Reduction of Copper(II)–Bleomycin: A Model for in Vivo Drug Activity†

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ABSTRACT: The effect of aliphatic thiols, including glutathione, cysteine, and dithiothreitol, on the anaerobic reduction of Cu(II)–bleomycin was examined. At neutral pH, cysteine is more efficient in reducing Cu(II)–bleomycin than either dithiothreitol or glutathione, while at alkaline pH the rate of reduction with all three reagents increases substantially. A kinetic treatment suggests that 2 equiv of thiol is required for each mole of Cu(II)–bleomycin reduced. Material balance studies verify this stoichiometry. If anaerobic reduction of Cu(II)–bleomycin is carried out in the presence of Fe(II), iron is chelated by the drug. This metal–drug complex is capable of degrading DNA when O_2 is introduced. The extent of DNA degradation, as measured by the release of malondialdehyde-like chromogens from the DNA, is directly dependent on the amount of Cu(II)–bleomycin reduced.

The bleomycins are a group of antineoplastic glycopeptides isolated from cultures of *Streptomyces verticillus* as Cu(II) complexes (Figure 1) (Umezawa et al., 1966a,b). Their therapeutic and cytotoxic activity is attributed to their ability to cleave DNA [see Hecht (1979); Umezawa (1980); Burger et al. (1981a)]. Bleomycin activity in vitro requires ferrous iron and molecular oxygen, both of which interact with the drug to produce a ternary complex whose formation is required for DNA cleavage (Sausville et al., 1976, 1978a; Burger et al., 1979a,b). Cu(II)–bleomycin does not degrade DNA in vitro, but Cu(II) is a potent inhibitor of the DNA breakage reaction, presumably because it displaces the Fe(II) required for activity (Sausville et al., 1978b). In contrast, in vivo studies in tissue culture and animals reveal that Cu(II)–bleomycin is as effective as metal-free bleomycin for the breakage of DNA. Cu(II)–bleomycin parallels metal-free bleomycin in its ability to inhibit growth of *Escherichia coli* (Suzuki et al., 1968) and of tumors in animals (Ishizuka et al., 1967; Crooke & Bradner, 1977; Rao et al., 1980) and to inhibit colony formation Chinese hamster V79 cells (Nunn & Lunec, 1978) and rat ascites hepatoma AH66 and AH66F cells (Takahashi et al., 1977).

In order to explain the discrepancy between the in vitro and in vivo activity of the Cu(II) complex, Umezawa and coworkers (Takahashi et al., 1977) hypothesized that in vivo, Cu(II)–bleomycin is activated in a series of reactions of which the first is a reduction of divalent copper to Cu(I) by intracellular reducing agents such as GSH,[1] CySH, NADPH, NADH, and ascorbate. It was further suggested that the Cu(I) produced by this reaction is sequestered by metal binding proteins within the cell, leaving the drug in a metal-free form that was thought to be capable of degrading DNA. It is now known that bleomycin reactivity in vitro requires Fe(II) for DNA breakage (Burger et al., 1981a).

In order to understand the reactivity of Cu(II)–bleomycin and a possible role for Fe(II)–bleomycin in vivo, we have studied the action of various physiological reductants on Cu(II)–bleomycin and the subsequent reaction of the drug with Fe(II). Using optical and EPR spectroscopies, we have shown that GSH and CySH reduce bleomycin-bound Cu(II) to Cu(I), while ascorbate, NADH, and NADPH do not. Furthermore, in the presence of thiol, Fe(II), if present, is rapidly chelated by the drug, leading to the formation of Fe(II)–bleomycin which, upon the addition of O_2, is capable of degrading DNA.

Experimental Procedures

Materials. Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories. It contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% other bleomycins. The molecular weight of bleomycin was assumed to be 1550. Solutions were prepared in either 20 mM potassium phosphate or 50 mM Hepes buffer, pH 7.0, or 0.11 M GlyGly buffer, pH 8.4.

Calf thymus DNA from Sigma was dissolved in 20 mM potassium phosphate buffer, pH 7.0, and the concentration was determined optically (ϵ_{260} = 6.6 (mM nucleotide)$^{-1}$ cm^{-1}). All other reagents were dissolved in distilled, deionized water. Anaerobic solutions of reagent-grade ferrous ammonium sulfate (Baker), CySH (Schwarz/Mann), GSH, and DTT (Sigma) were prepared shortly before use in rubber septum stoppered test tubes, which were then equilibrated for 15 min with water-saturated, ultrahigh-purity argon (99.999%) from Linde. All Cu(II)-containing solutions were prepared with cupric acetate (Mallinckrodt). Solutions of Cu(II)–bleomycin were prepared with ~10% molar excess drug.

The copper–amino acid complexes Cu(II)–$(Thr)_2$, Cu(II)–$(His)_2$, and Thr–Cu(II)–His were prepared as described by Sarkar & Kruck (1966). The tripeptide L-glycyl-L-histidyl-L-lysine was the generous gift of Dr. Boris Weinstein, University of Washington. The Cu(II)–tripeptide complex (Pickart et al., 1980) was prepared by adding a 5% molar excess of tripeptide to a 1.0 mM Cu(II) solution and adjusting the pH to 7.5. Copper–bovine serum albumin (Schwarz/Mann) was prepared by adding 1.0 mM Cu(II) to 1.1 mM protein and adjusting the pH to 7.5. Pig cerulo-

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[1] Abbreviations: RSH, alkyl thiol; GSH, glutathione (γ-L-glutamyl-L-cysteinylglycine); DTT, dithiothreitol; CySH, cysteine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GlyGly, glycylglycine; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EPR, electron paramagnetic resonance; BLM, bleomycin; MDA, malondialdehyde-like products of DNA degradation.

