MECHANISM OF ACTION OF BLEOMYCIN. STUDIES WITH THE GROWING CULTURE OF BACTERIAL AND TUMOR CELLS

HIDEO SUZUKI, KAZUO NAGAI, HIROSHI YAMAKI, NOBUO TANAKA and HAMAO UMEZAWA

Institute of Applied Microbiology, University of Tokyo

(Received for publication April 6, 1968)

Bleomycin was observed to inhibit DNA and protein syntheses in intact *Escherichia coli*, EHRLICH carcinoma and HeLa cells. DNA synthesis was more profoundly affected than protein synthesis. RNA synthesis was not significantly suppressed by the antibiotic. The activity of bleomycin was highly dependent on the number of cells and on the concentration of phosphate in the medium, being more pronounced at low concentration of phosphate and with fewer cells. Bleomycin complex, copper-free bleomycin A_2, copper-chelating bleomycin A_2 (Cu 2.6 % w/w) and copper-saturated bleomycin A_2 (Cu 5.1 %) were studied. All of them exhibited substantially the same activity. Bleomycin caused elongation of *E. coli* cells and enlargement of HeLa cells, with a reduction in the number of mitotic figures of growing HeLa cells, indicating that the antibiotic interfered with the mitotic process.

Phleomycin, a water-soluble antibiotic complex, was isolated from culture broth of a strain of *Streptomyces verticillus* by MAEDA *et al.* (1956)[1]. Later the antitumor activity of the antibiotic was demonstrated[2,3]. The mechanism of action of phleomycin was observed to be related to DNA and DNA synthesis. It was found to increase Tm* of dAT** polymer and inhibit DNA polymerase reaction[4~7].

More recently another antibiotic complex was discovered by UMEZAWA *et al.*(1966) and was designated bleomycin[8]. It was fractionated into bleomycins A and B. The former was further separated into 6 components and the latter into 5[9]. Bleomycin was observed to exhibit a significant antitumor and antibacterial activity[10,11].

The activity of bleomycin on the macromolecular synthesis was studied with the growing cells of *E. coli*, EHRLICH carcinoma and HeLa cells. DNA synthesis was most markedly inhibited by bleomycin complex, and by copper-containing or copper-free bleomycin A_2. These materials also interfered with protein synthesis. However, RNA synthesis was not significantly affected. The activity of bleomycin was highly dependent on the number of cells and on the concentration of phosphate in the medium. It was more pronounced at low concentration of phosphate and with fewer cells. The results are presented in this publication.

---

* Tm : Thermal melting of DNA.

** dAT : Deoxyadeninethymine.

Materials and Methods

Bleomycin complex 775 μg/mg (A_1 8 %, B_1 5 %, A_2 51 %, B_2 24 %, A_5 6 %, B_5 6.5 % and Cu 1.7 %, w/w), copper-chelating bleomycin A_2 (Cu 2.6 %), copper-saturated bleomycin A_2 (Cu 5.1 %), and bleomycin A_2 (copper-free) were prepared by T. TAKITA, Institute of Microbial Chemistry, Tokyo. Pluramycin was supplied by K. MAEDA, National Institute of Health, Tokyo. Mitomycin C was a product of Kyowa Hakko Kogyo, Co., Tokyo. Puromycin was kindly given by Lederle Lab., Pearl River, N. Y. L-Leucine-^{14}C (U), 198 mc/mM, was purchased from Daiichi Chemical Co., Tokyo. Thymidine-6-^{3}H (n), 1,850 mc/mM, uridine-^{3}H (G), 2,700 mc/mM, and adenine-8-^{14}C, 28.9 mc/mM, were products of Radiochemical Centre, Amersham, England.

^{14}C-Amino acids were a hydrolysate of *Chlorella* protein, 6.47 mc/mMC, and were a gift of Dr. B. MARUO, Institute of Applied Microbiology, University of Tokyo, Tokyo.

Culture of HeLa cells:

HeLa S-3 cells were grown in tubes or bottles in 1 ml of EAGLE's minimal essential medium[12], supplemented with 10 % calf serum. For the observation of effects of antibiotics on the growth, the media were changed for antibiotic-containing media on 0 day. At 2, 4, and 6 days, 3 tubes of a group were counted by the citric acid-crystal violet staining method and the average number of cells from each group is shown in Fig. 1.

