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Cobalt Chelate of Bleomycin. I. Physicochemical Properties and Distribution in Tumor Bearing Mice[1]

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Cobalt chelate of bleomycin(Co-BLM) was inert to ligand exchange reaction and showed absorption at 450 nm and 580 nm, which indicated cobalt is trivalent.

Co-BLM was accumulated in tumor tissue much more than BLM and other BLM metal chelates.

In urine of ^{67}Ga-BLM or ^{111}In-BLM injected mice, most of radioactivity was not bound to BLM. On the other hand, all the radioactivity was attributable to BLM bound ^{57}Co in urine of ^{57}Co-BLM injected mice.

In tumor tissue homogenate, most of Co-BLM was present in cell nuclei, while BLM, $CoCl_2$ and other BLM metal chelates distributed mainly in supernatant fractions.

Co-BLM may bind to deoxyribonucleic acid (DNA) in tumor cell.

From these results, we conclude Co-BLM to be superior as a tumor scanning agent than BLM and other BLM metal chelates.

Keywords——bleomycin; bleomycin-metal complexes; cobalt chelate; cobalt-57; radiopharmaceuticals; tumor scanning agent; distribution of labeled compounds in mice

There has been growing demand for the scintigraphic detection of cancer. A number of radioactive substances have been investigated to meet this demand.[3] However, success has been limited to this date. Recently we[4,5] and French workers[6] reported independently cobalt chelate of bleomycin(Co-BLM) as a promising tumor acanning agent. After the reports, various metal chelates of BLM have been examined for this purpose.[7–16] The distribution of metal-BLM in mice was studied using radioactive metal ions.

This paper describes physicochemical characteristics of Co-BLM as well as more detailed distribution studies of BLM and its chelates.

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3) K. Hisada, *Radioisotopes*, **24**, 204 (1975).
4) A. Kono, M. Kojima, and T. Maeda, *Radioisotopes*, **21**, 118 (1972).
5) T. Maeda, A. Kono, and M. Kojima, *Radioisotopes*, **21**, 436 (1972).
6) J.P. Nouel, H. Renault, J. Robelt, C. Jeanne, and L. Wicart, *La Nouvelle Press Medicale*, **8**, 95 (1972).
7) W.C. Eckelman, W.J. Rzeszotarski, B.A. Siegel, H. Kubota, M. Chelliah, J. Stevenson, and R.C. Reba, *J. Nucl. Med.*, **16**, 1033 (1975).
8) R.B. Grove, W.C. Eckelman, and R.C. Reba, *J. Nucl. Med.*, **14**, 917 (1973).
9) R.B. Grove, R.C. Reba, and W.C. Eckelman, *J. Nucl. Med.*, **15**, 386 (1974).
10) M.S. Lin, D.A. Goodwin, and S.L. Kruse, *J. Nucl. Med.*, **15**, 338 (1974).
11) T. Mori, K. Hamamoto, and K. Torizuka, *J. Nucl. Med.*, **14**, 431 (1973).
12) J.P. Nouel, J. Robelt, and A. Bertrand, In Proceeding of the First World Congress of Nuclear Medicine, Tokyo, Japan, p. 129, 1974.
13) J.J. Rasker, M.A.P.C. Van de Poll, H. Beekhuis, M.G. Woldring, and H.O. Nieweg, *J. Nucl. Med.*, **16**, 1058 (1975).
14) Y. Suzuki, K. Hisada, and T. Hiraki, *Radiology*, **113**, 139 (1974).
15) M. Tanaka and T. Maeda, *Jpn. J. Intern. Med.*, **32**, 881 (1973).
16) M.L. Thakur, M.V. Merric, and S.W. Gunasekera, Symposium on new Developements in Radiopharmaceuticals and Labeled Compound, IAEA-SM Copenhagen, March, 1973.

