DNA STRAND SCISSION INDUCED BY ADRIAMYCIN AND ACLACINOMYCIN A

AKIRA SOMEYA and NOBUO TANAKA

Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan

(Received for publication April 17, 1979)

The binding of adriamycin and aclacinomycin A with PM2 DNA, and the consequent cleavage of DNA have been demonstrated by agarose gel electrophoresis, using an ethidium bromide assay. Adriamycin was observed to induce a single strand scission of DNA in the presence of a reducing agent, but aclacinomycin A caused much less degree of DNA breaks. The DNA cleavage was enhanced by Cu^{2+} and Fe^{2+}, but not significantly by Ni^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, suggesting that reduction and auto-oxidation of the quinone moiety and H_2O_2 production participate in the DNA-cutting effect. The DNA degradation was dependent upon concentrations of the anthracyclines and $CuCl_2$. The degree of DNA cleavage at 0.04 mM adriamycin was similar to that at 0.4 mM aclacinomycin A in the presence of 1 mM NADPH and 0.4 mM $CuCl_2$. DNA was degraded to small fragments at 0.4 mM adriamycin and 0.2 mM $CuCl_2$. The anthracycline-induced DNA cleavage was stimulated by H_2O_2, but partially inhibited by potassium iodide, superoxide dismutase, catalase and nitrogen gas atmosphere. The results suggested that both free radical of anthracycline quinones and hydroxyl radical directly react with DNA strands.

We have studied the mechanism of action of aclacinomycin A, a new anthracycline antibiotic, and found that the drug binds to DNA and disturbs the template activity for RNA and DNA polymerases[1,2]. The chemoreceptor of anthracycline antibiotics seems to be DNA, and the interaction with DNA may lead to the inhibition of nucleic acid synthesis [*cf.* a review[3]]. Recently, adriamycin and daunorubicin have been reported to induce DNA strand scission *in vivo*[4,5] and *in vitro* in the presence of reducing agents[6]. LOWN *et al.*[6] have assumed that the DNA cleavage is induced by attack of hydroxyl radical, which is produced by reduction and auto-oxidation of the quinone moiety of the anthracyclines.

We have compared the DNA-cleaving activity of aclacinomycin A with that of adriamycin in the presence of reducing agents, $CuCl_2$, and/or other agents. From the current experiments we suggest that free radical of anthracycline quinone as well as hydroxyl radical attack DNA strands.

Materials and Methods

Adriamycin was generously given us by Kyowa Hakko Kogyo Co., Ltd., and aclacinomycin A by Sanraku-Ocean Co., Ltd. Superoxide dismutase and catalase were products of Sigma Chemical Co. PM2 DNA and NADPH were purchased from Boehringer Mannheim.

DNA cleavage and gel electrophoresis:

The reaction mixture contained 20 μg/ml PM2 DNA in 1 mM $NaBH_4$ and 50 mM Tris-HCl, pH 7.4 ($NaBH_4$-Tris buffer) or in 1 mM NADPH and 20 mM Tris-HCl, pH 7.4 (NADPH-Tris buffer). It was incubated at 30°C for 30 minutes in the presence or absence of anthracycline antibiotics, metal ions and/or other reagents, as indicated. Bromophenol blue (final 0.01%) and sucrose (final 20%) were added to the mixture, to give a final sample volume of 70~90 μl.

Agarose (0.7%) gel electrophoresis followed the procedure of HELLING *et al.*[8] The gels were ex-

truded into 0.5 or 1.0 μg/ml ethidium bromide solution, and the stained bands were visualized on Manasulu-Light. They were photographed with Polaroid type 667, using a red filter (Kodak No. 23A Wratten gelatin filter). In some cases, the gels were stained by methylene blue by the method of PEACOCK and DINGMAN[9].

Results

Interaction of Anthracycline Antibiotics with DNA

As illustrated in Fig. 1A, PM2 DNA showed 3 bands on agarose gel electrophoresis. According to AAIJ and BORST[10], the fastest moving band corresponded to the native form of covalently closed circular (ccc) DNA, the most slowly moving one the open circular form, and the intermediate the linear form.