Protein and nucleic acid syntheses in HeLa cells:

Antibiotic-containing media were added to the culture of HeLa S-3 cells 48 hours after inoculation, and the cells were incubated for an additional 4, 8, 24, and 48 hours. Then radioactive precursors were added to the media: ^{14}C-leucine 0.1 μc/ml, ^{3}H-thymidine 0.25 μc/ml and ^{3}H-uridine 0.1 μc/ml. After incubation for 30 minutes, the cells on the cover slips were chilled in cold saline, fixed in methanol, immersed in 2 % perchloric acid for 40 minutes to remove acid-soluble materials, rinsed in water, and dried. Radioactivity of the cells on each cover slip was determined in a windowless gas flow counter. The number of cells was determined by the citric acid-crystal violet staining method.

Observation of DNA synthesis in synchronized HeLa cells:

HeLa S-3 cells were synchronized by addition of aminopterine 10^{-6} M and adenosine 5×10^{-5} M to block endogenous synthesis of thymidine, according to the method of KAJIWARA *et al.*[13]. After 16 hours, the thymidine-less state was relieved by addition of thymidine 4×10^{-6} M. Then DNA synthesis was initiated and continued for 6 or 7 hours. The effect of bleomycin was examined at 3 different stages of DNA synthesis.

(1) The antibiotic was added to the culture 2 hours before the beginning of DNA synthesis, and the medium was changed to fresh medium, containing ^{3}H-thymidine 0.25 μc/ml at the initiation of DNA synthesis.

(2) The antibiotic and ^{3}H-thymidine were added to the culture at the time of thymidine rescue (the initiation of DNA synthesis).

(3) The antibiotic and ^{3}H-thymidine were added to the culture 5 hours after thymidine rescue.

The incubation with ^{3}H-thymidine was performed for an hour at 37°C, and the radioactivity was determined as described above.

Protein and nucleic acid syntheses in EHRLICH ascitic carcinoma cells:

EHRLICH carcinoma cells were contacted with bleomycin at 25°C for 30 minutes in a buffer (NaCl 1.4×10^{-4} M, Tris-HCl 2×10^{-2} M, and glucose 6×10^{-3} M, pH 7.2). Then radioactive precursors were added to the media: ^{3}H-thymidine 0.1 μc/ml, ^{3}H-uridine 0.02 μc/ml, and ^{14}C-leucine 0.02 μc/ml; and further incubated for an hour. The radioactivity of protein or nucleic acid fraction[14] was determined in a windowless gas flow counter.

Culture of *E. coli*:

E. coli B was incubated at 37°C with aeration in the following media. Medium I (HERSHEY): glucose 2 g, NaCl 5.4 g, NH_4Cl 1.1 g, $CaCl_2$ 11 mg, $MgCl_2$ 95 mg, KH_2PO_4 4.0 g,

Na_2SO_4 23 mg, $FeCl_3$ 0.16 mg, Tris 12.1 g, pH 7.4, per liter. Medium II: glucose 2 g, KCl 2.02 g, NH_4Cl 1.98 g, $CaCl_2$ 14.7 mg, $MgCl_2$ 142 mg, K_2HPO_4 142 mg, Na_2SO_4 398 mg, $FeCl_3$ 2.7 mg, Tris 12.1 g, pH 7.6, per liter.

Observation of protein and nucleic acid syntheses in the growing cells of *E. coli*:

The cells of *E. coli* B grown overnight in medium I or II supplemented with 0.1 % casamino acids were transferred to 20 volumes of fresh media and incubated at 37°C.

At the logarithmic phase of growth, the cell suspension was divided into 9.7 ml portions in L tubes at 0°C. After 10-minute incubation 0.5 μc of ^{14}C-amino acids or 1 μc of ^{14}C-adenine was added with 50 μg of adenine to the culture. The antibiotic was simultaneously introduced into the cells. The cell count of the suspension was approximately 3×10^8/ml. Growth was measured by turbidimetric method at 660 mμ and by viable cell count, using colony formation on nutrient agar. The nucleic acids and protein were fractionated from 3 ml of samples by the method of SCHMIDT and THANNHAUSER[14]. Determination of protein was carried out according to LOWRY *et al.*[15] Nucleic acid was estimated by the value of OD_{260}. The radioactivity was determined in a GM counter.