Experimental

Preparation of Metal-BLM——BLM-A_2 preparation which was kindly supplied from Nippon Kayaku Co. LTD was used in the present study. BLM-A_2 (^{14}C) was labeled at the sulfur bound methyl carbon (-S-$^{14}CH_3$). Metal complexes of BLM were prepared by mixing metal ions with an equimolar amount of BLM in aqueous solution and adjusting its pH to 6.5—7.0 with 0.1 N NaOH.[4] The chelating activity of BLM to each metal ions was proved by using radioisotopes such as ^{57}Co, ^{64}Cu, ^{67}Ga, ^{111}In, ^{65}Zn and ^{59}Fe. BLM chelates were separated by thin-layer chromatography (TLC) on silica gel plate (E. Merck F_{254}), with a solvent system, MeOH–10% CH_3COONH_4 in H_2O (1: 1). The radioactivity on TLC plates was detected with a Aloka JTC-201 TLC scanner. BLM was located with ultraviolet (UV)-irradiation (at 254 nm, Manasuru Co. LTD). The radioactivity of γ-emitters was measured with a TEN EA 14 well type scintillation counter and ^{14}C with a Aloka model 502 liquid scintillation counter.

The Measurements of Spectra——The absorption spectra of BLM and Co-BLM were measured in neutral aqueous solutions with Shimadzu Multipurpose model MPS-50L (240—800 nm) and Hitachi EPS-2 (800—1200 nm) spectrophotometers. Circular dichroism (CD) spectra were obtained in neutral aqueous solutions (BLM-A_2; 1.75×10^{-4} M) with a Nihon Bunko model ORD/UV-5 CD spectrometer.

Labeling of Metal-BLM——For ^{67}Ga labeling ^{67}Ga-citrate was converted into $^{67}GaCl_4^-$ ion in 6 N HCl and the anion was adsorbed on anion exchanger (Dowex 2-X8, 1×5 cm). After wash out of citric acid with 6 N HCl, ^{67}Ga(III) was eluted with distilled water as $^{67}GaCl_3$. $^{67}GaCl_3$ thus obtained was used for preparation of ^{67}Ga–BLM. Other radioisotopes used for labeling of metal-BLM were chloride forms in 0.1 N HCl solution.

Distribution of Metal-BLM in Tumor Bearing Mice——Male mice of ddN strain weighing about 25 g were used. Ehrlich ascites tumor cells, transplanted into the thigh of the mice, grew to the size of 1.0—1.5 cm in diameter in about 10 days. To these animals, BLM, metal-BLM or unchelated metal ions dissolved in 0.2 ml water (pH 6.5) were injected intraperitoneally (5 μCi to each mouse). The animals were anesthetized with ether 1 hr or 24 hr after the administration. Several tissues including tumor were taken out, weighed and their radioactivity was measured. The sera were dialyzed against 2000 ml of distilled water to remove the protein unbound radioactivity. The 20% tumor tissue homogenate in 0.25 M sucrose containing 3.3×10^{-3} M $CaCl_2$ was fractionated with refrigerated centrifuge (Hitachi 18PR-3) at $1000 \times g$ for 10 min. The deoxyribonucleic acid (DNA) contents of each fraction were determined by diphenylamine method.[17] Five ml of the homogenate of the tumor tissue from the ^{57}Co-BLM injected mouse, was lyophilyzed, and then extracted with 5 ml of methanol. The extract was analyzed by TLC. Whole body autoradiograms were taken according to the method described previously.[18]

Results

Chemical Study

Binding of Metal Ions to BLM——When radioisotopes of Cu(II), Co(II), In(III) and Ga(III) were mixed with BLM–A_2, the radioactivity of these ions was detected at the spot of BLM–A_2 on TLC plates. The typical thin-layer chromatograms of ^{57}Co–BLM and $^{57}CoCl_2$ are shown in Fig. 1, A and B. Other metal chelates of BLM gave almost the same chromatograms as Co–BLM. When BLM–A_2 was mixed with 1.3 equimolar amounts of $^{57}CoCl_2$, approximately 0.3 equimolar amounts of radioactivity was observed as free ion on TLC plate (Fig. 1, B). Citric acid prevented Ga(III) from binding to BLM–A_2. When citric acid was removed from ^{67}Ga-citrate with anion exchanger, Ga(III) bound to BLM(Fig. 1, C). However, Ga(III) dissociated from BLM in neutral and alkaline solution. Fe(III) and Zn(II) did not bind to BLM under the similar conditions (Fig. 1, D).

Ion Exchange and Ligand Substitution of Co-BLM——^{57}Co–BLM–A_2 was allowed to stand with either 4 equimolar amounts of disodium ethylenediaminetetraacetate(EDTA) or 10 equimolar amounts of $CoCl_2$ at room temperature. Radioactivity was detected only at the spot of Co–BLM–A_2 on TLC plates even after 30 days. Aqueous ^{57}Co–BLM–A_2 was mixed with 5% dithizone or 2% 8-hydroxyquinoline chloroform solution. No radioactivity was detected in the chloroform layer.

