INTRACELLULAR REDUCTION OF THE CUPRIC ION OF BLEOMYCIN COPPER COMPLEX AND TRANSFER OF THE CUPROUS ION TO A CELLULAR PROTEIN

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The cupric ion of the bleomycin copper complex has been shown to be reduced and transferred to a cellular protein by the following mechanism:

$$\text{Bleomycin} \sim Cu^{2+} \xrightarrow[X]{} \text{Bleomycin} + Cu^{+}$$

$$Cu^{+} + M \longrightarrow M \sim Cu^{+}$$

The intracellular reducing agents (X) are suggested to be sulfhydryl compounds, because their action is inhibited by N-ethylmaleimide. The active group of the cellular protein (M) that binds with the cuprous ion is suggested to be a sulfhydryl group. The action of the bleomycin copper complex in causing DNA fragmentation in cells can be explained by the mechanism presented in this paper. This mechanism in cells is also supported by the temperature dependency of the action of the bleomycin copper complex on cells.

Bleomycin (BLM) strongly binds with cupric ions and forms an equimolar bleomycin copper complex. Copper-free BLM when injected binds with cupric ions in the blood.[1] Both copper-free BLM and the BLM copper complex inhibit DNA synthesis in cells[2] and the growth of animal and bacterial cells[2,3,4,5,6]. However, the BLM copper complex does not cause scission of single strand DNA *in vitro*[7,8]. The scission of single strand *in vitro* by BLM is inhibited by addition of cupric ions to the reaction mixture.[9,10]

From these facts, it has been assumed that the cupric ion of the BLM copper complex should be removed in cells. In fact, DNA fragmentation occurs in rat ascites hepatoma AH66 cells, which were exposed to the BLM copper complex, almost at the same rate as when caused by copper-free BLM. This action of the BLM copper complex on intact cells was lowered by the addition of cupric sulfate to the medium[11]. The authors found that homogenates of rat ascites hepatoma AH66, AH66F cells and rat liver contained small molecular weight substance that reduce the cupric ions of the BLM copper complex and a protein that binds with cuprous ion[11]. In this paper, we wish to report the mechanism for removal of the cupric ions from the BLM copper complex in animal cells.

Materials and Methods

Chemicals

Copper-free BLM A_2'-c [BLM (−Cu)] and BLM A_2'-c copper complex [BLM (+Cu)] were used for the experiments. The C-terminal amine of A_2'-c is histamine. [Methyl-^{3}H]-thymidine (41 Ci/m mole) was purchased from The Radiochemical Centre, Amersham, neocuproin (2, 9-dimethyl-1, 10-phenanthroline) from Nakarai Chemicals, Ltd., Kyoto, Pronase E (1170 tyrosine units/mg) from Kaken-

kagaku Co., Ltd., Tokyo. BLM and other reagents were dissolved in 10 mM phosphate buffer, pH 7.2.

Preparation of the cytosol fraction of AH66 cells

AH66 cells which were collected from the abdominal cavity of rats (Donryu, female, 8-week-old) inoculated with rat ascites hepatoma AH66, were washed with cold saline and homogenized in 2 volumes of 10 mM phosphate buffer, pH 7.2, by Potter's homogenizer. The supernatant prepared by centrifugation of the homogenate at 105,000 *g* for 60 minutes was used as the cytosol fraction unless otherwise noted.

Determination of cuprous ion and copper

Cuprous ion was determined by a method using neocuproin which is a specific chelating agent for Cu^+. Neocuproin dissolved in dil.HCl was diluted to required concentrations with 10 mM phosphate buffer, pH 7.2 and the pH was adjusted to 7.2 with dil. NaOH. After mixing a neocuproin solution with BLM (+Cu) and cellular fractions, Cu^+ was determined spectrophotometrically at 455 nm (λ max of neocuproin Cu^+ chelate). A mixture without BLM (+Cu) was taken as a blank. Copper contents of eluates from column chromatography were determined by using an atomic absorption spectrophotometer Hitachi 208.

