A METABOLITE OF BLEOMYCIN A_5 IN RABBIT URINE

Sir:

As described previously,[1,2] bleomycins are rapidly inactivated in various organs of mice, more slowly in skin and lung than in the other organs. With 20-methylchoranthrene induced tumors, the rate of inactivation is lower in squamous cell carcinoma than in sarcoma. We also found an enzyme in mouse liver which hydrolyzes the carboxamide of the β-aminoalanine amide moiety of bleomycin.[2,3] Bleomycin A_5 was more resistant to this enzymatic hydrolysis than bleomycins A_2 and B_2.[1] However, when randomly tritiated bleomycin A_5 was subcutaneously injected into mice and the radioactivity and antibacterial activity of organ extracts were tested, more rapid reduction of the antibacterial activity of A_5 than of A_2 was observed. When 2.0 mg (4.86×10^6 dpm) of ^{3}H-bleomycin A_5 was injected into three mice which were sacrificed after 1 hour, the amount in urine by the radioactivity was 1,198 mcg, 1,237 mcg and 1,166 mcg, while the amount found by antibacterial assay with *Bacillus subtilis* was 288 mcg, 335 mcg and 310 mcg respectively. With bleomycin A_2, the concentration in urine by the radioactivity assay was substantially the same as that shown by antibacterial activity. As reported, bleomycin A_5 is different from A_2 in the terminal amine moiety which is spermidine in A_5 and 3-aminopropyldimethylsulfonium in A_2[1]. Thus, it was suggested that the spermidine moiety of A_5 can be rapidly metabolized.

After the subcutaneous injection of 20 mg of bleomycin A_5 in a rabbit, 130 ml of urine was collected in 24 hours. After the pH was adjusted to 6.8, the urine was passed through a column of IRC-50 (H^+) which was then eluted with 0.1 N HCl-methanol (1:1). The fraction (60 ml) which showed the characteristic absorption of bleomycin at 292 nm was collected, neutralized with IR-45 (OH^-), and evaporated to dryness. It was dissolved in 2.0 ml of 0.05 M sodium chloride and chromatographed on CM-Sephadex C-25 with a gradient of sodium chloride from 0.05 M to 1.0 M. The elution pattern is shown in Fig. 1. A bleomycin A_5-derived compound in the largest peak was adsorbed on 10 ml of XAD-2 resin and eluted with 0.02 N HCl-methanol (1:1). The eluate was neutralized with IR-45 resin and evaporated to dryness, yielding 4.4 mg of a blue powder.

The compound thus obtained showed a single spot in high voltage electrophoresis (3,500 V, pH 1.8 in formic acid-acetic acid-water 25:75:900, 15 minutes). Its lower basicity compared to bleomycin A_5 was shown by lower mobility toward the cathode. It gave a negative ninhydrin that indicating an intact carboxamide group. Total hydrolysis of the compound with 6 N HCl for 20 hours gave the same hydrolysis products as from A_5 except for spermidine. Instead of spermidine, a more acidic product was found with high voltage electrophoresis.

This hydrolysis product derived from the spermidine moiety of bleomycin A_5 was compared with the synthetic compounds shown in Table 1. In high voltage electrophoresis,

Fig. 1. CM-Sephadex C-25 chromatorgaphy of a urine extract after subcutaneous injection of bleomycin A_5

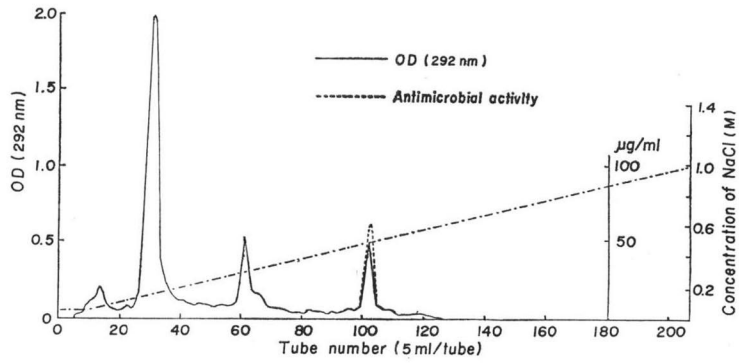


Table 1. High-voltage electrophoresis of various compounds related to the oxidation product of spermidine

