Granum PE, An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal Biochem* **109:** 156–159, 1980.
23. Grassetti DR and Murray JF, Determination of sulfhydryl groups with 2,2′- or 4,4′-dithiopyridine. *Arch Biochem Biophys* **119:** 41–49, 1967.
24. Seglen PO, Preparation of isolated rat liver cells. *Methods Cell Biol* **13:** 29–83, 1976.
25. Gebhardt R and Burger HJ, Selective loss of hormonal induction of glutamate transport in primary cultures of hepatocytes from rats treated with CCl_4. *J Hepatol* **4:** 381–389, 1987.
26. Malcolm ADB, Temperature dependence of the spectroscopic properties of NADH. *Methods Enzymol* **66:** 8–11, 1980.
27. Anderson ME, Enzymatic and chemical methods for the determination of glutathione. In: *Glutathione, Chemical, Biochemical, and Medical Aspects, Part A* (Eds. Dolphin D, Poulson R and Avramovic O), pp. 339–365. John Wiley and Sons, New York, 1989.
28. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
29. Deneke SM and Fanburg BL, Regulation of cellular glutathione. *Am J Physiol* **257:** L163–L173, 1989.
30. Tonda K and Hirata M, Effect of L-methionine on 2-carboxybenzaldehyde reductase induction by phenobarbital in primary cultures of rat hepatocytes. *Chem Biol Interactions* **77:** 149–158, 1991.
31. Schubert R, Comparison of octanol/buffer and liposome/buffer partition coefficients as models for the in vivo behavior of drugs. *Proc MoBBEL* **8:** 11–20, 1994.
32. Carmagnol F, Sinet PM and Jerome H, Selenium-dependent and non-selenium-dependent glutathione peroxidases in human tissue extracts. *Biochim Biophys Acta* **159:** 49–57, 1983.
33. Coon MJ, Blake RC, White RE and Nordblom GD, Assays for cytochrome P-450 peroxygenase activity. *Methods Enzymol* **186:** 273–278, 1990.
34. Reed CJ and De Matteis F, Cumene hydroperoxide-dependent oxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine and 7-ethoxycoumarin by cytochrome P-450. *Biochem J* **261:** 793–800, 1989.
35. Vaz AD and Coon MJ, Reductive cleavage of hydroperoxides by cytochrome P-450. *Methods Enzymol* **186:** 278–282, 1990.
36. Davies MJ, Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with heme-proteins by electron spin resonance spectroscopy. *Biochim Biophys Acta* **964:** 28–35, 1988.
37. Rush JD and Koppenol WH, Reactions of Fe(II)-ATP and Fe(II)-citrate with t-butyl hydroperoxide and cumyl hydroperoxide. *FEBS Lett* **275:** 114–116, 1990.
38. Davies MJ and Slater TF, Studies on the metal-ion and lipoxygenase-catalysed breakdown of hydroperoxides using electron-spin-resonance spectroscopy. *Biochem J* **245:** 167–173, 1987.
39. Weiss RH and Estabrook RW, The mechanism of cumene hydroperoxide-dependent lipid peroxidation: the function of cytochrome P-450. *Arch Biochem Biophys* **251:** 348–360, 1986.
40. Da Costa Ferreira AM, Ciriolo MR, Marcocci L and Rotilio G, Copper(I) transfer into metallothionein mediated by glutathione. *Biochem J* **292:** 673–676, 1993.
41. Ciriolo MR, Desideri A, Paci M and Rotilio G, Reconstitution of Cu, Zn-superoxide dismutase by the Cu(I) glutathione complex. *J Biol Chem* **265:** 11030–11034, 1990.
42. Brouwer M and Brouwer-Hoexum T, Glutathione-mediated transfer of copper(I) into American lobster apohemocyanin. *Biochemistry* **31:** 4096–4102, 1992.
43. Schulz GE, Schirmer RH, Sachsenheimer W and Pai EF, Structure of the flavoenzyme glutathione reductase. *Nature* **273:** 120–124, 1978.
44. Rafter GW, Copper inhibition of glutathione reductase and its reversal with gold thiolates, thiol, and disulfide compounds. *Biochem Med* **27:** 381–391, 1982.