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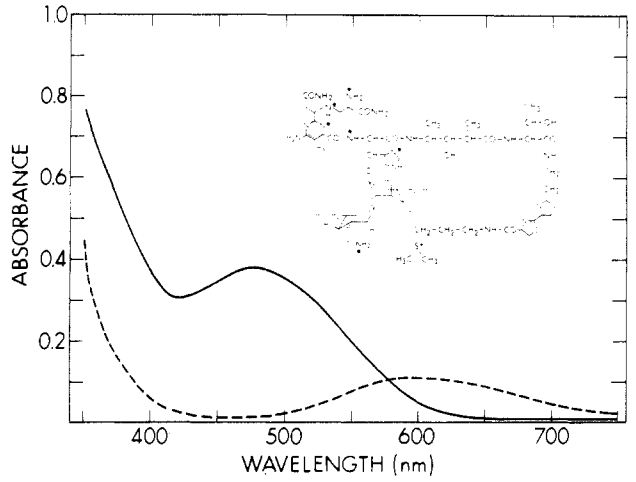

FIGURE 1: Optical spectra of metallobleomycin complexes. Solutions of Fe(II)–bleomycin (—) and Cu(II)–bleomycin (– –) contained 1.1 mM bleomycin and 1.0 mM metal ion in 0.1 M GlyGly buffer, pH 8.4. For the Fe(II) complex, a solution of bleomycin was made anaerobic, as described under Experimental Procedures, prior to metal addition. The inset contains the structure of bleomycin A_2. The asterisks denote the ligands that are thought to be involved in Cu(II) binding (Iitaka et al., 1978; Dabrowiak et al., 1978).

plasmin was prepared by the method of Peisach & Levine (1965) and stored at 77 K until used. Bovine superoxide dismutase and dopamine β-hydroxylase were purchased from Sigma.

Cuprous chloride was prepared by reducing cupric chloride with sodium sulfite. Briefly, 20 mL of 3.8 M $CuCl_2$ was mixed with 50 mL of 1.6 M Na_2SO_3. This was then added to 1 L of a stock solution containing 2.0 mL of concentrated HCl and 1 g of Na_2SO_3. After the white cuprous chloride precipitate was allowed to settle, the supernatant was removed by decantation. The precipitate was collected by filtration on a Büchner funnel and washed with 500 mL of stock solution. This was followed by a series of washings: 5 times with 20 mL of glacial acetic acid, 2 times with 50 mL of absolute ethanol, and finally 6 times with 15 mL of anhydrous diethyl ether. The precipitate was heat dried at 90 °C and stored in a vacuum desiccator. During the washing steps, care was taken to maintain liquid on top of the cuprous chloride precipitate so as to prevent unnecessary exposure to air. Anaerobic cuprous chloride solutions were freshly prepared in aqueous acetonitrile (2:1 v/v).

Optical Studies. Optical absorption spectral studies were performed on a Cary Model 14R spectrophotometer with 1 cm path length cuvettes. All experiments were carried out at room temperature (~25 °C) except where noted. For experiments performed at other temperatures, a Cary constant-temperature cell holder connected to a Lauda K2/R water bath was used. Spectra, recorded under anaerobic conditions, were obtained with the use of special Thunberg cuvettes equipped with rubber septum stoppered side arms.

In a typical experiment, the main compartment of the Thunberg cuvette contained 3.0 mL of 1.0 mM Cu(II)–bleomycin in GlyGly buffer (except where indicated). This solution was equilibrated with water-saturated argon for a minimum of 15 min. A Hamilton gas-tight syringe was used to inject a 0.3 M anaerobic solution of a reducing agent via the septum-stoppered side arm of the cuvette.

EPR Studies. All EPR spectra were obtained with a Varian E-12 spectrometer equipped with a Varian E-231 multipurpose cavity. The magnetic field strength and frequency were measured with a Varian NMR gaussmeter and a Systron-Donner frequency counter, respectively. The samples, studied in quartz EPR tubes at 77 K, contained 50% (v/v) glycerol or ethylene glycol so as to ensure good glass formation. For anaerobic samples, transfer was effected by injecting the sample directly into EPR tubes under an atmosphere of purified argon. The magnetic parameters $g_\parallel$, $g_\perp$, and $A_\parallel$ were determined by direct observation of the EPR absorption derivative spectrum (Peisach & Blumberg, 1974).

Degradation of DNA. All reactions were carried out at room temperature in 15 mM phosphate buffer, pH 7.0. Reaction mixtures were prepared anaerobically in septum-stoppered glass test tubes (9 × 75 mm) equipped with a septum-stoppered 0.5-mL side arm. The chemical reduction of Cu(II)–bleomycin was initiated when the contents of the side arm (70 μL) containing 4.3 mM DNA and 4.3 mM CySH were mixed with the contents of the main chamber (236 μL) containing 0.30 mM Cu(II), 0.26 mM bleomycin, and 0.26 mM Fe(II). The tubes were opened at the indicated times and vigorously aerated for 30 s so that the degradation of DNA could commence (Burger et al., 1979b). After an additional 30 s, a 100-μL aliquot was added to 700 μL of a solution containing 1 mM EDTA and 32 mM 2-thiobarbituric acid. The extent of DNA breakage was assessed from the formation of a complex at 90 °C between malondialdehyde-like chromogens (Burger et al., 1980), which are products of DNA degradation, and 2-thiobarbituric acid ($\epsilon_{532} = 1.6 \times 10^5$ M^{-1} cm^{-1}) (Waravdekar & Saslaw, 1959). After the tubes were cooled to room temperature, optical absorptions were measured with a Zeiss PMQ spectrophotometer. None of the components of the reaction mixture interfered with the assay using authentic malondialdehyde standards (Kwon & Watts, 1963).

Calculations. An iterated least-squares analysis of the reaction kinetics was carried out by fitting the data to a first-order equation of the form $Y = A(1 - e^{-B(t-C)})$, where Y is the concentration of the reaction component at time t, A the concentration limit after prolonged incubation, B a rate constant ($t_{1/2} = 0.69/B$), and C the lag preceding the first-order phase of the reaction. The data were also fit to a type I second-order equation (Benson, 1960) of the form $A = (1/t)(1/Y - 1/B)$. Here Y is the concentration at time t, A the rate constant, and B the initial concentration ($t_{1/2} = 1/(AB)$).

Results

Copper Uptake by Bleomycin. Addition of bleomycin to those Cu(II) complexes listed in Table I that are thought to mediate copper transport in serum (Neumann & Sass-Kortsak, 1967; Pickart, 1980) leads to the fast chelation of the copper by drug. This was demonstrated optically and with EPR. When 1.0 mM bleomycin was added to a Cu(II) complex (1.0 mM) of an amino acid, peptide, or albumin, there was a shift in the λ_{max} (Table I) and an increase in absorption characteristic of Cu(II)–bleomycin formation. In an analogous EPR study, Cu(II) chelation by bleomycin was demonstrated from changes in the magnetic parameters $g_\parallel$ and $A_\parallel$ for the Cu(II)–peptide, Cu(II)–amino acid, or Cu(II)–albumin complexes to those characteristic of Cu(II)–bleomycin. In all cases, bleomycin chelated all the Cu(II).