The treatment of PM2 DNA with adriamycin gave more slowly migrating and broader bands of all the 3 forms of DNA, indicating the complex formation of the antibiotic with the 3 types of DNA (Fig. 1B). Fluorescence of ethidium bromide, staining DNA, became weaker, probably because adriamycin, intercalating the double strand DNA, interfered with the binding of ethidium bromide.

In the presence of a reducing agent, $NaBH_4$, the same treatment gave more intensive and broader band of open circular form DNA, indicating that adriamycin caused a single strand scission of ccc DNA (Fig. 1C).

Under the same conditions, aclacinomycin A did not significantly alter the migration rates of the 3 forms of PM2 DNA, and gave less amounts of open circular form DNA in the presence of $NaBH_4$, indicating that less degree of a single strand break was induced by aclacinomycin A than by adriamycin (Fig. 1D).

Fig. 1. Agarose (0.7%) gel electrophoresis pattern of PM2 DNA and mobility changes of DNA induced by anthracyclines in the absence or presence of $NaBH_4$.

The buffer used was $NaBH_4$-Tris buffer.

A : Control.
B : +0.4 mM adriamycin. $NaBH_4$ was omitted.
C : +0.4 mM adriamycin.
D : +0.4 mM aclacinomycin A.

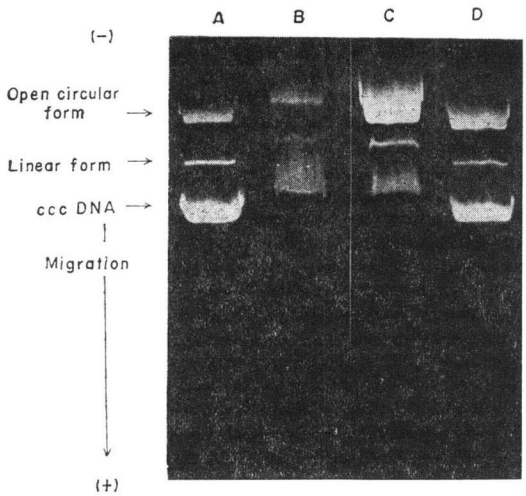
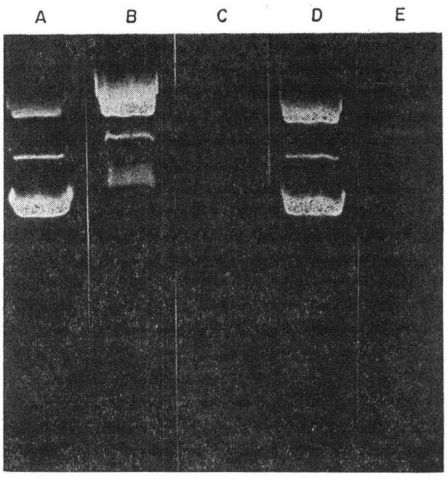


Fig. 2. The effect of $CuCl_2$ on the anthracycline-induced DNA cleavage.

$NaBH_4$-Tris buffer.

A : Control.
B : +0.4 mM adriamycin.
C : +0.4 mM adriamycin and 0.4 mM $CuCl_2$.
D : +0.4 mM aclacinomycin A.
E : +0.4 mM aclacinomycin A and 0.4 mM $CuCl_2$.



The Effect of Metal Ions on Anthracycline-induced DNA Cleavage

CONE *et al.*[7] have shown the important role of divalent metal ions in complexing with the reduced quinone moiety of streptonigrin for the auto-oxidation process. Since anthracycline antibiotics also contain quinone groups in the molecules, an attempt was made to correlate the metal ion efficiency for auto-oxidation of the anthracyclines and H_2O_2 production with the DNA-cleaving ability of adriamycin and aclacinomycin A.

Of metal ions tested, ferrous ion (Fe^{2+}) were found to be the most potent in stimulating degradation of DNA. However, the effect of the anthracyclines was ambiguous in the reaction mixture containing Fe^{2+}, because Fe^{2+} with reducing agents led to degradation of DNA at metal concentration of 1.0 mM even in the absence of the anthracyclines. On the contrary, cupric ion (Cu^{2+}) with reducing agents did not cleave DNA to a significant extent in the absence of the antibiotics. Fig. 2 shows the enhancement of DNA degradation by $CuCl_2$ in the presence of the anthracyclines and $NaBH_4$. Adriamycin was observed to cause DNA cleavage more markedly than aclacinomycin A in the absence of $CuCl_2$. Both drugs induced a similar, strong degradation of DNA in the presence of $CuCl_2$; *i.e.* little or faint fluorescence of DNA bands was detected by ethidium bromide assay on the gels, indicating that DNA was degraded to small fragments. Ni^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+} did not significantly stimulate the anthracycline-induced cleavage of DNA.