Determination of BLM (−Cu) and BLM (+Cu)

Unless otherwise noted, the reaction mixture which contained BLM (+Cu), cellular fractions and neocuproin, was incubated at 37°C, and shaken successively with equal volumes of isoamylalcohol and chloroform to remove proteins and neocuproin. In order to recover BLM (−Cu) derived from original BLM (+Cu), the water layer was applied to a column (1×16 cm) of CM-Sephadex C-25 equilibrated with 10 mM phosphate buffer (pH 7.2) and the adsorbed BLM (−Cu) and BLM (+Cu) were eluted with a linear gradient of 10 and 50 mM phosphate buffer, pH 7.7. During incubation, BLM (−Cu) was hydrolyzed to desamide-BLM by BLM hydrolase contained in the cytosol, and this desamide-BLM was eluted in the fraction of pH from 7.2 to 7.7. BLM (−Cu) and BLM (+Cu) eluted from a column were determined spectrophotometrically at 290 nm.

Separation of neocuproin Cu^+ chelate and EDTA Cu^{2+} chelate

A mixture containing these chelates in 20% dimethylsulfoxide solution was applied to a column of Sephadex G25 equilibrated with 10 mM phosphate buffer, pH 7.2, containing dimethylsulfoxide at 20%. EDTA Cu^{2+} chelate was eluted by the same solution used for equilibration and thereafter neocuproin Cu^+ chelate was eluted by 10 mM phosphate buffer, pH 7.2, containing dimethylsulfoxide at 50%.

Determination of DNA fragmentation

AH66 cells suspended in EAGLE's MEM medium with 10% calf serum (1×10^5 cells/ml) were incubated with [methyl-^{3}H]-thymidine (0.2 μCi/ml) for about 20 hours at 37°C. The cells were collected, washed and resuspended in the same medium (1×10^5 cells/ml).

For determination of BLM-induced DNA fragmentation, an alkaline elution method described by KOHN *et al* was employed with a slight modification[12]. After exposure to BLM (−Cu) or BLM (+Cu) AH66 cells were filtered onto a 25 mm diameter, 0.8 μm pore size cellulose triacetate filter (Gelman, Metricel GA-4) and washed with 10 ml of cold saline. The cells were lysed on the filter at room temperature with 10 ml of a solution containing 2 M NaCl, 0.02 M Na_3-EDTA, 0.2% sodium N-lauroylsarcosinate and 0.08% sodium deoxycholate at pH 8.2, and washed with 5 ml of 1 mM Na_3-EDTA at a flow rate of 3 ml/min. The filter was then eluted with 0.018 M tetrabutylammonium hydroxide - 0.02M tetrabutylammonium salt of EDTA, pH 12.8, at a flow rate of 1 ml/min. The radioactivity of eluted fractions and the filter were determined by a Packard liquid scintillation spectrometer Tri-Carb. 3380 with 10 ml of triton toluene scintillation fluid.[13]

Results

Production of Cu^+ and BLM (−Cu) from BLM (+Cu) by the Cytosol Fraction of AH66 Cells

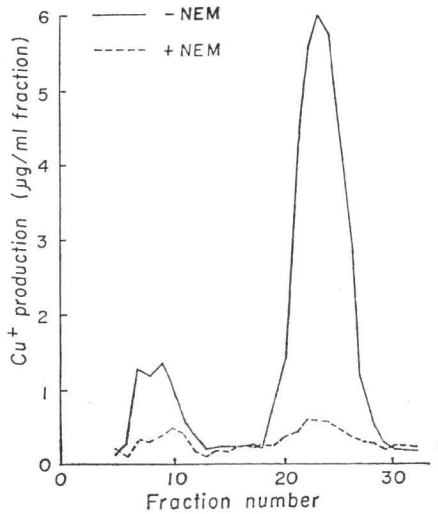
When the cytosol fraction of AH66 cells was incubated with BLM (+Cu) in the presence of neocuproin at 37°C, the production of free Cu^+ was observed: the absorbance at 455 nm characteristic of the

Table 1. Correlation of Cu^+ with BLM (−Cu) produced from BLM (+Cu)

Time (min.)	Cu^+ (μmole)	BLM(−Cu) (μmole)	Ratio of Cu^+ to BLM(−Cu)
22	0.693	0.641	1.09
33	0.793	0.684	1.16

To 10 ml of the cytosol of AH66 cells, 1 ml of BLM (+Cu) solution (3 mg/ml) and 9 ml of 1 mM neocuproin were added for incubation at 37°C. Cuprous ion and BLMs were determined as described in Materials and Methods.