When 1.0 mM bleomycin was added to copper proteins such as ceruloplasmin (0.21 mM), dopamine β-hydroxylase (3.1 μM), or superoxide dismutase (3.1 μM), the EPR spectrum for each of these did not change (Table I), even after incubation for 10 min at 37 °C. This indicates that bleomycin does not chelate tightly bound Cu(II) as found in these copper proteins but does chelate the Cu(II) weakly bound to albumin.

Reduction of Cu(II)–Bleomycin by Thiols: Optical Studies. Attempts were made to study the anaerobic reduction of Cu(II)–bleomycin (1.0 mM) with a 10-fold molar excess of

Table I: EPR and Optical Parameters of Cu(II) Complexes and Cu(II) Proteins

	pH	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ ($cm^{-1} \times 10^{-3}$)	λ_{max} (nm)	$\epsilon(\lambda_{max})$ (M^{-1} cm^{-1})
copper complex						
Cu(II)-$(His)_2$	7.6	2.24	2.06	18.8	625	79.3
Cu(II)-$(Thr)_2$	7.9	2.27	2.06	19.1	615	84.3
His-Cu(II)-Thr	7.9	2.25	2.06	18.9	617	76.2
Cu(II)-Gly-His-Lys	7.5	2.22	2.07	21.8	595	41.0
Cu(II)-GlyGly	8.4	2.26	2.06	17.7	635	5.5
Cu(II)-aquo	5.6	2.36	2.08	15.9	768	29.2
Cu(II)-albumin	7.5	2.19	2.05	19.2	ND[a]	ND
copper proteins						
superoxide dismutase	7.5	2.27	2.06	14.8	680	300[b]
ceruloplasmin [type I Cu(II)]	7.5	2.21	2.06	7.75	610	11300[c]
dopamine β-hydroxylase	7.0	2.27	2.04	18.0	ND	ND
copper bleomycin						
Cu(II)-bleomycin	7.5	2.22	2.06	18.6	603	118.0
RS^--Cu(II)-bleomycin	8.2	2.22	2.06	18.6	595	ND

[a] ND, not determined. [b] After McCord & Fridovich (1969). [c] After Malkin & Malmström (1970).

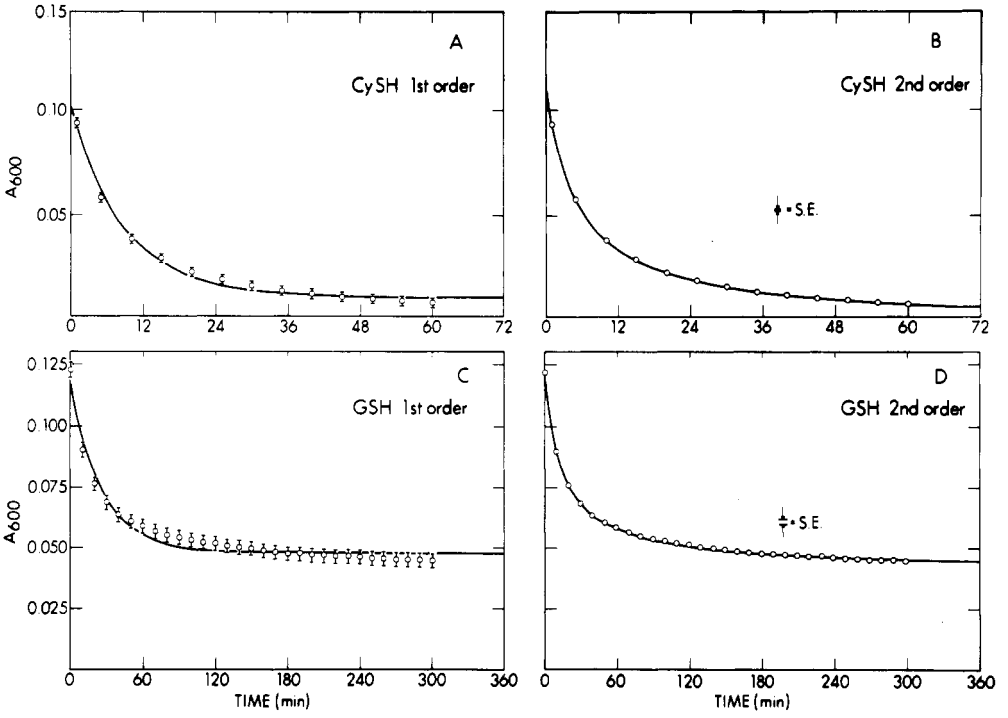

FIGURE 2: Kinetic analysis of Cu(II)-bleomycin reduction by a 2-fold molar excess of thiol-containing physiological reductants. Reaction mixtures contained 1.1 mM bleomycin and 1.0 mM Cu(II) in 0.11 M GlyGly buffer, pH 8.4 in 3.0 mL. Solutions were made anaerobic in septum-stoppered Thunberg cuvettes by equilibration with water-saturated argon. The reactions were initiated when the contents of the side arm, 20 μL of 0.3 M anaerobic CySH (panels A and B) or GSH (panels C and D), were mixed with the Cu(II)-bleomycin solution in the main chamber of the cuvette. The reduction of CySH was studied at 25 °C and of GSH at 37 °C. The optical absorption at 600 nm, A_{600}, as a function of time, was fit either to a first-order rate equation (panels A and C) or a second-order rate equation (panels B and D) as described under Experimental Procedures. The standard errors (S.E.) show the deviation of the data from the computer-generated function. As can be seen, the data are fit better to a second-order rate equation than to a first-order equation. Analogous results were obtained when the molar ratio of reductant to Cu(II)-bleomycin was varied from 1 to 10.

ascorbate, NADH, or NADPH[2] at pH 8.4. Even after 60 min, no reduction of Cu(II) could be recognized optically or magnetically. However, Cu(II)-bleomycin reduction could be effected with thiol-containing reagents (Table II).

When an excess of a thiol such as GSH, CySH, or DTT was added to an anaerobic solution of Cu(II)-bleomycin, there was an immediate shift of λ_{max} from 603 to 595 nm, suggestive of the formation of a ternary thiol-Cu(II)-bleomycin complex. This shift in λ_{max} took place prior to reduction, which was monitored by following the decrease of absorption near 600

[2] The NADH and NADPH experiments were performed in the presence of 0.1 mM phenazine methosulfate.