Dependency of DNA Degradation on Anthracycline and $CuCl_2$ Concentrations

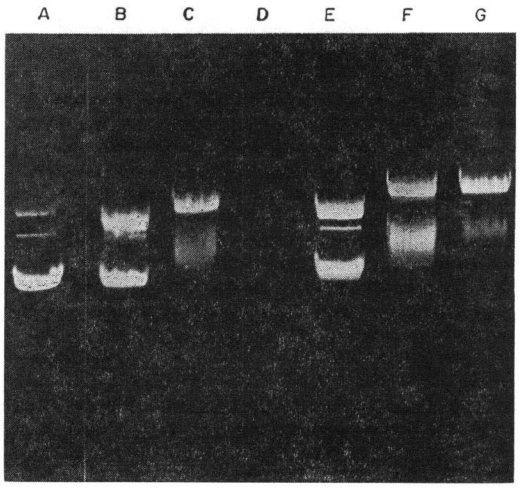
PM2 DNA was treated with various concentrations of adriamycin or aclacinomycin A in the presence of NADPH and $CuCl_2$. The migration rates were dependent upon drug concentrations in a range of 0.004~0.4 mM adriamycin (Fig. 3B~D). DNA seemed to be completely degraded to small fragments at 0.4 mM adriamycin, and no DNA bands were detected by ethidium bromide assay (Fig. 3D) as well as by methylene blue staining (data are not shown). On the other hand, at corresponding concentrations of aclacinomycin A, the extent of DNA cleavage was distinctly smaller (Fig. 3E~G). Densitometric measurements showed that the degree of DNA strand scission at 0.04 mM adriamycin (Fig. 3C) was similar to that at 0.4 mM aclacinomycin A (Fig. 3G).

The extent of DNA degradation by adriamycin was observed to increase with increasing concentrations of $CuCl_2$, reaching a plateau at a molar ratio of 2:1 (the drug: $CuCl_2$) in $NaBH_4$-Tris buffer (Fig. 4).

Fig. 3. Antibiotic concentration dependency of PM2 DNA degradation in the presence of NADPH and $CuCl_2$.

The assay was performed in NADPH-Tris buffer containing 0.4 mM $CuCl_2$.

A : Control.
B : +0.004 mM adriamycin.
C : +0.04 mM adriamycin.
D : +0.4 mM adriamycin.
E : +0.004 mM aclacinomycin A.
F : +0.04 mM aclacinomycin A.
G : +0.4 mM aclacinomycin A.



Effects of Hydrogen Peroxide, Potassium Iodide, Superoxide Dismutase, Catalase, and Nitrogen-gas Atmosphere on Anthracycline-induced DNA Cleavage

The addition of hydrogen peroxide (H_2O_2) to the reaction mixture stimulated DNA cleavage induced by adriamycin and $CuCl_2$, even at low concentrations of H_2O_2, which themselves did not significantly cause DNA degradation (Fig. 5C, D). Potassium iodide (KI), a hydroxyl radical ($OH^{\cdot}$) scavenger, partially blocked DNA degradation induced by aclacinomycin A and $CuCl_2$ (Fig. 6C, D). The results appeared to support the assumption[6] that anthracycline-induced strand scission of DNA may result from the hydroxyl radical ($OH^{\cdot}$) attacking on DNA.

DNA cleavage, induced by adriamycin or aclacinomycin A in the presence of a reducing agent and $CuCl_2$, was partially but not completely reversed by superoxide dismutase or catalase (Fig. 7C~H). The effect of both anthracyclines on DNA was partially restored under nitrogen gas (N_2) atmosphere (Fig. 8D, E). The results indicated that superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), producing hydroxyl radical ($OH^{\cdot}$), may partially participate in DNA strand scission, but the free quinone radical may also directly react with DNA and induce DNA cleavage.