Fig. 1. Sephadex G25 elution profile of the activity contained in the cytosol fraction of AH66 cells to reduce the cupric ion of BLM (+Cu).
The cytosol (58 ml) fraction of AH66 cells was applied to a column (2.64×70 cm) of Sephadex G25 and eluted with 10 mM phosphate buffer, pH 7.2, at 5°C at a flow rate of 38 ml/hour. For the determination of Cu^+ production in the presence or absence of N-ethylmaleimide (shown as NEM in the figure), 0.5 ml of each fraction was pre-incubated with 1 ml of 1 mM N-ethylmaleimide or 10 mM phosphate buffer, pH 7.2, for 30 minutes at 37°C and thereafter 0.1 ml of BLM (+Cu) solution (3 mg/ml) and 0.5 ml of 1 mM neocuproin were added and incubated for 30 minutes at 37°C.



neocuproin Cu^+ chelate gradually increased. The amounts of Cu^+ and BLM (−Cu) produced from BLM (+Cu) were determined and the amounts after 22-minute and 33-minute incubation are shown in Table 1. Cu^+ and BLM (−Cu) were produced in an equimolar ratio (1.09~1.16).

The cytosol fraction of AH66 cells was fractionated by Sephadex G25 column chromatography and Cu^+ from BLM (+Cu) produced by each fraction was determined in the presence of neocuproin. As shown in Fig. 1, the amount of Cu^+ produced by small molecular weight fractions was 4.85 times more than that by high molecular weight fractions during incubation for 30 minutes at 37°C. This production of Cu^+ was inhibited by the addition of N-ethyl-maleimide at 0.66 mM.

The substances eluated by Sephadex G25 column chromatography shown in Fig. 1 were divided into 2 fractions: a high molecular weight fraction (fraction No . 6~15) and a small molecular weight fraction (fraction No. 19~29), and the production of BLM (−Cu) from BLM (+Cu) by these 2 fractions and by a mixture of these fractions were determined. When 3 mg of BLM (+Cu) was incubated with 35 ml of the three fractions for 60 minutes at 37°C, 243 μg, 930 μg of BLM (−Cu) were produced by the high molecular fraction and by the mixture of both fractions, respectively. But no BLM (−Cu) was produced by the small molecular weight fraction alone, although this fraction produced the highest amount of Cu^+ in the presence of neocuproin.

However, when nitrogen gas was bubbled through the incubation mixture to avoid oxidation of Cu^+ produced to Cu^{2+} by oxygen, the small molecular fraction could produce BLM (−Cu) from BLM (+Cu) (Fig. 2).

Cu^+ Bound to the High Molecular Weight Cellular Material

Almost all the copper removed from BLM (+Cu) by the cytosol fraction of AH66 cells was found as its complex with a high molecular weight cellular fraction as measured by an atomic absorption

Fig. 2. Production of BLM(−Cu) from BLM(+Cu) by the small molecular weight fraction from Sephadex G25 column chromatography.
An 1 ml of BLM (+Cu) solution (3 mg/ml) was incubated with 15 ml of the small molecular weight fraction for 30 minutes at 37°C aerobically or anaerobically. The mixture incubated anaerobically was added to a suspension of CM-Sephadex under bubbling N_2 gas and 5 ml of CM-Sephadex which bound BLMs, was overlaid on a column of 30 ml of CM-Sephadex. The mixture incubated aerobically was treated in the same way without bubbling N_2 gas. Bleomycins were eluted as described in Materials and Methods.