Table II: Rate of Cu(II)-Bleomycin Reduction[a]

molar equivalents of reducing agent	apparent $t_{1/2}$ (min) of reduction with		
	cysteine	dithiothreitol	glutathione
1	5.9	28.5	
2	6.9	28.8	3200
5	3.1	13.7	280
10	2.2	9.6	80

[a] Anaerobic reaction mixtures contained 1.1 mM bleomycin and 1.0 mM Cu(II) in 3.0 mL of 0.11 M GlyGly buffer, pH 8.4 at 25 °C. Small volumes of 0.3 M reductant were added to achieve the final concentrations. A_{600} was studied as a function of time, and the data were fit to a first-order (DTT) or second-order (CySH; GSH) rate equation in order to compute $t_{1/2}$.

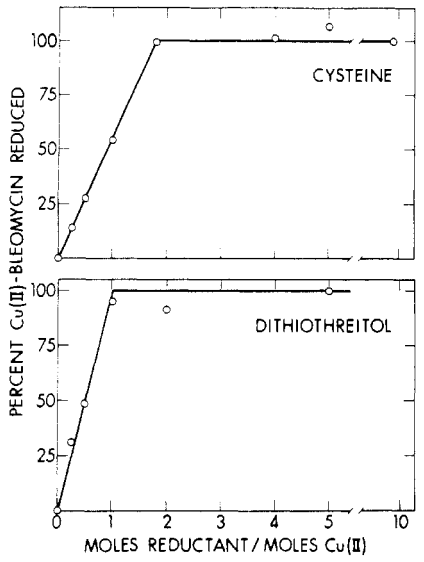

FIGURE 3: Stoichiometry of Cu(II)–bleomycin reduction by cysteine and dithiothreitol. The stoichiometry was determined by titrating Cu(II)–bleomycin with CySH (upper panel) or DTT (lower panel) and allowing the reaction to go to completion as evidenced by constancy of optical absorption at 600 nm. Anaerobic reaction mixtures, prepared in Thunberg cuvettes, contained 1.1 mM bleomycin and 1.0 mM Cu(II) in 0.11 M GlyGly buffer, pH 8.4. The reaction was initated when the reducing agent was mixed with the Cu(II)–bleomycin solution.

nm, leading to the formation of a colorless product.

The reduction of Cu(II)–bleomycin by GSH, CySH, or DTT was examined under a variety of conditions. The molar ratio of thiol to Cu(II)–bleomycin was varied, as were the pH and temperature. Computer-aided analysis (Figure 2) demonstrated that the kinetics of reduction with either GSH or CySH were fit better to a second-order rate equation rather than to a first-order equation. In the former case, the mean weight error was 50–70% smaller. Thus, a kinetic treatment suggested that the chemical reduction of Cu(II)–bleomycin is second order with respect to thiol and further suggested that 2 equiv of thiol is required. This stoichiometry was verified from the material balance study shown in Figure 3. When an equimolar quantity of CySH or GSH was added to Cu(II)–bleomycin, 50% of the Cu(II) was reduced, even after long-term incubation. When 0.5 equiv of CySH was added, only 25% reduction of Cu(II) was seen while the addition of 2 molar equiv of GSH or CySH was required for complete reduction. With DTT, which contains two sulfhydryl groups, 1 molar equiv of DTT was required for the reduction of 1 mol of Cu(II)–bleomycin. These observations demonstrate that 2 equiv of thiol is required for the reduction of 1 equiv of Cu(II)–bleomycin.

Although each of these thiol-containing reducing agents reacted with Cu(II)–bleomycin, they did so differently. For example, the $t_{1/2}$ for the reduction of Cu(II)–bleomycin at 25 °C was 2.2 min with a 10-fold excess of CySH at pH 8.4. With DTT and GSH, under the same conditions, the $t_{1/2}$ was 9.6 and 80 min, respectively (Table II). When the molar ratio of thiol to Cu(II)–bleomycin was decreased, the $t_{1/2}$ of Cu(II) reduction increased (Table II). With a 5-fold molar excess of CySH, the $t_{1/2}$ was 3.1 min. With stoichiometric CySH the $t_{1/2}$ (computed from initial rates) increased to 5.9 min, although in this case, complete reduction of Cu(II) did not take place.

The pH of the system was also shown to have an effect on the rate of reduction. At pH 7.0 there was no detectable reduction of Cu(II)–bleomycin by a 5-fold molar excess of GSH even after 100 min, while with CySH, the $t_{1/2}$ was 13.1 min. At pH 8.4, the $t_{1/2}$ for CySH and GSH was 3.1 and 280 min, respectively.

Temperature also influenced the rates of reduction; as the temperature increased, the $t_{1/2}$ decreased. The activation energies for Cu(II)–bleomycin reduction by CySH, DTT, and GSH, as determined by Arrhenius plots, are 10.5 ± 0.03, 13.3 ± 0.06, and 15.9 ± 0.12 kcal/mol, respectively. The activation energies for the reduction of Cu(II)–bleomycin by thiols are within a range of physiological reactions that can occur spontaneously in vivo (White et al., 1978). We find under a variety of different conditions CySH is a better reductant than DTT, which is in turn better than GSH.

EPR Studies. A 10-fold molar excess of CySH was added to an anaerobic 50% ethylene glycol solution containing 1.0 mM Cu(II)–bleomycin and 55 mM GlyGly buffer, pH 8.4. At various time intervals, samples were removed and the EPR spectrum of each was determined. An analysis of the decrease in amplitude of the EPR feature at $g = 2.06$ attributable to Cu(II)–bleomycin indicated that Cu(II) reduction took place with apparent second-order kinetics and with a $t_{1/2}$ of 1.8 min. During the course of the reduction, the line shape of the spectrum did not change, suggesting that cysteinyl sulfur was not bound equatorially to the Cu(II) (Getz & Silver, 1974; Peisach & Blumberg, 1974) in the ternary Cu(II)–bleomycin–cysteine complex, whose formation was demonstrated optically (Table I).