UV and CD Spectra——When Co(II) and an equimolar amount of BLM–A_2 were mixed, new absorption bands appeared at 450 nm and 580 nm(Fig. 2). Molar extinction coefficients

17) K. Burton, *Biochem. J.*, **62**, 315 (1956).
18) A. Kono, M. Kojima, and T. Maeda, *Radioisotopes*, **19**, 507 (1970).

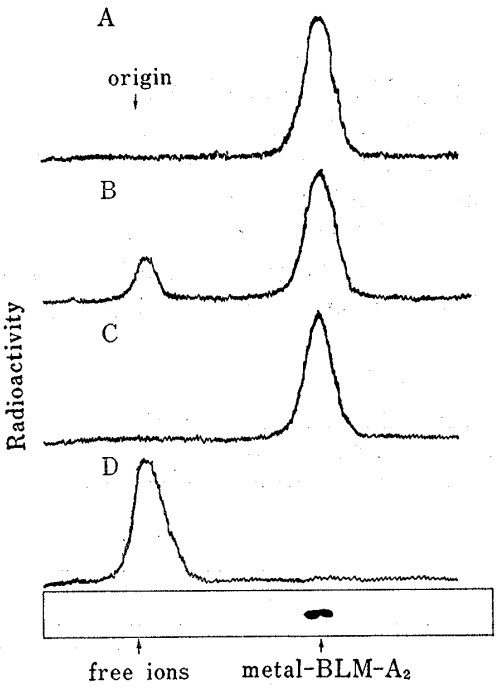

Fig. 1. Thin-Layer Chromatograms of BLM Metal Chelates

$BLM-A_2$; 7×10^{-7} mol in neutral aqueous solution (pH 6.8)
A) $^{57}CoCl_2$, 7×10^{-7} mol. B) $^{57}CoCl_2$, 9.1×10^{-7} mol.
C) $^{67}GaCl_3$, 7×10^{-7} mol. D) $^{65}ZnCl_2$, 7×10^{-7} mol.
TLC; silica gel.
solvent system, MeOH: H_2O containing 10% CH_3COONH_4 (1:1)
detection, Radiochromatoscanner and 254 nm Irradiation lamp.

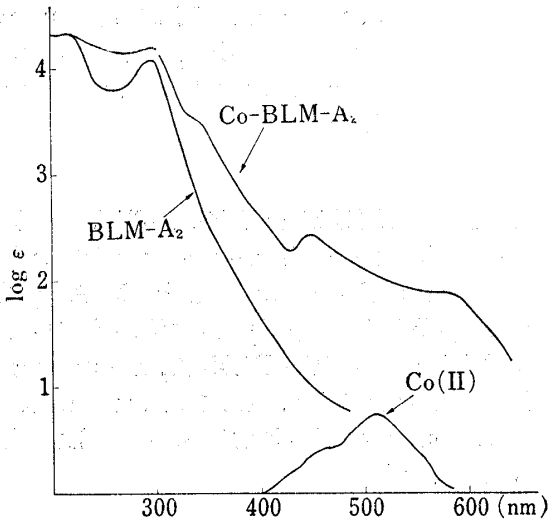

Fig. 2. UV Absorption Spectra of BLM, Co-BLM, and $CoCl_2$ in Neutral Aqueous Solution (PH6.8)

$BLM-A_2$; 3.3×10^{-5}M (220—400 nm).
$Co-BLM-A_2$; 3.3×10^{-5}M (220—400 nm)
2.9×10^{-3}M (400—700 nm).
$CoCl_2$; 5.7×10^{-2}M (350—700 nm).

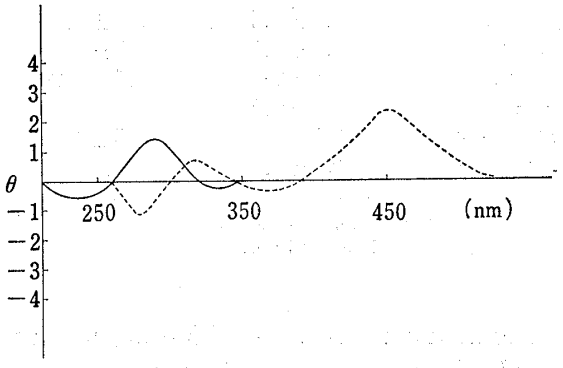

Fig. 3. CD Spectra of BLM and Co-BLM in Neutral Aqueous Solution (pH 6.8)

$BLM-A_2$, $Co-BLM-A_2$; 1.75×10^{-4}M.
------: $Co-BLM-A_2$. ——: $BLM-A_2$.