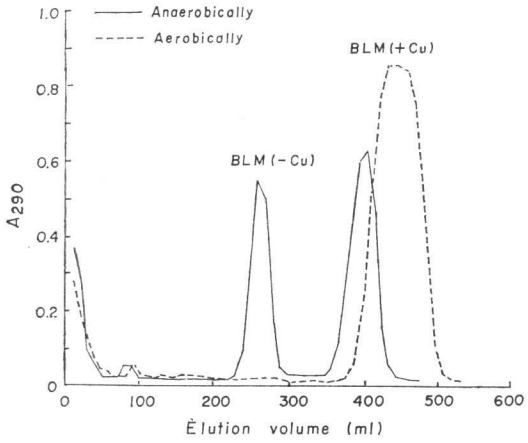
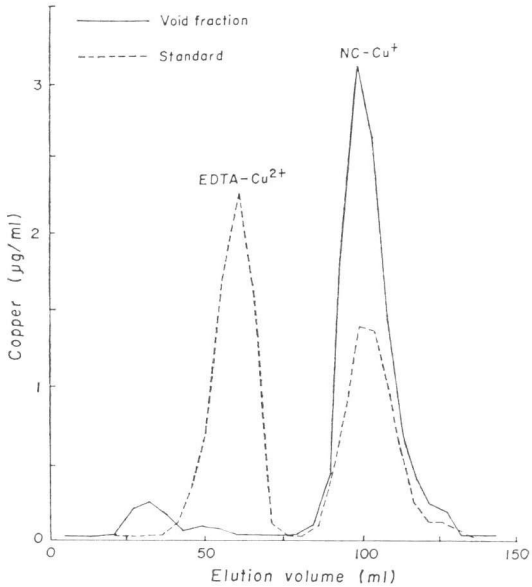


Fig. 3. Sephadex G25 elution profile of EDTA Cu^{2+} chelate and neocuproin Cu^{+} chelate.
A 7 ml of the void fraction through CM-Sephadex chromatography was incubated with 1 ml of 0.1 M N-ethylmaleimide, 2 ml of 50 mM neocuproin (shown as NC in the figure) dimethylsulfoxide solution and 103.6 mg of Pronase E in 10 mM phosphate buffer, pH 7.2, under bubbling N_2 gas for 30 minutes at 0°C and for 2 hours at 37°C succesively. After the incubation, 0.5 ml of 0.2 M Na_4-EDTA was added and the mixture was applied to a column of Sephadex G25 to determine Cu^{+} and Cu^{2+}. In order to prepare a standard solution of neocuproin Cu^{+} chelate and EDTA Cu^{2+} chelate, a mixture of 2 ml of 50 mM neocuproin dimethylsulfoxide solution, 2 ml of cupric sulfate solution (12 μg/ml as Cu^{2+}), and 2 ml of 4 mM ascorbate for the formation of neocuproin Cu^{+} chelate was added to another mixture of 0.5 ml of 0.2 M Na_4-EDTA, 2 ml of cupric sulfate solution (12 μg/ml as Cu^{2+}), and 1.5 ml of 10 mM phosphate buffer, pH 7.2, for the formation of EDTA Cu^{2+} chelate.



spectrophotometer. In order to determine the electronic charge of the copper bound to the high molecular weight cellular material, after the reaction, it was collectd and incubated with Pronase E, N-ethylmaleimide and neocuproin in 20% dimethylsulfoxide solution at 37°C for 2 hours under bubbling N_2 gas and applied to a column of Sephadex G25 after the addition of EDTA. As shown in Fig. 3, 91.8% of the total copper appeared with the peak of the neocuproin Cu^{+} chelate. No peak of the EDTA Cu^{2+} chelate was observed. In other experiments, 85.4% of the total copper was extracted by isoamylalcohol as the neocuproin Cu^{+} chelate, after 3 ml of the high molecular weight fraction was incubated with 2 ml of 2.5 mM neocuproin for 1 hour at 37°C under bubbling N_2 gas.

Proteinaceous Nature of the High Molecular Weight Cellular Fraction Binding the Cu^{+}

When the homogenate of AH66 cells was incubated with BLM (+Cu) in the presence of N-ethylmaleimide, the production of BLM (−Cu) was almost completely inhibited (Fig. 4). The copper removed from BLM (+Cu) was recovered in the void volume after application of the reaction mixtures to columns of CM-Sephadex. The copper was present as a complex with cellular material and was then

Fig. 4. Effect of N-ethylmaleimide on the production of BLM (−Cu) from BLM (+Cu) by the homogenate of AH66 cells.
After 10 ml of the 4 volumes homogenate of AH66 cells was preincubated with 9 ml of 2.2 mM N-ethylmaleimide or 10 mM phosphate buffer, pH 7.2, for 10 minutes at 37°C, 1 ml of BLM (+Cu) solution (3 mg/ml) was added and incubated for 60 minutes at 37°C. The supernatants prepared by 105,000 *g* centrifugation of the mixtures were applied to a column of CM-Sephadex to determine BLMs.