Reduction of Cu(II)–Bleomycin with Thiol and Subsequent Formation of Fe(II)–Bleomycin. To an anaerobic solution of 1.0 mM Cu(II)–bleomycin in GlyGly buffer, 1.0 mM Fe(II) and 5.0 mM CySH were anaerobically added. The optical spectrum was examined every 4 min. With time the absorption of cysteine–Cu(II)–bleomycin (λ_{max} = 595 nm) decreased concomitant with an increase in absorption attributable to Fe(II)–bleomycin formation (λ_{max} = 476 nm; $t_{1/2}$ = 3.3 min; 25 °C) (Figure 1). The amount of iron bound was the same whether Fe(II) was present during the reduction or added after reduction had taken place. It appears then that Fe(II) is rapidly bound to the drug once the Cu(II) is reduced by thiol.

In another study, Cu(II)–bleomycin was optically titrated with CySH. When the spectrum no longer changed, Fe(II) stoichiometric with the copper was added. The amount of Fe(II) bound by the drug was determined from the absorption at 476 nm. It was found that 2 equiv of CySH was required for the drug to bind all the added Fe(II).

Substitution of Cu(II) for the Fe(II) Bound to Bleomycin. Cu(II) (1.0 mM) was added anaerobically to a solution of 1.0 mM Fe(II)–bleomycin in GlyGly buffer. The absorption of Fe(II)–bleomycin at 476 nm decreased, while the absorption of Cu(II)–bleomycin near 600 nm increased (Figure 1). A kinetic analysis of the optical change at 476 nm was fit to a first-order rate equation, and the $t_{1/2}$ was found to be 0.8 min. Although bleomycin has a greater affinity for Cu(II) than for Fe(II) (Sugiura et al., 1979; Oppenheimer et al., 1981), the exchange time of Cu(II) for Fe(II) already bound to the drug is slow.

Cu(I)–Bleomycin and Thiol Interactions. Cu(I)–bleomycin was prepared anaerobically either by the addition of Cu(I) to the drug or by chemical reduction of Cu(II)–bleomycin with dithionite. Cu(I)–bleomycin is a yellow complex having an end absorption in the ultraviolet (Figure 4A). It is distinct from the colorless product that is formed when Cu(II)–bleomycin is reduced with excess thiol (Figure 4B).

When Cu(I)–bleomycin was treated with CySH, the yellow color decreased. For complete optical bleaching, 2 equiv of

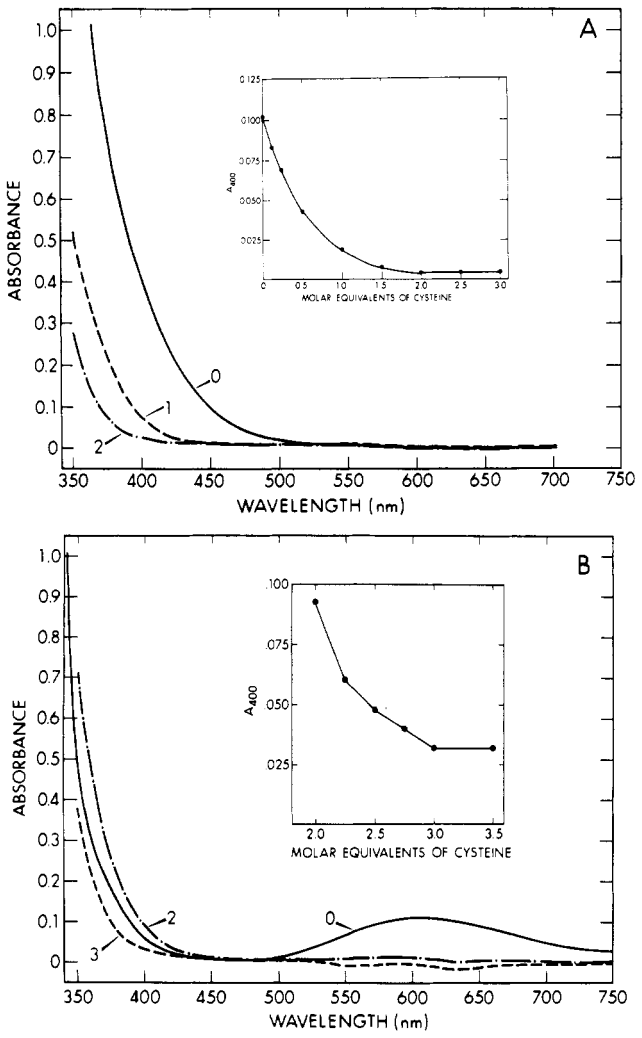

FIGURE 4: Copper-bleomycin and thiol interaction. Anaerobic reaction mixtures contained 1.1 mM bleomycin and either 1.0 mM Cu(I) (A) or 1.0 mM Cu(II) (B) in 0.11 GlyGly buffer, pH 8.4. (A) Optical absorption spectra of Cu(I)-bleomycin in the absence (—) and presence of 1 (--) and 2 (-·-) molar equiv of CySH. The inset plots A_{400} vs. the molar concentration of CySH added to Cu(I)-bleomycin. A total of 2 equiv of CySH was required to achieve complete bleaching of the Cu(I) chromophore. (B) Optical absorption spectra of Cu(II)-bleomycin (—), 90 min after the addition of 2 equiv of CySH (-·-) and after the addition of a third equivalent of CySH (--). The inset shows the titration of the end absorbance (A_{400}) vs. the equivalents of CySH added. Cu(I)-bleomycin requires 2 equiv of CySH to effect a complete spectral change, while Cu(II)-bleomycin requires 3.

CySH was required (Figure 4A). It is suggested that this loss of color represents the removal of copper from the drug. When Cu(II)-bleomycin was reduced with 2 equiv of CySH, the solution lost its blue color, but there was a residual end absorbance in the near-ultraviolet (Figure 4B). When this solution was anaerobically titrated with CySH, 1 additional equiv of thiol was required to abolish the near-ultraviolet absorption (Figure 4B, inset). This suggests that a total of 3 equiv of thiol may be required for the reduction of Cu(II) and its removal from the drug.

Substitutions of Fe(II) for the Cu(I) Bound to Bleomycin. When 1.0 mM Fe(II) was added to a solution containing 1.0 mM Cu(I)-bleomycin prepared from cuprous chloride in 50 mM Hepes buffer, pH 7.0, the yellow color of Cu(I)-bleomycin persisted, confirming the findings of Oppenheimer et al. (1981) that Fe(II) does not completely displace Cu(I) bound to the drug. When this same solution was titrated with CySH, 1 equiv of thiol was required for the formation of Fe(II)-bleomycin as determined from the absorption generated at 476 nm. Excess CySH added at this point had no effect on the complexation of Fe(II) by the drug. The Fe(II)-bleomycin formed from Cu(I)-bleomycin and CySH was optically identical with that formed by direct addition of Fe(II) to the metal-free drug.