TABLE I. Distribution of Radioactivity in Ehrlich Solid Tumor Bearing Mice 1 hr after *i.p.* Injection of $^{64}Cu-BLM-A_2$

Tissue	Distribution ratio (%)[a,b]	Tissue	Distribution ratio (%)[a,b]
Tumor	0.34±0.07	Lung	0.62± 0.05
Liver	0.82±0.12	Heart	0.31± 0.06
Kidney	1.67±0.46	Testis	0.24± 0.07
Spleen	0.23±0.01	Blood	0.52± 0.07
Stomach[c]	1.47±0.74	Urine	26.6±10.
Intestine[c]	2.95±0.98		

a) $\frac{\text{radioactivity of tissue}}{\text{total radioactivity injected}\times\text{tissue weight (g)}}\times100$.
b) Means of 3 mice.
c) With its contents.

of Co–BLM–A_2 were 256 and 72 at 450 nm and 580 nm, respectively. The CD profile of Co–BLM–A_2 consisted of a main positive band at 450 nm, a minor positive band at 320 nm and two negative bands at 370 nm and 280 nm, whereas BLM–A_2 had a single positive band at 285 nm(Fig. 3).

Biological Study

Distribution of BLM and Metal-BLM in Tumor Bearing Mice——Cu–BLM: Distribution ratios of radioactivity in tissues of mice are shown in Table I. There is a marked difference between ^{64}Cu–BLM–A_2 and Cu–BLM–A_2 (^{14}C) injected mice. Tumor to liver concentration ratios of Cu–BLM–A_2 (^{14}C) and ^{64}Cu–BLM–A_2 were 1.24 and 0.41, respectively.

Co–BLM: In ^{57}Co–BLM–A_2 administered mice, 3.6 and 1.8% of the radioactivity were found in tumor at 1 hr and 24 hr after injection, respectively. All the radioactivity in urine was at the spot of Co–BLM–A_2 on TLC plate. Table II shows %-radioactivity found in organs of the mice.

Table II. Distribution of Radioactivity in Ehrlich Solid Tumor Bearing Mice 1 hr after and 24 hr after *i.p.* Injection of ^{57}Co–BLM–A_2

Tissue	Distribution ratio (%)[a,b]		Tissue	Distribution ratio (%)[a,b]	
	1 hr	24 hr		1 hr	24 hr
Tumor	3.6±1.1	1.8±0.43	Lung	1.7±0.4	0.15±0.15
Liver	2.0±0.5	1.7±0.46	Heart	1.2±0.4	0.10±0.05
Kidney	9.5±2.9	1.7+0.20	Testis	1.3±0.4	0.10±0.03
Spleen	1.2±0.5	0.8±0.30	Blood	2.4±0.7	0.07±0.02
Stomach[c]	1.5±0.4	0.5±0.20	Urine	65.3±7.0	66.5±3.3
Intestine[c]	1.7±0.7	1.8±0.60	Feces	—	15.0±6.6

a, c) Same as Table I.
b) Means of 7 mice.

The distribution of Co–BLM–A_2 (^{14}C) in mice 1hr after the administration is shown in Table III. The distribution of radioactivity was nearly the same in ^{57}Co–BLM–A_2 and in Co–BLM–A_2 (^{14}C). The distribution of chloride form of ^{57}Co(II) in the tumor bearing mice was completely

Table III. Distribution of Radioactivity in Ehrlich Solid Tumor Bearing Mice 1 hr after *i.p.* Injection of BLM–A_2(^{14}C) or Co–BLM–A_2(^{14}C)

Tissue	Distribution ratio (%)[a,b]		Tissue	Distribution ratio (%)[a,b]	
	BLM–A_2(^{14}C)	Co–BLM–A_2(^{14}C)		BLM–A_2(^{14}C)	Co–BLM–A_2(^{14}C)
Tumor	0.90±0.11	2.24±0.58	Lung	1.02±0.51	1.01±0.25
Liver	0.63±0.23	0.85±0.25	Heart	0.51±0.21	0.35±0.05
Kidney	5.82±3.11	3.02±0.40	Testis	0.72±0.38	0.25±0.05
Spleen	0.62±0.32	1.15±0.61	Blood	1.42±0.45	1.31±0.60
Stomach[c]	0.55±0.26	1.91±0.60	Urine	66.3±5.0	65.4±4.2
Intestine[c]	0.76±0.28	2.95±1.40			

a, b, c) Same as Table I.

