THE DISTRIBUTION OF ^{3}H-BLEOMYCIN IN MOUSE TISSUE

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A bleomycin mixture which contained A_2 as the main component and B_2 as the second component was tritiated. The ^{3}H-bleomycin thus prepared was subcutaneously injected to mice and the distribution of the bleomycin among organs of mice was determined. The results indicated a high concentration in skin and peritoneum. The concentrations determined by radioactivity were higher than those determined by the antibacterial activity suggesting that tissue contains some substance which reduces the bacterial activity against *B. subtilis* or inactivates the bleomycin.

Bleomycins contained in organs of mice after injection were determined by the cylinder plate method using *B. subtilis* as the test organism and reported in previous papers by ISHIZUKA *et al.*[1] and ICHIKAWA *et al.*[2] These authors reported that the bleomycins were found in high concentrations in lung and skin. Distribution of individual bleomycins A_1, A_2, A_2', A_5, B_1, B_2, B_4 and B_5 was studied by UMEZAWA *et al.*[3] Bleomycin A_2, the main component of bleomycin mixtures studied clinically, gives a high concentration in skin of mice and the therapeutic effect of bleomycin on squamous cell carcinoma in humans is considered to be related to its high distribution in skin.

However, the concentration shown by the antibacterial activity may not show the real concentration of the antibiotic in the organs. The antibacterial activity gives the concentration of the antibiotic in the active form which is extracted from the organs. If the antibiotic is not sufficiently extracted from an organ, if an organ contains a component which interferes the antibacterial activity, or if the antibiotic is inactivated in the organ, then the concentration shown by the antibacterial activity does not indicate the real concentration of the antibiotic in organs. The amount of the antibiotic taken up by tissues, cells or organs can be found by injecting a radioactive form and determining the radioactivity. The results of measuring the distribution of tritiated bleomycin among organs of mice and the relation to the distribution shown by the antibacterial activity are reported in this paper.

Materials and Methods

Copper-containing ^{3}H-bleomycin: Copper-containing bleomycin mixture was tritiated by the usual method and after tritiation was purified by CM Sephadex chromatography

as reported by UMEZAWA *et al.*[4] The contents of each bleomycin were determined by the same chromatography. It contained 11.8 % A_1, 48.5 % A_2, 3.9 % A_2', 2.0 % A_5, 3.4 % B_1, 27.0 % B_2, 3.4 % B_4.

Determination of concentration of bleomycin in organs of mice: One mg of the copper-containing ^{3}H-bleomycin described above and 1.6 mg of cold copper-containing bleomycin of similar composition were dissolved in distilled water and the total volume was made to 0.65/ml, that is, 4.0 mg of bleomycin/ml. From this solution 0.05 ml was taken and the radioactivity of 0.1 ml of the 100 times diluted solution was measured by a liquid scintillation counter [CPM-200(LS-II) Beckman Instruments, Inc.]. The radioactivity of 1.0 mg of the bleomycin mixture which was injected was 4.75×10^6 dpm.

Distribution of ^{3}H-bleomycin among organs: A half ml of the bleomycin solution above described containing 2.0 mg of the bleomycin was injected to two mice subcutaneously. The syringe was washed with distilled water and the water used for washing was determined for the radioactivity. The radioactivity which remained in the syringe was 0.87×10^6 dpm and therefore the total radioactivity of the bleomycin which was actually introduced into two mice was 8.63×10^6 dpm ($9.50\times10^6-0.87\times10^6$). One hour after the injection, the two mice were sacrificed. Each organ was removed and the same organs taken from two mice were combined, cut into pieces and homogenized in 1/10 M phosphate buffer of pH 6.8. It was centrifuged and the residue was washed with methanol of about equal volume and centrifuged. The supernatants of the centrifugation were combined and dried. The dried material thus obtained was dissolved in 1/10 M pH 6.8 phosphate buffer, using a volume about equal to the volume of the organs and the radioactivity was assayed. The residue which was obtained by centrifugation of the homogenate was heated in 1 N HCl at 100°C for 16 hours in a sealed tube. The hydrolysate was neutralized with sodium hydroxide and the radioactivity of the solution was determined by a liquid scintillation counter.