Reoxidation. Various paramagnetic species are detectable in these experiments, each with distinct magnetic parameters. Thus, detection of specific compounds can be accomplished by direct observation of the EPR absorption derivative spectrum. At the completion of a 2-h anaerobic incubation at room temperature, a reaction mixture consisting of Cu(II)-bleomycin, excess thiol, and Fe(II) in GlyGly buffer contained no EPR-detectable material. When air was then introduced, a number of paramagnetic species could now be seen. After 5-10-s aeration, three different ferric-bleomycin complexes were noted: low-spin Fe(III)-bleomycin (g = 2.45, 2.18, and 1.89) (Burger et al., 1979b), activated bleomycin (g = 2.26, 2.17, and 1.94) (Burger et al., 1981b; Kuramochi et al., 1981), and RS^{-}-Fe(III)-bleomycin (g = 2.34, 2.20, and 1.94) (Antholine & Petering, 1979). The EPR of Cu(II)-bleomycin only began to appear after longer aeration (~20 s). With extensive aeration, ~2 min, the only EPR-detectable metal-drug complex present was Cu(II)-bleomycin.

DNA Degradation. We have demonstrated that Cu(II)-bleomycin can degrade DNA in the presence of thiols and Fe(II). The reduction of Cu(II)-bleomycin by thiols is difficult to study under aerobic conditions because the Cu(I) product reoxidizes. Yet, the breakage of DNA by Fe(II)-bleomycin does not occur under anaerobic conditions. For these reasons, DNA degradation in the presence of Cu(II)-bleomycin was examined in two steps: the first, the anaerobic reduction of Cu(II)-bleomycin by thiols in the presence of Fe(II) leading to Fe(II)-bleomycin formation and, the second, the rapid aerobic reaction of this complex leading to DNA breakage.

In a reaction mixture containing Fe(II), Cu(II)-bleomycin, and DNA, the addition of a 5-fold molar excess of CySH led to the production of malondialdehyde-like products (Burger et al., 1980) when the vessel was opened to air (Figure 5A). The yield of MDA was directly proportional to the amount of Cu(II)-bleomycin reduced. The apparent $t_{1/2}$ for Cu(II)-bleomycin reduction at pH 7.0 was 15.9 min while the $t_{1/2}$ for MDA production was 18.2 min (Figure 5A). In a plot of the percent Cu(II)-bleomycin reduced vs. the percent of the maximal yield of MDA (Figure 5B), the slope of the line is near unity. This demonstrates that the amount of Cu(II)-bleomycin reduced is directly proportional to the production of MDA from DNA. Calculations show that 1 mol of MDA was formed for every 3.8 mol of Cu(II)-bleomycin reduced. Thus the efficiency of the DNA degradation reaction is approximately 25%. This number correlates well with the yield of MDA produced from DNA reacted with Fe(II)-bleomycin in a single turnover experiment (Burger et al., 1979b). In the absence of Fe(II) or thiol, no MDA was produced from DNA and Cu(II)-bleomycin.

Discussion

It has been proposed that the activity of Fe(II)-bleomycin depends on the generation of "activated bleomycin", the form of the drug kinetically competent with DNA cleavage (Kuramochi et al., 1981; Burger et al., 1981b). In vivo studies have demonstrated that a variety of metal ions including Zn(II), Co(II), and Cu(II) are potent inhibitors of DNA degradation by bleomycin (Sausville et al., 1978b). Since both Cu(II)-bleomycin and metal-free bleomycin are antitumor agents whose in vivo activity is believed to be DNA cleavage, we have studied the interaction of Cu(II)-bleomycin with iron

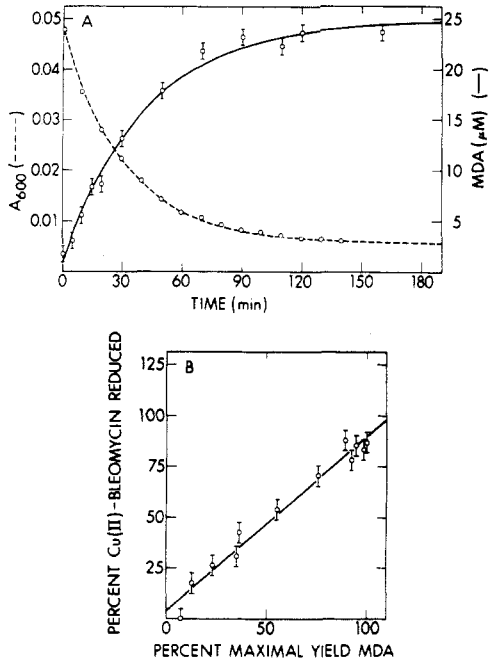

FIGURE 5: Correlation between Cu(II)–bleomycin reduction and DNA degradation. (Panel A) Production of malondialdehyde-like compounds (MDA) (—) was assayed with anaerobic reaction mixtures prepared in septum-stoppered side arm test tubes. The solutions contained 1.0 mM DNA, 0.2 mM bleomycin, 0.2 mM Fe(II), 0.22 mM Cu(II), and 1.0 mM CySH in 15 mM potassium phosphate buffer, pH 7.0. At the times indicated the tubes were opened and aerated for 1 min. The absorbance at 600 nm, A_{600} (---), which is directly proportional to the concentration of Cu(II)–bleomycin, was fit to a second-order rate equation, while the yield of MDA was fit to a first-order rate equation as described under Experimental Procedures. It should be noted that any excess Cu(II) present in the reaction mixture is not bound to the drug [0.22 mM Cu(II):0.2 mM bleomycin] and does not affect the overall reaction. (Panel B) The data presented in panel A were normalized relative to the theoretical limits at infinite time of the amount of Cu(II)–bleomycin reduced and the maximal yield of MDA produced, as determined from the kinetic analysis. Data were fit to a straight-line function of the form $y = Ax + B$. The standard error bars indicate the deviation of the data from this function.

and potential physiological reductants.