different from that of ^{57}Co–BLM 1 hr and 24 hr after injections(Table IV). By 24 hr dialysis of the serum of mouse administered with ^{57}Co–BLM for 1 hr, 63% of radioactivity was lost. The loss was 19% in $^{57}CoCl_2$ injected mouse.

Table IV. Distribution of Radioactivity in Ehrlich Solid Tumor Bearing Mice 1 hr and 24 hr after Injection of $^{57}CoCl_2$

Tissue	Distribution ratio (%)[a,b] 1 hr	24 hr	Tissue	Distribution ratio (%)[a,b] 1 hr	24 hr
Tumor	2.3±0.9	1.3±0.2	Lung	3.1±0.9	1.3± 0.3
Liver	10.5±5.0	3.0±0.4	Heart	2.7±0.7	1.2± 0.2
Kidney	8.7±4.3	2.5±0.6	Testis	2.6±1.1	0.6± 0.04
Spleen	5.7±4.0	1.2±0.2	Blood	6.5±2.1	1.3± 0.5
Stomach[c]	6.1±4.0	1.6±0.2	Urine	5.0±2.3	38.0±12.0
Intestine[c]	5.7±3.1	7.2±5.0	Feces	—	9.0± 3.0

a, b, c) Same as Table II.

The increase of BLM dose caused a high accumulation of radioactivity of ^{57}Co in tumor tissue and other organs. However, the tumor to organs and tumor to blood ratios were unaffected. The urinary excretion of radioactivity(^{57}Co) increased with an increase of BLM dose.

As seen from the whole body autoradiograms(Fig. 4), the tumor tissues are clearly distinguished from surrouding tissues, and radioactivity(^{57}Co) is located only in actively living area in tumor tissues.

^{67}Ga–BLM and ^{111}In–BLM: When the urine of mice was analyzed by TLC 1 hr after injection of ^{67}Ga–BLM or ^{111}In–BLM, 40—50% of radioactivity was present at different spots from BLM(Fig. 5). When ^{67}Ga-citrate was injected to tumor bearing mice, radioactivity of ^{67}Ga in the tumor tissue was about five times more than that of ^{67}Ga–BLM at 24 hr. However, the tumor to liver and the tumor to blood ratios were almost the same with these two ^{67}Ga preparations.

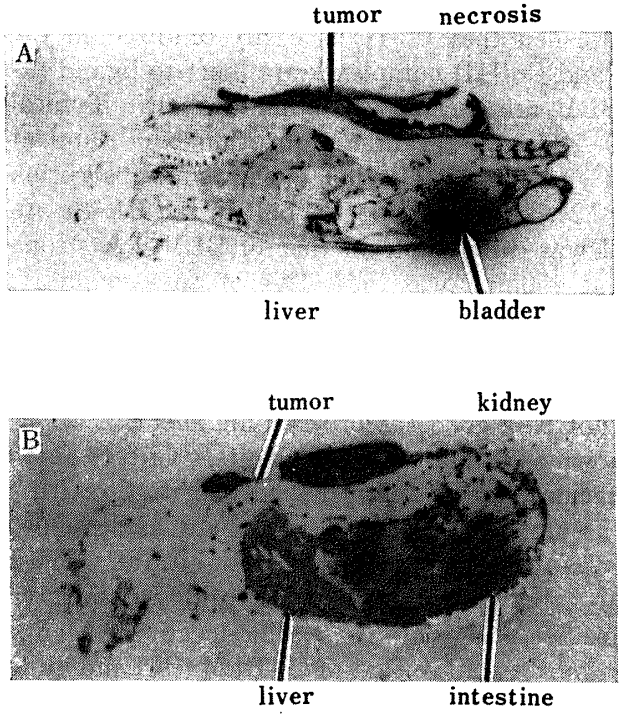

Fig. 4. Whole Body Autoradiograms of ^{57}Co–BLM–A_2 Injected Mice Bearing Ehrlich Solid Tumor

A) 1 hr after injection.
B) 24 hr after injection.