Results

Effects of Bleomycin on the Growth of HeLa Cells and *E. coli*

Bleomycin was observed to inhibit the growth of HeLa S-3 cells and *E. coli* B. It was significantly inhibitory at concentrations higher than 1 μg/ml, when the antibiotics were introduced into the culture of HeLa cells at the loagrithmic phase of growth in EAGLE's minimal essential medium supplemented with 10 % calf serum. The results are presented in Fig. 1. They are in accordance with those reported in the previous paper[16]. The activity of bleomycin was highly dependent on the number of cells. More marked inhibition was observed with fewer cells than illustrated in Fig. 1. No significant difference was demonstrated of the activity of copper-chelating and copper-free bleomycin.

The antibiotic was observed to inhibit the growth of *E. coli* B at concentrations higher than 20 μg/ml, when it was introduced into the

Fig. 1. Effects of bleomycins on growth of HeLa S-3 cells

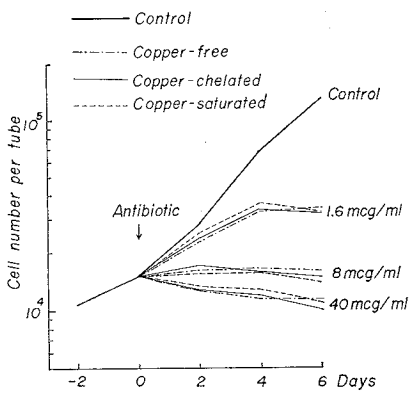
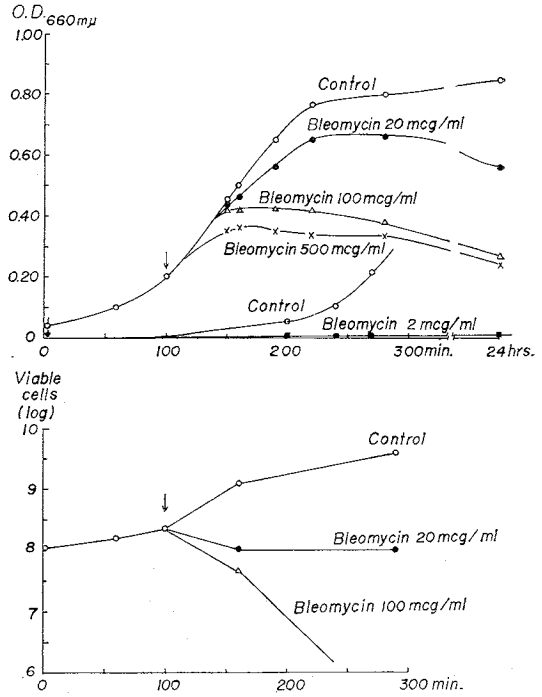


Fig. 2. Effects of bleomycin on growth of *E. coli* B in medium I



culture at the logarithmic phase of growth in medium I.

The activity seemed to be bactericidal, because it decreased the number of viable cells. The results are summarized in Fig. 2. The activity of bleomycin was highly dependent on the cell number and phosphate concentration in the medium. The same tendency was observed as in the case of HeLa cells.

Microscopic Observation of Cells Treated with Bleomycin

Bleomycin was found to cause elongation of *E. coli* B, when it was added to the culture at the logarithmic phase of growth. Elongated cells of various sizes appeared 3 hours after the addition of the antibiotic at a concentration of 20 μg/ml (Plate 1).

Marked morphological changes were observed in the growing HeLa cells treated with bleomycin complex or bleomycin A_2 (copper-free). The nucleus became larger, and the cytoplasm was also enlarged and spread on glass by the treatment with bleomycin. The number of mitotic figures was markedly decreased and the shrinkage of chromosomes was observed. The number of polynuclear and mononuclear giant cells of various sizes was significantly increased by the treatment with bleomycin at the concentration of 40 μg/ml for 24 hours or longer (Plate 2).

These observations indicated that bleomycin interfered with mitosis.

Plate 1. Elongation of cells of *E. coli* by bleomycin

The cells of *E. coli* Q 13 were treated overnight in a medium containing 20 μg/ml of bleomycin.