In this paper we have also examined the interaction of metal-free bleomycin with various Cu(II) complexes that are thought to be accessible to the drug in the circulation. The Cu(II)–amino acid and Cu(II)–peptide complexes studied, which represent only a few of those present in human serum, are believed to function by mediating Cu(II) and Zn(II) transport into cells (Neumann & Sass-Kortsak, 1967; Harris & Sass-Kortsak, 1967; Hallman et al., 1971; Giroux & Henkin, 1972; Pickart et al., 1980). The addition of metal-free bleomycin to complexes including Cu(II)–Gly-His-Lys, Cu(II)–$(His)_2$, Cu(II)–$(Thr)_2$, His–Cu(II)–Thr, and Cu(II)–albumin leads to the removal and chelation of Cu(II) by the drug. The ability of bleomycin to sequester Cu(II) from amino acid and peptide complexes is due to the stability of the polydentate chelate structure that is formed (Iitaka et al., 1978; Dabrowiak et al., 1978). The reported binding constant of Cu(II) with bleomycin (Sugiura et al., 1979; Solaiman et al., 1979) far exceeds the binding constants of the various amino acid and peptide complexes used in this study (Sharma, 1967; Perrin & Sharma, 1967; Sarkar et al., 1968). Our experiments suggest that when metal-free bleomycin is administered in vivo, it may become bound to Cu(II). The isolation of Cu(II)–bleomycin from the urine of rabbits to which metal-free bleomycin was administered is consistent with this suggestion (Hori et al., 1974).

As Cu(II)–bleomycin does not break DNA in vitro, we examined its conversion to Fe(II)–bleomycin, an active form of the drug. This was accomplished by the interaction of Cu(II)–bleomycin with physiological reductants. Of those studied, only thiols were capable of reducing Cu(II)–bleomycin, permitting Fe(II) to be bound. Although the redox potentials of reduced pyridine nucleotides (−320 mV) are lower than those for CySH or GSH (−230 mV), neither NADH nor NADPH reduces Cu(II)–bleomycin. Thus, the redox potential of the reductant is not the single governing factor in Cu(II)–bleomycin reduction. Glutathione and CySH have a second property, complex formation, which permits the reduction of bleomycin-bound Cu(II) to take place. Although both of these thiol-containing reductants have the same redox potential, CySH reduces bleomycin-bound Cu(II) faster (Table II).

The interaction between thiols and Cu(II)–bleomycin reveals three distinct features: (1) thiol addition to Cu(II)–bleomycin causes an immediate change in the optical absorption spectrum without altering the EPR spectrum; (2) 2 equiv of thiol is required to reduce 1 equiv of Cu(II) bound to the drug; (3) the rate of reduction depends upon the thiol agent used. Under similar conditions of pH, concentration, and temperature, the order of reactivity is CySH > DTT > GSH.

On the basis of our findings, we propose a model for the reduction of Cu(II)–bleomycin by thiol-containing reducing agents:

$$\text{Cu(II)–BLM} + \text{RSH} \rightarrow \text{RS}^{-}\text{–Cu(II)–BLM} + \text{H}^{+} \quad (1)$$

In eq 1 a thiol binds to Cu(II)–bleomycin, altering the optical but not the EPR properties. There is an immediate change in the optical spectrum, shifting λ_{max} from 603 to 595 nm. As the EPR properties of mononuclear Cu(II) complexes depend, for the most part, on the nature of the equatorial ligands and not on the axial ligands (Getz & Silver, 1974; Peisach & Blumberg, 1974), the thiol must react with the Cu(II) axially, thereby affecting the optical properties but not affecting the magnetic properties. The existence of a ternary complex with a thiol has been reported for Fe(III)–bleomycin (Antholine & Petering, 1979).

The reduction of Cu(II)–bleomycin to Cu(I)–bleomycin is the rate-limiting step (eq 2). Our experiments demonstrate

$$\text{RS}^{-}\text{–Cu(II)–BLM} + \text{RSH} \rightarrow \text{RS}^{-}\text{–Cu(I)–BLM} + \text{RS}\cdot + \text{H}^{+} \quad (2)$$

that 2 mol of RSH either supplied by 2 mol of GSH or CySH or by 1 mol of DTT is required to reduce 1 mol of Cu(II)–bleomycin (Figure 3). Each of the two thiols has a different function: first, the formation of a ternary thiol–copper–drug complex and, second, the electron transfer reaction. It is interesting to note that 2 equiv of thiol is also required for the reduction of the Cu(II) complex of 3-ethoxy-2-oxobutyl-aldehyde bis(thiosemicarbazone) (Petering, 1972), a chelator having a high affinity for Cu(II).

Another product of Cu(II)–bleomycin reduction is RS·, a thiol radical. This radical can be terminated as in eq 3 (Kolthoff & Stricks, 1951).

$$\text{RS}\cdot \rightarrow {}^{1}/_{2}\text{RS–SR} \quad (3)$$

Once the RS^{-}–Cu(I)–bleomycin complex is formed as in eq 2, it can react with another thiol molecule

$$\text{RS}^{-}\text{–Cu(I)–BLM} + \text{RSH} \rightarrow \text{Cu(I)–}(^{-}\text{SR})_2 + \text{BLM} + \text{H}^{+} \quad (4)$$

effectively dissociating Cu(I) from the drug (Figure 4). Thus, 3 equiv of thiol is required to reduce and dissociate the metal

ion from Cu(II)–bleomycin, as shown in the overall reaction given in eq 5.

$$\text{Cu(II)–BLM} + 3\text{RSH} \rightarrow \text{Cu(I)–}(^{-}\text{SR})_2 + {}^1/_2\text{RSSR} + \text{BLM} + 3\text{H}^+ \quad (5)$$

Any Fe(II) present is now readily sequestered by metal-free drug:

$$\text{Fe(II)} + \text{BLM} \rightarrow \text{Fe(II)–BLM} \quad (6)$$

If Cu(I)–bleomycin is produced in the absence of thiol, such as by reacting the Cu(II)–bleomycin complex with dithionite

$$\text{Cu(II)–BLM} + 1e^- \rightarrow \text{Cu(I)–BLM} \quad (7a)$$

or by the addition of cuprous chloride to the metal-free drug

$$\text{Cu}^{\text{I}}\text{Cl} + \text{BLM} \rightarrow \text{Cu(I)–BLM} + \text{Cl}^- \quad (7b)$$

yellow, cuprous bleomycin is formed. Fe(II) does not completely displace Cu(I) from this complex, even after long incubation. However, metal-free bleomycin can now be prepared from cuprous bleomycin by the additon of 2 equiv of thiol (Figure 4A)

$$\text{Cu(I)–BLM} + 2\text{RSH} \rightarrow \text{Cu(I)–}(^{-}\text{SR})_2 + \text{BLM} + 2\text{H}^+ \quad (8)$$

and Fe(II), if present, can bind to the metal-free drug (eq 6).