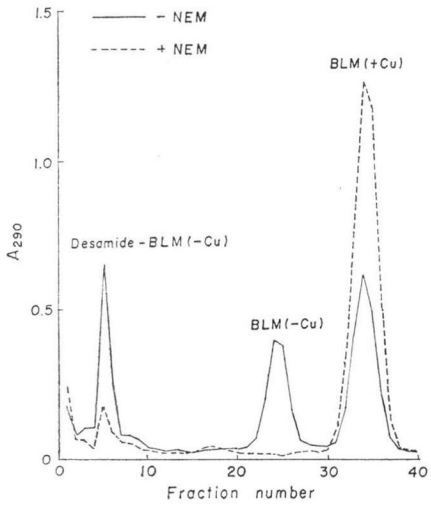
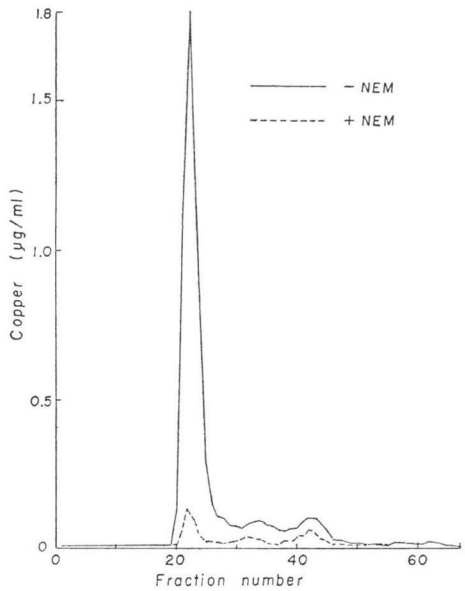


Fig. 5. Sephadex G75 elution profile of copper removed from BLM (+Cu) by the homogenate of AH66 cells.
The void fractions obtained by CM-Sephadex column chromatography described in the legend of Fig. 4 were lyophilized, dissolved in 5 ml of water and applied to a column (2.64×45 cm) of Sephadex G75. They were eluted with 10 mM phosphate buffer, pH 7.8, at 5°C at a flow rate of 15 ml/hour.



chromatographed on columns of Sephadex G75 (Fig. 5). In the experiments with N-ethylmaleimide present no copper was found bound to high molecular weight material.

The production of BLM (−Cu) from BLM (+Cu) by the homogenate of AH66 cells was inhibited also by the pretreatment of the homogenate with Pronase E (Fig. 6).

The void fraction from a column of CM-Sephadex was incubated with EDTA or EDTA and N-ethylmaleimide to detect release of copper from the complex of copper with high molecular weight material. The total released copper was 6.2% in the absence of EDTA and N-ethylmaleimide, 29.2% in the presence of EDTA and 53.0% in that of EDTA and N-ethylmaleimide (Fig. 7). This result suggested that a high molecular weight material with sulfhydryl groups is involved in the binding of Cu^+ derived from BLM (+Cu). Therefore, the high molecular weight fraction of AH66 cells which was pretreated with N-ethylmaleimide and separated from excessive N-ethylmaleimide by Sephadex G25 column chromatography was incubated with BLM (+Cu) and 1 mM cysteine for 30 minutes at 37°C under bubbling N_2 gas to avoid oxidation of cysteine by oxygen. As shown in Fig. 8, the mixture of cysteine and the high molecular weight fraction pretreated with N-ethylmaleimide could not produce BLM (−Cu).