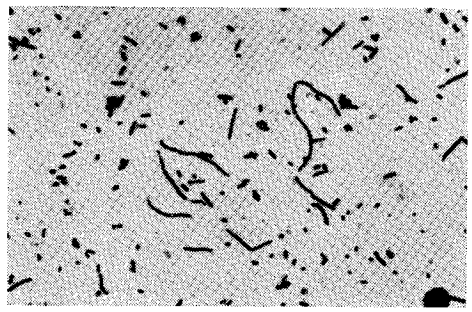
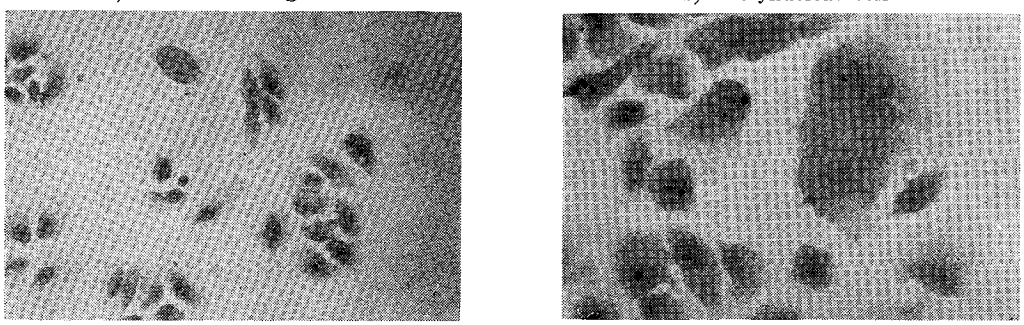


Plate 2. Morphological changes in growing HeLa S-3 cells treated with bleomycin A_2 (copper-free)

a) Mononuclear giant cell

b) Polynuclear cell



The cells were cultured in a medium containing 40 μg/ml of bleomycin A_2 (copper-free) for 24 hours in (a), and 48 hours in (b), respectively.

Effects of Bleomycin A_2 (Copper-free) on the Biosynthesis of Macromolecules of HeLa Cells

The incorporation of ^{3}H-thymidine into DNA of HeLa cells was inhibited by the presence of 40 μg/ml of bleomycin. The inhibition was more marked with longer contact of cells and the antibiotic. The incorporation of ^{3}H-uridine into RNA and that of ^{14}C-leucine into protein was not significantly influenced by bleomycin. The

Table 1. Effect of copper-free bleomycin A_2 on protein and nucleic acids syntheses in growing HeLa S-3 cells

Bleomycin A_2		Cell number $\times 10^4$	Incorporation of					
			^{3}H-thymidine		^{3}H-uridine		^{14}C-leucine	
Incubation hr.	Concentr. μg/ml		cpm*	%	cpm	%	cpm	%
4	0	2.8	2,050	100	324	100	584	100
	40	2.8	1,626	79	304	93	644	110
8	0	2.8	2,060	100	524	100	476	100
	40	2.8	1,550	75	538	103	494	104
24	0	3.6	2,140	100	514	100	584	100
	40	2.9	1,069	50	455	89	628	107
28	0	4.8	2,320	100	783	100	416	100
	40	2.1	1,000	43	1,295	165	576	138

* cpm : counts per minute per 10^5 cells.

phosphate concentration in the medium was 7.8×10^{-4} M. The results are presented in Table 1.

Effects of Bleomycin A_2 on DNA Synthesis of HeLa Cells

As summarized in Table 2, the inhibition of DNA synthesis of HeLa cells was more marked with copper-free bleomycin than with copper-chelating or copper-saturated bleomycin A_2. The effects of bleomycin were studied with cultures synchronized by treatment with aminopterine. Bleomycin inhibited DNA synthesis to the same degree when given before, at the time of or after the initiation of DNA synthesis. The results are presented in Table 3.

Table 2. Effects of bleomycins on DNA synthesis in growing HeLa cells

		Incorporation of ^{3}H-thymidine	
		cpm/tube	% Inc.
Control		755	100
Bleomycin A_2 (copper-free)	40 μg/ml	402	53
	8	443	59
	1.6	628	83
Bleomycin A_2 (copper-chelate)	40 μg/ml	414	55
	8	552	73
	1.6	764	101
Bleomycin A_2 (saturated with copper)	40 μg/ml	445	59
	8	734	97
	1.6	802	106

The cells were pretreated with bleomycin at 37°C for 4 hours.

Table 3. Effects of copper-free bleomycin A_2 on DNA synthesis of synchronized HeLa cells

Time of addition of bleomycin A_2	cpm/cover slip		
	Without bleomycin A_2	With bleomycin A_2	Ratio of incorporation*
2 hours before DNA synthesis	617	368	60
At initiation of DNA synthesis	617	349	57
5 hours after DNA synthesis	426	269	63

* (With bleomycin A_2/without bleomycin A_2). Bleomycin A_2 : 40 μg/ml.

Effects of Bleomycin A_2 on Macromolecular Synthesis of Ehlrich Carcinoma Cells

As shown in Table 4, DNA synthesis and protein synthesis were inhibited by the pre-treatment with bleomycin A_2 (copper-free) for 30 minutes before the addition of radioactive precursors, but RNA synthesis was not significantly affected.