Although Fe(II) does not completely displace Cu(I) bound to the drug, the addition of a single thiol to the cuprous complex permits Fe(II) binding to take place (eq 9).

$$\text{Cu(I)–BLM} + \text{Fe(II)} + \text{RSH} \rightarrow \text{Cu(I)–}^{-}\text{SR} + \text{Fe(II)–BLM} + \text{H}^+ \quad (9)$$

If O_2 is introduced into the reaction mixture, any Cu(I) present will be oxidized to Cu(II) (eq 10) and will ultimately displace any iron bound to the drug (eq 11 and 12).

$$\text{Cu(I)–}(^{-}\text{SR})_2 + \text{O}_2 + 4\text{H}^+ \rightarrow \text{Cu(II)} + \text{RSSR} + 2\text{H}_2\text{O} \quad (10)$$

$$\text{Cu(II)} + \text{Fe(II)–BLM} \rightarrow \text{Cu(II)–BLM} + \text{Fe(II)} \quad (11)$$

$$\text{Cu(II)} + \text{Fe(III)–BLM} \rightarrow \text{Cu(II)–BLM} + \text{Fe(III)} \quad (12)$$

The formation of a disulfide in the presence of Cu(I) and O_2 (eq 10) has been documented (Kolthoff & Stricks, 1951; Stricks & Kolthoff, 1951). The displacement of Fe(II) by Cu(II) (eq 11), as demonstrated in this paper, and the displacement of Fe(III) by Cu(II) (eq 12) (Povirk, 1979) are slow.

In a fast reaction, Fe(II)–bleomycin is activated by O_2 (Kuramochi et al., 1981; Burger et al., 1981b), and if DNA is present, the DNA-cleavage reaction ensues.

$$\text{Fe(II)–BLM} + \text{DNA} \xrightarrow{\text{O}_2} \text{Fe(III)–BLM} + \text{DNA}^* \quad (13)$$

Here DNA* represents degraded DNA (Burger et al., 1980).

The series of reactions presented here have considerable bearing on the probable course of events subsequent to the in vivo administration of bleomycin. Our studies suggest that when the drug is administered, it chelates available Cu(II). As long as Cu(II) is bound, oxidative inactivation as takes place with Fe(II) in the absence of DNA cannot occur (Burger et al., 1980). Therefore, there is a marked advantage for bleomycin to bind Cu(II) in vivo. Once Cu(II)–bleomycin is formed, reducing agents such as cysteine, glutathione, or even possibly cysteinyl residues in a protein such as metallothionein are able to complex and reduce the copper, removing it from the drug. Metal-free bleomycin generated by the above scheme is capable of chelating Fe(II), producing an active form of the drug, which in the presence of O_2 undergoes redox reactions and degrades DNA.

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Size of Acetylcholine Receptors in the Membrane. An Improved Version of the Radiation Inactivation Method†

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ABSTRACT: The radiation inactivation method was used to study the size of acetylcholine receptors in the intact membrane-bound state. This technique was reinvestigated, and modifications were made which remove substantial difficulties affecting previous applications of it to such proteins. The molecular size was deduced here by reference to a set of protein standards: an inactivation ratio was defined relative to a given internal enzyme molecular weight standard, and a linear calibration plot for the inactivation ratios of the protein standards was constructed and applied. The acetylcholine receptor in *Torpedo* electric organ, cat denervated muscle, and chick embryonic muscle was found by this method to exist in the membrane as a homogeneous population of the same size in each case. This receptor, when identified thus as the α-neurotoxin-binding target structure, has an apparent molecular weight of 300 000 or a molecular volume of about 350 nm^3. In comparison, the molecular weight of the cat muscle receptor when solubilized, as analyzed by gel electrophoresis after extensive cross-linking, was found to be 270 000 ± 20 000. These two values are thought to be equivalent by virtue of the situation and structure of the receptor protein in the cell membrane. If a disulfide-bridge dimeric receptor exists in the membrane (as other evidence has indicated for *Torpedo*), each monomer acts independently there in binding α-neurotoxin, since the monomers can be inactivated independently by irradiation in the *Torpedo* membrane. In the muscle membrane no evidence for the existence of receptor dimers, of any kind, has been found.

The molecular size of nicotinic AcCh[1] receptors has hitherto been studied only in detergent solutions. The receptor, as extracted thus from *Torpedo* electric organ, exists as stable monomeric and dimeric molecules, with sedimentation coefficients of about 9 S and 13 S and Stokes' radii of 7 and 9 nm, respectively; when extracted from eel electric organ, it is found only as the smaller of these [for references, see Karlin (1980)]. The *Torpedo* receptor dimer in detergent solution contains those 9S monomers joined by a specific disulfide bridge (Chang & Bock, 1977; Hucho et al., 1978; Hamilton et al., 1977); reduction (e.g., with dithiothreitol) converts the dimers present to monomers. The dimers have been clearly seen in electron microscopy of the 13S fraction (Wise et al., 1981). Reduction of *Torpedo* membranes (Witzemann & Raftery, 1978), and chemical reoxidation thereof (Hamilton et al., 1979), produces (in subsequent analysis in detergent solution) those same monomers and dimers, respectively. The extent or significance of the dimer occurrence in the native membrane is uncertain.

For the related AcCh receptors of mammalian skeletal muscles, only the 9S/7-nm form is found as the native form in detergent solutions (Chiu et al., 1973; Brockes & Hall, 1975; Merlie et al., 1978; Shorr et al., 1981). The treatments with dithiothreitol or *N*-ethylmaleimide which change the size distribution of the *Torpedo* AcCh receptors (Chang & Bock, 1977) do not change this single native size of muscle AcCh receptors (Barnard et al., 1978; Lo et al., 1981).

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[1] Abbreviations: AcCh, acetylcholine; ADH, alcohol dehydrogenase; α-BuTX, α-bungarotoxin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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