Fig. 4. $CuCl_2$ concentration dependency of PM2 DNA cleavage induced by adriamycin.

The reaction was assayed in $NaBH_4$-Tris buffer containing 0.4 mM adriamycin.

A : +0.05 mM $CuCl_2$.
B : +0.1 mM $CuCl_2$.
C : +0.2 mM $CuCl_2$.
D : +0.4 mM $CuCl_2$.
E : +1.0 mM $CuCl_2$.

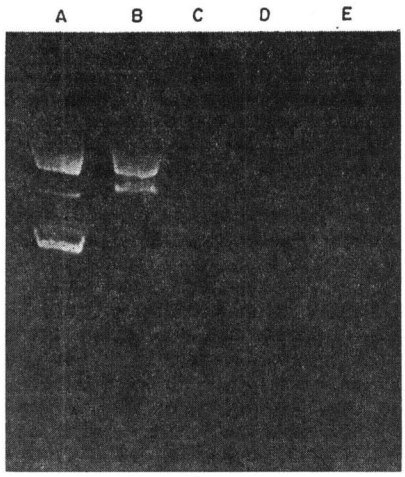
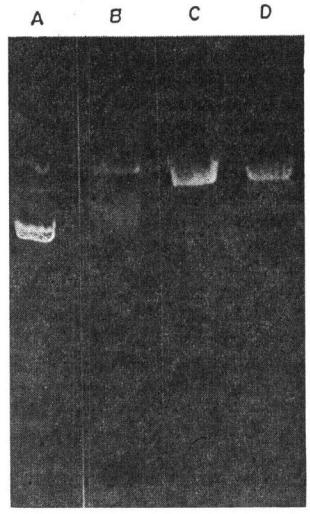


Fig. 5. Stimulation of PM2 DNA strand scission by hydrogen peroxide. NADPH-Tris buffer.

A : Control.
B : +0.05 mM adriamycin and 0.4 mM $CuCl_2$.
C : +0.05 mM adriamycin, 0.4 mM $CuCl_2$, and 0.3 mM H_2O_2.
D : +0.05 mM adriamycin, 0.4 mM $CuCl_2$, and 0.6 mM H_2O_2.



Discussion

In the current experiments, DNA strand scission by anthracycline antibiotics has been demonstrated by agarose gel electrophoresis, which is a simple and sensitive method for determining conformational alteration and degradation of DNA. Reduced mobilities of DNA in the gels may be due to strand breakage, conformational changes (including super-helical relaxation), and/or alteration of electrostatic characteristics of DNA, caused by the bound antibiotics. It remains to be determined whether the reduced migration rate is also attributed to dimer or polymer formation of DNA strands

Fig. 6. Partial inhibition of the anthracycline-induced DNA degradation by potassium iodide (KI).

$NaBH_4$-Tris buffer.

A : Control. $NaBH_4$ was omitted.

B : +0.4 mM aclacinomycin A and 0.4 mM $CuCl_2$.

C : +0.4 mM aclacinomycin A, 0.4 mM $CuCl_2$, and 4 mM KI.

D : +0.4 mM aclacinomycin A, 0.4 mM $CuCl_2$, and 8 mM KI.

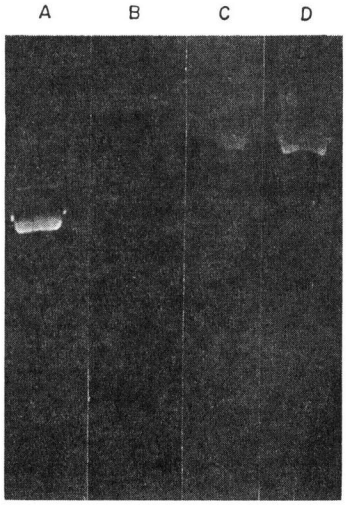


Fig. 8. Partial inhibition of the anthracycline-induced DNA degradation under nitrogen gas atmosphere.

The assay was performed in the reaction mixture containing 0.4 mM $CuCl_2$ in $NaBH_4$-Tris buffer. The mixtures were bubbled by nitrogen gas, and the test tubes were filled with nitrogen gas and sealed with stoppers in lanes D and E.

A : Control. $NaBH_4$ was omitted.