Effects of Bleomycin A_2 on Macromolecular Synthesis of Growing Cells of *E. coli*

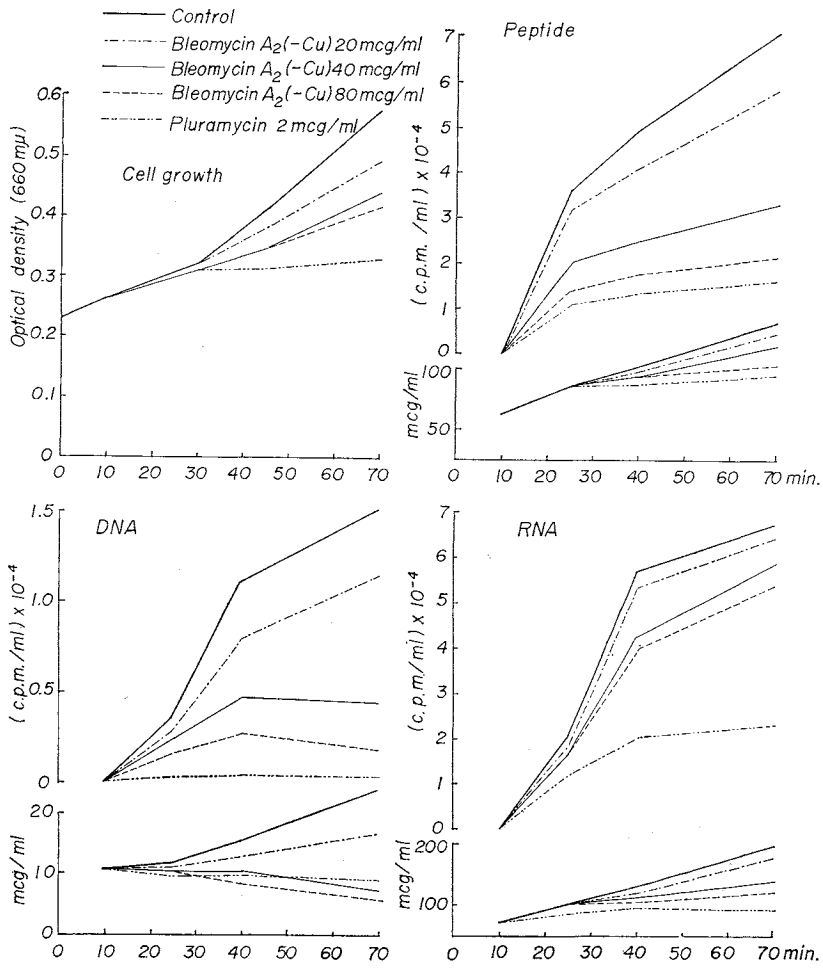
Table 4. Effects of copper-free bleomycin A_2 on the syntheses of DNA, RNA and protein in EHRLICH ascitic carcinoma cells

	Incorporation (cpm/tube)		
	^{3}H-thymidine	^{3}H-uridine	^{14}C-leucine
Control	1,321	1,398	634
Bleomycin A_2 40 μg/ml	990 (70%)	1,529	456 (72%)
Mitomycin C 50 μg/ml	1,284 (91%)	1,634	
Puromycin 5 μg/ml		1,734	136 (21%)

The incorporation of ^{14}C-amino acids into protein of the growing cells of *E. coli* B was inhibited by bleomycin A_2 (copper-free) in medium II, which contained a low concentration of phosphate. However, at the higher concentration of phosphate, when medium I (HERSHEY) was used, much less inhibition was observed in the presence of bleomycin A_2.

The incorporation of ^{14}C-adenine into DNA was more markedly inhibited by bleomycin A_2 (copper-free) in media I and II, which contained high and low concentration of phosphate respectively. The incorporation of ^{14}C-adenine into RNA was

Fig. 3. Effects of copper-free bleomycin A_2 on the growth and macromolecular syntheses of *E. coli* B



not significantly affected by the presence of the antibiotic. In conclusion, DNA synthesis was most profoundly affected by bleomycin, and less grade of inhibition of protein synthesis was observed. RNA synthesis was not significantly affected. The results are illustrated in Fig. 3.