Temperature Dependency of Reduction of Copper and DNA Fragmentation of AH66 Cells Caused by BLMs

The reduction of Cu^{2+} of BLM (+Cu) by the cytosol fraction of AH66 cells, cysteine and gluta-

Fig. 6. Effect of Pronase E on the production of BLM (−Cu) from BLM (+Cu) by the homogenate of AH66 cells.
To inactivate BLM hydrolase, the 4 volumes homogenate of AH66 cells was incubated for 1 minute at 100°C and centrifuged for 40 minutes at 15,000 *g*. After 10 ml of the supernatant was pretreated with 5 ml of Pronase E solution (300 mg) or 5 ml of 10 mM phosphate buffer, pH 7.2, for 60 minutes at 37°C, 5 ml of BLM (+Cu) solution (0.6 mg/ml) was added for incubation of 60 minutes at 37°C. The incubation mixtures were directly applied to a column of CM-Sephadex without isoamylalcohol and chloroform treatment.

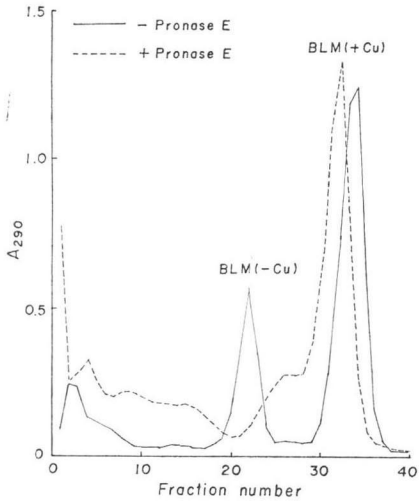
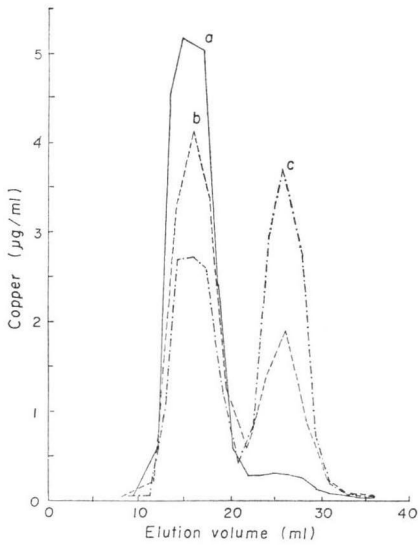


Fig. 7. Sephadex G25 elution profile of copper released from a complex of copper with a high molecular weight material by N-ethylmaleimide. A 4 ml of the void fraction from CM-Sephadex column chromatography was incubated for 30 minutes at 37°C with the following mixtures: (a) 2 ml of 10 mM phosphate buffer, pH 7.2, (b) 2 ml of 30 mM Na_4-EDTA, (c) 1 ml of 60 mM Na_4-EDTA and 1 ml of 60 mM N-ethylmaleimide. In (b) and (c), pH was adjusted to 7.2 by dil.H_3PO_4. After the incubation, these mixtures were applied to a column (1×45 cm) of Sephadex G25 and eluted with 10 mM phosphate buffer, pH 7.2.



thione was temperature dependent as shown in Fig. 9. The amount of Cu^+ produced by the cytosol at 1°C was 15.4% of that at 37°C for 30 minutes. Cysteine reduced Cu^{2+} of BLM (+Cu) 20 times more than glutathione did during 30 minutes at 37°C.

From the temperature dependency of the reduction, it was presumed that BLM (+Cu) would not cause fragmentation of the intracellular DNA of AH66 cells at 0°C. As shown in Fig. 10, when AH66 cells were incubated with 500 μg/ml of BLM (−Cu) or BLM (+Cu) for 30 minutes at 0°C, BLM (−Cu) caused DNA fragmentation, but BLM (+Cu) did not and the same elution profile of DNA as that of the control was obtained. On the other hand, when AH66 cells, which were treated with BLMs at 0°C as described above, were washed with the medium and incubated again in the medium without BLMs for 30 minutes at 37°C, BLM (+Cu) could cause DNA fragmentation at the almost same rate as BLM (−Cu) did at 0°C.