Determination of the antibacterial activity: The extracts of homogenized organs obtained as described above were measured for antibacterial activity by the cylinder plate method using *B. subtilis* as the test organism and cold bleomycin as the standard.

Results and Discussion

The data shown in Table 1 indicate the amounts and the concentrations of the bleomycin which was extracted with phosphate buffer from homogenized organs. The radioactivity of the injected bleomycin was 4.75×10^6 dpm/mg, and using this value, the amounts and the concentrations of the bleomycin in each organ was calculated. A total of 65 % of the injected bleomycin was recovered in the extract of organs.

The radioactivity which remained in homogenized organs after the extraction was determined by testing the radioactivity of an acid hydrolysate. The results are shown in Table 2. Only 2.5 % of the injected bleomycin remained in the organs after the extraction. It indicates that bleomycin in organs can be extracted with phosphate buffer from their homogenates.

The total bleomycin which was actually introduced into mice was 1.8 mg and 1.17 mg was recovered in the supernatant of the homogenate and 0.05 mg was recovered in the residue, that is, totally 1.22 mg (68 % of the injected amount) was recovered. Therefore, 32 % of the injected bleomycin was not recovered. In this experiment, the excreted urine was not collected and therefore, unrecovered bleomycin is thought to be excreted in urine.

Table 1. Distribution of ^{3}H-bleomycin among organs of two mice

	dpm/ml of the extract ($\times 10^3$)	Volume (ml) of the extract	Total dpm of the extract ($\times 10^3$)	Bleomycin (mcg) in total	Weight (mg) of each organ	Bleomycin (mcg)/g of organ
Liver	13.9	7.0	97.3	20.4	2,739	7.4
Spleen	9.6	1.8	17.3	3.6	638	5.6
Kidney	94.8	1.8	170.6	35.9	577	62.2
Testis	9.2	1.0	9.2	1.9	130	14.6
Urinary bladder	16.0	0.8	12.8	2.6	16	162.5
Stomach	8.6	1.3	11.2	2.3	294	7.8
Large intestine	12.1	3.1	37.5	7.8	1,116	6.9
Small intestine	25.4	7.8	198.1	41.7	3,245	12.8
Heart	8.0	1.0	8.0	1.6	150	10.6
Lung	14.1	1.2	16.9	3.5	367	9.5
Brain	4.2	1.8	7.6	1.6	724	2.2
Tongue	10.5	0.8	8.4	1.7	103	16.5
Skin	51.1	14.6	746.0	157.0	6,941	22.6
Bone	11.6	4.2	48.7	10.2	1,270	8.0
Muscle	12.0	13.8	165.6	34.8	5,770	6.0
Peritoneum	30.1	3.0	90.3	19.0	896	21.2
Diaphragm	6.0	0.9	5.4	1.1	95	11.5
Feces	3.4	19.0	64.6	13.6		
Urine	974.0	3.9	3,798.6	799.7		
Blood	32.7	1.7	55.6	11.7		
Total			5,569.7	1,171.6		
Recovery			64.5 %	65.0 %		

The activity of the injected bleomycin: 4.75×10^6 dpm/mg.
Total radioactivity injected: 8.63×10^6 dpm, that is, 1.8 mg of bleomycin/2 mice.

Comparing the amount of the bleomycin which remained after the extraction to that extracted, the ratio is 47 : 1,172 indicating that the bleomycin in the organ is easily extracted with neutral buffer. After extraction with phosphate buffer methanol was added to the residue to dissolve the soluble bleomycin, but most of bleomycin was found to be extracted with the buffer. As shown in fifth column of Table 2, the ratios of the amount remaining in the homogenate after extraction to the total amount recovered were in the following order: spleen>lung>muscle>peritoneum>bone>kidney>stomach>large intestine>diaphragm>brain>heart>skin>urinary bladder>tongue>testis>small intestine. This series does not parallel the concentration in the organs. It is uncertain whether these ratios indicate a varying organ affinity for bleomycin or are the result of experimental variation.