B : +0.4 mM aclacinomycin A.

C : +0.4 mM adriamycin.

D : +0.4 mM aclacinomycin A. Nitrogen gas atmosphere.

E : +0.4 mM adriamycin. Nitrogen gas atmosphere.

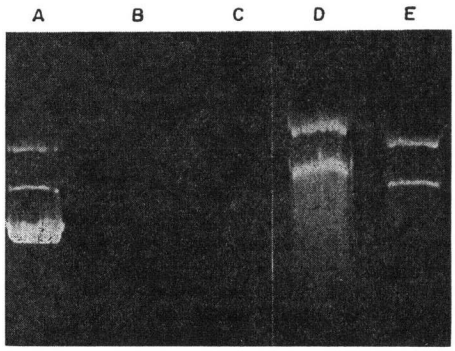
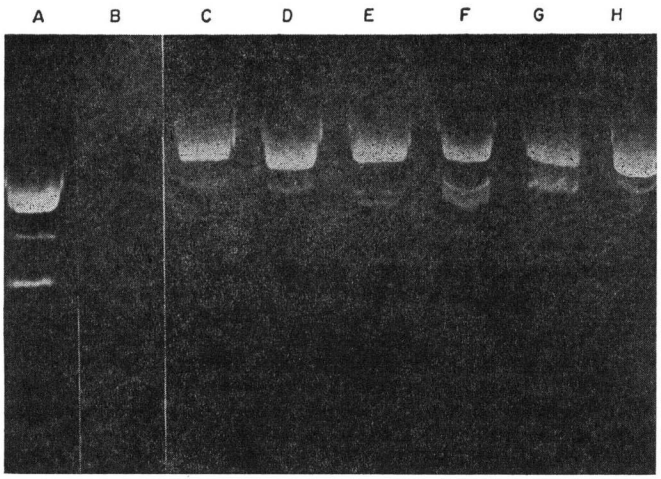


Fig. 7. Partial block of the anthracycline-induced DNA cleavage by catalase and superoxide dismutase (SOD).

The reaction mixture contained 0.4 mM $CuCl_2$ in $NaBH_4$-Tris buffer.



A: Control.

B: +0.4 mM adriamycin.

C: +0.4 mM adriamycin, 0.2 mg/ml catalase and 0.1 mg/ml SOD.

D: +0.4 mM aclacinomycin A, 0.2 mg/ml catalase, and 0.1 mg/ml SOD.

E: +0.4 mM adriamycin and 0.2 mg/ml catalase.

F: +0.4 mM aclacinomycin A and 0.2 mg/ml catalase.

G: +0.4 mM adriamycin and 0.1 mg/ml SOD.

H: +0.4 mM aclacinomycin A and 0.1 mg/ml SOD.

by cross-links with the antibiotics.

Adriamycin shows more marked capacity for inducing DNA cleavage than aclacinomycin A. The results seem to be in accordance with the report of UMEZAWA *et al.*[12] that adriamycin is a potent mutagen but aclacinomycin A is not.

The mechanism of DNA cleavage by anthracycline antibiotics is still not certain. Fe^{2+} and Cu^{2+} have been observed to enhance the anthracycline-induced DNA strand scission in the current experiments, but Cu^{2+} may change to Cu^{+} in the presence of reducing agents. Anthracycline drugs form chelates with many cations, suggesting a possibility of coordination of O_2 molecule for metal chelate compounds and increase of efficiency of auto-oxidation and H_2O_2 production[7,14]. The observed effects of H_2O_2, KI, superoxide dismutase, catalase, and N_2 atmosphere on the anthracycline-induced DNA cleavage appear to support the following hypothesis (HABER-WEISS reaction), which has been also suggested by CONE *et al.*[7] with streptonigrin and by LOWN *et al.*[8] with adriamycin and daunorubicin.