Discussion

It has been shown that the cytosol fraction of AH66 cells removes copper from BLM (+Cu) by the following mechanism:

$$\mathrm{BLM}\sim\mathrm{Cu}^{2+} \xrightarrow[X]{} \mathrm{BLM}+\mathrm{Cu}^{+} \quad (1)$$

$$\mathrm{Cu}^{+} + \mathrm{M} \longrightarrow \mathrm{M}\sim\mathrm{Cu}^{+} \quad (2)$$

The reaction of removal of copper from BLM (+Cu) is initiated by reduction of Cu^{2+} bound to BLM to Cu^{+}. This reduction is caused by intracellular reductants shown as X in the equation (1). This process could be shown by the result that BLM (−Cu) and Cu^{+} were produced in an equimolar ratio when neocuproin was added to fix Cu^{+} (Table 1).

The agents (X) were mainly separated into a small molecular weight fraction by Sephadex G25 column chromatography and reduction of Cu^{2+} bound to BLM was inhibited 93.4% by N-ethylmaleimide (Fig. 1). This result suggests that the cellular reducing agents (X) are sulfhydryl compounds such as cysteine and glutathione. Which sulfhydryl compound is the main reductant for Cu^{2+} bound to BLM, remains to be proven, but it was found that cysteine had about 20 times stronger activity than reduced glutathione to reduce Cu^{2+} bound to BLM (Fig. 9). Cupric ion bound to BLM was also reduced well by ascorbate, NADH and NADPH. Therefore, the slight production of Cu^{+} seen in the presence of N-ethylmaleimide may be attributed to such reducing agents.

Fig. 8. Effect of N-ethylmaleimide treatment of a high molecular weight material on the production of BLM (−Cu) from BLM (+Cu).
After the incubation of the cytosol fraction (20 ml) of AH66 cells with or without 1 ml of 0.21 M N-ethylmaleimide for 15 minutes at 37°C, they were separated from excessive N-ethylmaleimide by Sephadex G25 column chromatography. Their 16 ml were incubated with 1 ml of BLM (+Cu) solution (3 mg/ml) and 3 ml of 6.7 mM cystein for 30 minutes at 37°C under bubbling N_2 gas.

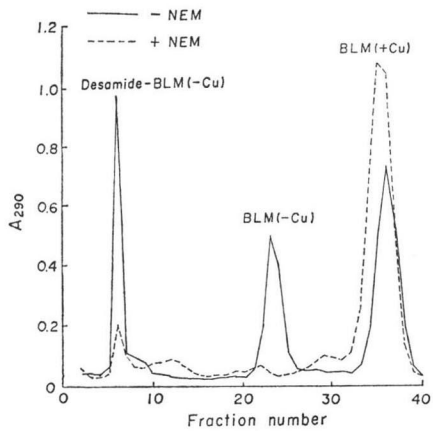


Fig. 9. Temperature dependency of reduction of cupric ion bound to BLM by cellular reducing agents.
For the production of Cu^{+}, 1 ml of 1 mM neocuproin and 1 ml of BLM (+Cu) solution (0.3 mg/ml) were incubated with 1 ml of reducing agents: the cytosol fraction of AH66 cells, 1 mM cysteine or 1 mM reduced glutathione, for 30 minutes at 1, 12, 21, 30, 37 and 47°C.

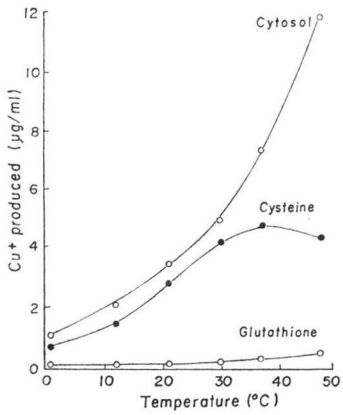
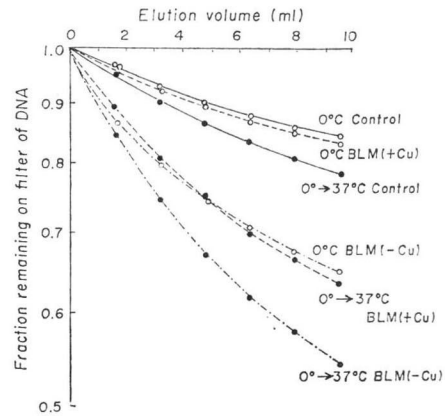