As described above, most of the bleomycin in the organs can be extracted with neutral buffer. Therefore, the concentration of bleomycin in each organ shown in Table 1 gives the real distribution among organs of mice except in kidney and bladder. As shown in Table 1, of 1,171.6 mg recovered, 799.7 mcg was found in urine. Therefore, even a small amount of urine in kidney and bladder must have a big influence on the concentration of bleomycin in these organs. The values in kidney and bladder shown in Table 1 do not show the real concentrations of bleomycin in these organs. The data in Table 1 indicate a high concentration of bleomycin in skin, peritoneum and tongue. As reported in a previous paper[3], bleomycin A_2, the main component

of the bleomycin employed for the present experiment, shows a high concentration in skin and peritoneum when determined by antibacterial activity. As reported in the same paper, bleomycin A_2 does not give as high concentration in lung as a bleomycin mixture. The concentration in lung varies with on each bleomycin and the ^{3}H-bleomycin which contained A_2 at 48.5%, B_2 27.0%, A_1 11.8% and A_2', A_5 and B_4 less than 4.0% was found to show lower concentration in lung.

Amounts of bleomycin in each organ determined by the antibacterial activity are shown in Table 3. In the same table the amounts determined by radioactivity are shown for comparison. It is surprising that the amounts shown by the antibacterial activity were much less than those shown by the radioactivity. The ratios of the amounts determined by the antibacterial activity to those determined by the radioactivity is shown in fourth column of the same table. The ratio was less than 0.06 except for skin (0.283), muscle (0.198), lung (0.109) and peritoneum (0.089). Even in urine, the amount determined by the antibacterial activity was about 1/3 that determined by the radioactivity. These results suggest that all organs contain some substances which reduce the antibacterial activity or inactivate the bleomycin. The ratio of the bleomycin in urine determined by the antibacterial activity to that determined by the radioactivity in another experiment was 0.722. Thus, this ratio is different depending on the experiment and is considered to be due to some substance in urine influencing the activity.

Table 2. Amounts of bleomycins* which remained in homogenates of organs after the extraction

Organs	Weight (mg)	Total bleomycin (mcg) in residue	Residual bleomycin (mcg)/g of tissue	Amount remaining after extr. / Total amount recovered**
Liver	2,739	2.10	0.76	0.010
Spleen	638	1.20	1.88	0.250
Kidney	577	7.30	12.65	0.170
Testis	130	0.08	0.61	0.040
Urinary bladder	16	0.13	8.12	0.047
Stomach	294	0.47	1.59	0.169
Large intestine	1,116	1.40	1.25	0.152
Small intestine	3,245	1.60	0.49	0.037
Heart	150	0.14	0.93	0.080
Lung	367	0.95	2.58	0.213
Brain	724	0.20	0.27	0.111
Tongue	103	0.08	0.77	0.042
Skin	6,941	13.10	1.88	0.078
Muscle	5,770	10.90	1.88	0.212
Peritoneum	896	5.02	5.60	0.209
Diaphragm	95	0.16	1.68	0.126
Bone	1,270	2.46	2.05	0.195

* Determined by the radioactivity of the acid hydrolysates of residues homogenates after the extraction.

** The total amount was calculated from the values (a) in fifth column of Table 1 and the values (b) in the third column as follows; b/a+b.

Total amount is that found in extract plus the amount remaining in the residue.

Contrary to other organs, the amount of bleomycin in blood shown by antibacterial activity was higher than that shown by radioactivity. ^{3}H-bleomycin injected as described above is a mixture of bleomycins and if a bleomycin such as A_5 which shows much higher antibacterial activity than A_2, as described in a previous paper[3], is contained in high ratio in the blood, then the amount shown by the antibacterial activity becomes higher than that shown by the radioactivity.