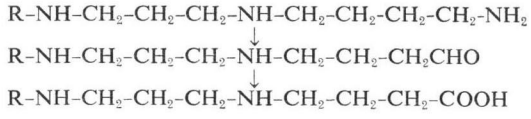
Compounds	Rm value*
NH_2-CH_2-CH_2-COOH	1.48
NH_2-CH_2-CH_2-CH_2-COOH	1.71
Compound I**	1.90
Compound II***	1.90
NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-COOH	1.90
NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-CH_2OH	2.00
Spermidine [NH_2-$(CH_2)_3$-NH$(CH_2)_4$-NH_2]	2.67

* Rm value of alanine as 1.00.
** Compound I was obtained by hydrolysis of a metabolite of bleomycin A_5.
*** Compound II was obtained by reduction of the metabolite of bleomycin A_5 with $NABH_4$ followed by the hydrolysis.

the hydrolysis product behaved similarly to N-(3-aminopropyl)-4-aminobutyric acid. Their identity was confirmed by silica gel thin-layer chromatography: RF 0.28 with *n*-propanol-pyridine-acetic acid-water (15:10:3:12, v/v); Rf 0.18 with *n*-butanol-acetic acid-water, 4:1:5; Rf 0.14 with *n*-butanol-ethanol-chloroform-17% aqueous ammonia, 4:5:2:3; Rf 0.08 with ethyl acetate-pyridine-acetic acid-water, 5:5:1:3; Rf 0.03 with *n*-butanol-acetic acid-water, 4:1:2.

N-(3-Aminopropyl)-4-aminobutyric acid is a known compound,[4] but it has not been well characterized. It was synthesized as follows: A chloroform solution of 4-bromobutyronitrile was added to 1, 3-diaminopropane at room temperature. After stirring for 30 minutes, the reaction mixture was refluxed for an hour. A precipitate was removed by filtration and the filtrate was evaporated. The residue was hydrolyzed with 6 N HCl at 125°C for 4 hours. The hydrolyzate was dried, dissolved in water, and applied to column of Dowex 1×2 (50~100 mesh, OH^-). After washing with water, the product was eluted with 0.1 N acetic acid. The eluate was dried after addition of HCl and N-(3-aminopropyl)-4-aminobutyric acid dihydrochloride was recrystallized from the residue with methanol-ethylacetate, m.p. 201°C.

Thus, it is suggested that the following oxidation of bleomycin A_5 occurs *in vivo*.



Spermidine oxidase which was crystallized from serum by YAMADA *et al.*[5] oxidatively deaminates the other amine of spermidine.[6] Thus, it is not certain whether this spermidine oxidase is involved in oxidative deamination of the supermidine moiety of bleomycin A_5. Moreover, it is not certain, whether the last step, oxidation of aldehyde to carboxyl, requires a specific enzyme or not.

As described above, the metabolic product of bleomycin A_5 extracted from urine was obtained as a blue powder. Treatment with hydrogen sulfide removed the blue color indicating that this compound was excreted in the copper-chelated form. Thus after subcutaneous injection of copper-free material, bleomycin A_5 or the metabolic product chelates cupric ion from tissues.

SENJI HORI
TSUTOMU SAWA
TAKEO YOSHIOKA
TOMOHISA TAKITA
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23, Kamiosaki, Shinagawa-ku,
Tokyo, Japan

(Received March 13, 1974)

References

1) UMEZAWA, H.: Natural and artificial bleomycins. Chemistry and antitumor activity. Pure Appl. Chem. 28: 665~680, 1971
2) UMEZAWA, H.; T. TAKEUCHI, S. HORI, T. SAWA, M. ISHIZUKA, T. ICHIKAWA & T. KOMAI: Studies on the mechanism of antitumor effect of bleomycin on squamous cell carcinoma. J. Antibiotics 25: 409~420, 1972
3) TAKITA, T.; Y. MURAOKA, T. YOSHIOKA, A. FUJII, K. MAEDA & H. UMEZAWA: The chemistry of bleomycin. IX. The structures of bleomycin and phleomycin. J. Antibiotics 25: 755~758, 1972.
4) REPPE, W. & Mitarbeitern: Athinylierung. Ann. Chem. 596: 158~224, 1955
5) YAMADA, H. & K. T. YASUNOBU: Monoamine

oxidase. I. Purification, crystallization and properties of plasma monoamine oxidase. J. Biol. Chem. 237: 1511~1516, 1962

6) TABOR, C. W.; H. TABOR & U. BACHRACH: Identification of the aminoaldehydes produced by the oxidation of spermine and spermidine with purified plasma amine oxidase. J. Biol. Chem. 239: 2194~2203, 1964