Fig. 10. Temperature dependency of DNA fragmentation caused by BLM(−Cu) and BLM(+Cu).
In order to investigate temperature dependency of BLM induced DNA fragmentation, 2 kinds of incubation were carried out: (1) A 2 ml of AH66 cells' suspension (1×10^{5} cells/ml) was incubated with 0.1 ml of BLM (−Cu) or BLM (+Cu) solution (10.5 mg/ml) for 30 minutes at 0°C. (2) After the same incubation as described in (1), the cells were washed 2 times with 5 ml of cold medium by centrifugation, and thereafter incubated again in fresh medium without BLMs for 30 minutes at 37°C. The fragmentation of DNA was determined by the alkaline elution as described in Materials and Methods.



A high molecular weight fraction from Sephadex G25 column chromatography also produced Cu^+ from BLM (+Cu), though the amount was about one fifth of that produced by the small molecular weight fraction (Fig. 1). This reduction may be caused by proteins with sulfhydryl groups, because it was inhibited by N-ethylmaleimide.

The small molecular weight fraction from Sephadex G25 column chromatography could not produce BLM (−Cu) from BLM (+Cu) under aerobic conditions, but it could produce BLM (−Cu) under-anaerobic conditions (Fig. 2). This result shows that Cu^+ is easily oxidized by oxygen to Cu^{2+} which readily binds to BLM (−Cu). Therefore, Cu^+ produced by the reducing agents (X) must be bound by cellular materials (M) as shown in equation (2).

The fact that the copper bound to the cellular material was Cu^+ (Fig. 3), showed that this Cu^+ reductively removed from BLM (+Cu) was caught without oxidation by the cellular material (M).

A complex of copper removed from BLM (+Cu) with the cellular material was separated as a void fraction in CM-Sephadex column chromatography and this complex was eluted at the void volume in Sephadex G75 column chromatography (Fig. 5). Pretreatment of the homogenate of AH66 cells with Pronase E inhibited the production of BLM (−Cu) from BLM (+Cu) (Fig. 6). These results suggest that the cellular material which binds Cu^+, is a protein.

WINGE *et al.* reported that *p*-chloromercuribenzoate caused release of Cu^{2+} from a protein: named as copper-chelatin in which sulfhydryl groups serve as ligands for copper.[14] Similarly, N-ethylmaleimide caused release of copper from a complex of Cu^+ with the cellular material (Fig. 7). Pretreatment of the high molecular weight fraction from Sephadex G25 column chromatography with N-ethylmaleimide inhibited the production of BLM (−Cu) (Fig. 8). Therefore, sulfhydryl groups of a protein may be necessary to bind Cu^+.

That the mechanism of copper removal is acting in mammalian cells, is supported by the temperature dependency of DNA fragmentation caused by BLM (+Cu) (Fig. 10). EHRLICH ascites carcinoma cells take up BLM at 0°C at about a third lower rate than at 37°C[15]. Therefore, AH66 cells were treated at 0°C with BLMs in high enough concentration to determine fragmentation of DNA by the alkaline elution method. BLM (−Cu) could cause fragmentation of DNA at 0°C, because BLM (−Cu) causes single strand scission of DNA almost independently of temperature[10]. But BLM (+Cu) could not degrade DNA at 0°C, because reduction of Cu^{2+} bound to BLM, which is the first step of the mechanism of copper removal, was dependent on temperature (Fig. 9). When the temperature of incubation was shifted up to 37°C after BLMs had been removed from the medium by washing the cells, BLM (+Cu) could cause fragmentation of DNA, because Cu^{2+} of BLM (+Cu) which had been taken up by the cells at 0°C, was reduced to Cu^+ at 37°C.

From the results shown above, it is suggested that, when BLM (−Cu) are injected, they bind partially or completely with Cu^{2+} in the blood as reported by KANAO *et al.*,[1] and that BLM (+Cu) which are formed in the blood and taken up by cells, show their anti-tumor activity as BLM (−Cu) by the mechanism of copper removal.

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