The influence of the homogenate on the antibacterial activity was examined in another experiment. Two kidneys (weights 577 mg), one half of two livers combined (1,000 mg), two lungs (356 mg) and peritonea taken from two mice (760 mg) were homogenized in 1/10 M pH 6.8 phosphate buffer and 2 ml of buffer containing 200 mcg of

^{3}H-bleomycin were added and incubated for 30 minutes. After centrifugation, the radioactivity and the antibacterial activity of the supernatants were determined. The results are shown in Table 4. The radioactivity recovery was 77~89 %. The recovery of antibacterial activity was much less than that of radioactivity except in the case of lung. These results indicate that liver, kidney and peritoneum contain some substances which reduce the antibacterial activity or inactivate the bleomycins.

The data shown above indicate that the bleomycin concentrates in skin and peritoneum. They also show that the amount found by the antibacterial activity is less than the actual amount. The smaller amount shown by antibacterial activity is considered to be due to substances in homogenates which reduce the antibacterial activity or inactivate the bleomycin. This will be clarified by using a single radioactive bleomycin such as ^{3}H-bleomycin A_2, and looking for metabolic products.

Table 3. The ratios of amount of bleomycin determined by antibacterial activity to that determined by radioactivity

Organs	Bleomycin (mcg) by radioactivity (a)	Bleomycin (mcg) by antibacterial activity (b)	b/a
Liver	20.3	<0.50	<0.024
Spleen	3.6	<0.11	<0.030
Kidney	35.5	1.10	0.031
Testis	1.9	0.10	0.052
Urinary bladder	2.6	0.07	0.027
Stomach	2.3	<0.08	<0.034
Large intestine	7.8	<0.20	<0.025
Small intestine	41.3	1.06	0.026
Heart	1.6	0.01	0.006
Lung	3.5	0.38	0.109
Brain	1.6	<0.13	<0.081
Tongue	1.8	0.05	0.028
Skin	154.8	43.80	0.283
Bone	10.1	0.44	0.044
Muscle	44.5	8.80	0.198
Peritoneum	18.9	1.68	0.089
Diaphragm	1.1	<0.03	<0.027
Urine	791.3	273.00	0.345
Feces	13.3	<1.90	<0.143
Blood	11.6	25.5	2.198
Total	1,171.6	355.99	

Table 4. Recovery of bleomycin in homogenates of various organ *in vitro*

	Weight	Volume of the mixture of the homogenate and ^{3}H-bleomycin (ml)	Volume(ml) of the supernatant	Recovery in the supernatant by radioactivity	Recovery % by antimicrobial activity
Kidney	577	4.05	3.3	85.0 %	30.0 %
Liver	1,000	4.5	3.35	83.5	24.7
Lung	356	4.1	3.7	89.0	92.0
Peritoneum	760	4.0	3.2	77.0	40.0
Bleomycin (200 mcg)	—		2.0	100.0	100.0

The mixture of bleomycin and homogenate was incubated at 37°C for 30 minutes.

References

1) Ishizuka, M.; H. Takayama, T. Takeuchi & H. Umezawa : Activity and toxicity of bleomycin. J. Antibiotics, Ser. A 20 : 15~24, 1967.
2) Ichikawa, T.; A. Matsuda, K. Miyamoto, M. Tsubosaki, T. Kaihara, K. Sakamoto & H. Umewawa : Biological studies on bleomycin A. J. Antibiotics, Ser. A 20 : 149~155, 1967
3) Umezawa, H.; M. Ishizuka, K. Kimura, J. Iwanaga & T. Takeuchi : Biological studies on individual bleomycins. J. Antibiotics 21 : 592~602, 1968
4) Umezawa, H.; Y. Suhara, T. Takita & K. Maeda : Purification of bleomycins. J. Antibiotics, Ser. A 19 : 210~215, 1966.