(1) $A+2H \longrightarrow AH_2$
(2) $AH_2+O_2 \longrightarrow AH^{\cdot}+HO_2^{\cdot}$ (2′) $AH^{\cdot}+DNA \longrightarrow$ strand scission
(3) $HO_2^{\cdot} \longrightarrow H^{+}+O_2^{-}$
(4) $2O_2^{-}+2H^{+} \longrightarrow H_2O_2+O_2$
(5) $2H_2O_2 \longrightarrow 2H_2O+O_2$
(6) $O_2^{-}+H_2O_2 \longrightarrow OH^{\cdot}+OH^{-}+O_2$ (6′) $OH^{\cdot}+DNA \longrightarrow$ strand scission

The current results suggest that both the free radical of anthracycline quinone ($AH^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$) may be generated by the reduction and auto-oxidation of the anthracycline quinone, and react with DNA. LOWN *et al.*[6] have concluded that DNA is attacked by the hydroxyl radical, but have not specified the role of free radical form of anthracyclines. The discrepancy of their results and ours seem to be derived from difference of sensitivity of the assay systems employed. The analysis with electrophoresis is more sensitive than the fluorescence assay[6]. BACHUR *et al.*[11] have proposed a general mechanism for microsomal activation of quinone antitumor agents to free radicals which possess high affinity for nucleic acid, protein and lipid.

The free radical form of anthracyclines and hydroxyl radical may also participate in the interaction with actin and heavy meromyosin[13].

Acknowledgements

The current work was supported by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. HAMAO UMEZAWA, Institute of Microbial Chemistry, for his kind advice and cooperations throughout the present experiments.

References

1) YAMAKI, H.; H. SUZUKI, T. NISHIMURA & N. TANAKA: Mechanism of action of aclacinomycin A. I. The effect on macromolecular syntheses. J. Antibiotics 31: 1149～1154, 1978
2) MISUMI, M.; H. YAMAKI, T. AKIYAMA & N. TANAKA: Mechanism of action of aclacinomycin A. II. The interaction with DNA and with tubulin. J. Antibiotics 32: 48～52, 1979
3) DI MARCO, A.; F. ARCAMONE & F. ZUNINO: Daunomycin (daunorubicin) and adriamycin and structural analogues: Biological activity and mechanism of action. Antibiotics. III. Mechanism of action of antimicrobial and antitumor agents. pp. 101～128, Springer-Verlag, Berlin-Heidelberg-New York, 1974
4) SCHWARTZ, H. S.: DNA breaks in P-288 tumor cells in mice after treatment with daunorubicin and adriamycin. Res. Commun. Chem. Pathol. Pharmacol. 10: 51～64, 1975
5) ROSS, W. E.; D. L. GLAUBIGER & K. W. KOHN: Protein-associated DNA breaks in cells treated with adriamycin or ellipticine. Biochim. Biophys. Acta 519: 23～30, 1978
6) LOWN, J. W.; S.-K. SIM, K. C. MAJUMDAR & R.-Y. CHANG: Strand scission of DNA by bound adriamycin and daunorubicin in the presence of reducing agents. Biochem. Biophys. Res. Commun. 76: 705～710, 1977
7) CONE, R.; K. HASAN, J. W. LOWN & A. R. MORGAN: The mechanism of the degradation of DNA by streptonigrin. Canad. J. Biochem. 54: 219～223, 1976
8) HELLING, R. B.; H. M. GOODMAN & H. W. BOYER: Analysis of endonuclease R-Eco RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virology 14: 1235～1244, 1974
9) PEACOCK, A. C. & C. W. DINGMAN: Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7: 668～674, 1968
10) AAIJ, C. & P. BORST: The gel electrophoresis of DNA. Biochim. Biophys. Acta 269: 192～200, 1972

11) BACHUR, N. R.; S. L. GORDON & M. V. GEE: A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res. 38: 1745~1750, 1978
12) UMEZAWA, K.; M. SAWAMURA, T. MATSUSHIMA & T. SUGIMURA: Mutagenicity of aclacinomycin A and daunomycin derivatives. Cancer Res. 38: 1782~1784, 1978
13) SOMEYA, A.; T. AKIYAMA, M. MISUMI & N. TANAKA: Interaction of anthracycline antibiotics with actin and heavy meromyosin. Biochem. Biophys. Res. Commun. 85: 1542~1550, 1978
14) YESAIR, D. W.; S. MCNITT & L. BITTMAN: Proposed mechanism for the reductive glycosidic cleavage of daunomycin (NSC 82151) and adriamycin (NSC 123127). Microsomes and drug oxidations. pp. 688~697, Pergamon Press, New York, 1977