Discussion

Bleomycin was observed to inhibit DNA and protein syntheses in the intact cells of *E. coli*, EHRLICH carcinoma and HeLa cells. DNA synthesis was more markedly affected than protein synthesis. No substantial difference in the activity was demonstrated with bleomycin complex and bleomycin A_2, both copper-containing and copper-free. The primary site of action of bleomycin has been further investigated in our laboratory, using cell-free systems. Bleomycin A_2 and A_5 did not significantly affect protein synthesis in cell-free systems. However, bleomycin complex inhibited protein synthesis in ribosomal systems by interfering with the formation of aminoacyl-sRNA, particularly with those of arginyl- and lysyl-sRNA. The results indicated that, besides bleomycin A_2 and A_5, another factor, inhibiting protein synthesis, was contained in bleomycin complex. Phleomycin was demonstrated to increase Tm of dAT polymer or DNA[6,17]. In contrast to phleomycin, bleomycin A_2 was observed to decrease Tm of DNA[17]. The results indicated that bleomycin A_2 bound with DNA and labilized the double strand structure of DNA. The most plausible primary site of action of bleomycin A_2 seems to be involved in DNA and its function, including DNA synthesis and mitosis.

The activity of bleomycin was highly dependent on the number of cells and the concentration of phosphate in the medium resembling the activity of streptomycin. However the detailed mechanism of action remains to be determined.

Acknowledgement

This investigation is supported in part by Public Health Research Grant CA 05082-07, from the National Cancer Institute, U. S. A.

References

1) MAEDA, K.; H. KOSAKA, K. YAGISHITA & H. UMEZAWA : A new antibiotic, phleomycin. J. Antibiotics, Ser. A 9 : 82～85, 1956
2) BRADNER, W. T. & M. H. PINDELL : Antitumor properties of phleomycin. Nature 196 : 682～683, 1962
3) UMEZAWA, H.; M. HORI, M. ISHIZUKA & T. TAKEUCHI : Studies on antitumor effect of phleomycin. J. Antibiotics, Ser. A 15 : 274～275, 1962.
4) TANAKA, N.; H. YAMAGUCHI & H. UMEZAWA : Mechanism of action of phleomycin, a tumor-inhibitory antibiotic. Biochem. Biophys. Res. Comm. 10 : 171～174, 1963
5) TANAKA, N.; H. YAMAGUCHI & H. UMEZAWA : Mechanism of action of phleomycin. I. Selective inhibition of the DNA synthesis in *E. coli* and in HeLa cells. J. Antibiotics, Ser. A 16 : 86～91, 1963
6) FALASCHI, A. & A. KORNBERG : Phleomycin, an inhibitor of DNA polymerase. Federation Proc. 23 : 940～945, 1964
7) TANAKA, N. : Effect of phleomycin on DNA polymerase of tumor origin. J. Antibiotics, Ser. A 18 : 111, 1965
8) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI : New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19 : 200～209, 1966
9) UMEZAWA, H.; Y. SUHARA, T. TAKITA & K. MAEDA : Purification of bleomycins. J. Antibiotics, Ser. A 19 : 210～215, 1966
10) ISHIZUKA, M.; H. TAKAYAMA, T. TAKEUCHI & H. UMEZAWA : Activity and toxicity of bleomycin. J. Antibiotics, Ser. A 20 : 15～24, 1967

11) ICHIKAWA, T.; A. MATSUDA, K. MIYAMOTO, M. TSUBOSAKI, T. KAIHARA, K. SAKAMOTO & H. UMEZAWA : Biological studies on bleomycin A. J. Antibiotics, Ser. A 20 : 149～155, 1967
12) EAGLE, H. : Amino acid metabolism in mammalian cell cultures. Science 130 : 432～437, 1959
13) MUELLER, G. C.; K. KAJIWARA, E. STUBBLEFIELD & R. R. RUECKERT : Molecular events in the reproduction of animal cells. I. The effect of puromycin on the duplication of DNA. Cancer Res. 22 : 1084～1090, 1962
14) SCHMIDT, G. & S. J. THANNHAUSER : A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. 161 : 83～89, 1945
15) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL : Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193 : 265～275, 1951
16) KUNIMOTO, T.; M. HORI & H. UMEZAWA : Modes of action of phleomycin, bleomycin and formycin on HeLa S-3 cells in synchronized culture. J. Antibiotics 20 : 277～281, 1967
17) NAGAI, K.; H. YAMAKI, H. SUZUKI, N. TANAKA & H. UMEZAWA : Mechanism of action of bleomycin. II. Effects of bleomycin A_2 on thermal denaturation of DNA. (in preparation)