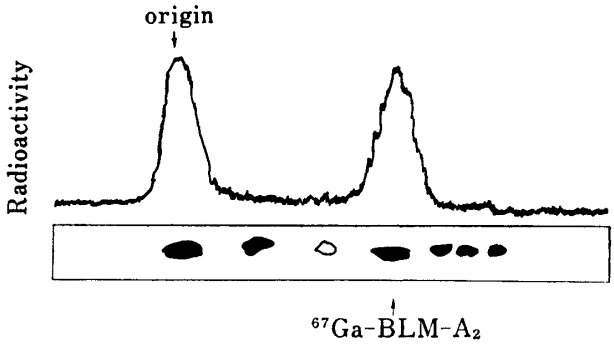

Fig. 5. Thin-Layer Chromatograms of the Urine of the ^{67}Ga–BLM–A_2 Injected Mouse

Urine; collected at 1 hr after injection of 7×10^{-7}M ^{67}Ga-BLM-A_2.
TLC conditions; same as Fig. 1.

Distribution of Co-BLM or Co(II) in Solid Tumor Tissue——Radioactivity found in nuclear fraction of tumor homogenate is listed in Table V. The ratio of the radioactivity to DNA

content in the nuclear fraction was almost constant during the purification steps of the nuclei. In $^{57}CoCl_2$ injected mice, the ratio became about an half of crude nuclear fraction.

Table V. Distribution Ratios of Radioactivity in the Nuclear(N.) Fraction of Ehrlich Solid Tumor Tissue Homogenate

	Radioactivity in N. Fraction (%)[a]	DNA content in N. fraction (%)[b]
^{57}Co-BLM-A_2	68.5	70.3
Co-BLM-A_2(^{14}C)	77.6	71.6
Cu-BLM-A_2(^{14}C)	19.0	—
BLM-A_2(^{14}C)	22.5	82.3
$^{57}CoCl_2$	29.5	—

a) Ratios to total radioactivity in the 20% homogenate.
b) Ratios to total DNA content in the homogenate.

Discussion

Co(II) formed a BLM chelate of an equal stability to Cu(II). Zn(II) did not bind to BLM. It has been reported that small amounts of Zn(II) and Co(II) affect the DNA binding[19] and DNA single stand scission[20] activities of BLM. Hence, it may be concluded that Zn(II) and Co(II) affect the biological activity of BLM in different ways. Ga-BLM and In-BLM showed small complex stability constants and are assumed to dissociate in physiological conditions. From Co(II), 1:1 Co-BLM complex was formed. Ligand exchange reaction of the complex was extremely slow. It is well established that Co(II) is easily oxidized in the presence of air and powerful complexing agent and Co(III) complexes are inert to ligand exchange.[21] Bands at 450 and 580 nm of Co-BLM are ascribed to bound cobalt. Typical Co(III) chelates have absorption bands at 350—400 nm, the first absorption band, and at 500—600 nm, the second absorption band.[22] Generally, Co(II) chelate shows an absorption band at 1000—1200 nm[23] which lacked in Co-BLM. These support cobalt in the BLM complexes is trivalent. The CD profile of Co-BLM was different from that of BLM. A strong positive band associated with the absorption at 450 nm, was indicative of an asymmetric interaction of cobalt and BLM.

The higher accumulation of radioactivity in the liver of ^{64}Cu-BLM than Cu-BLM(^{14}C) indicated that Cu(II) dissociated from BLM and deposited in the liver. The tumor affinity of ^{64}Cu-BLM was not large, so ^{64}Cu-BLM may not be a good tumor scanning agent.

^{67}Ga-citrate and ^{111}In-citrate have been known as tumor scanning agents. Citrate anion is used for a protection from radiocolloid formation, so this anion has nothing to do with a tumor affinity. ^{67}Ga-BLM and ^{111}In-BLM are assumed to be unstable in a body as described above. The distribution ratio of ^{111}In-BLM was almost the same as ^{111}In-citrate. These suggested that most of residual radioactivity in a body was attributable to ions dissociated from BLM. Then ^{67}Ga-BLM and ^{111}In-BLM are not said as tumor specific localizing agents.

The distribution pattern of ^{57}Co-BLM was almost equal to that of Co-BLM (^{14}C) and quite different from that of $^{57}CoCl_2$ 1 hr after injection. The results of the dialysis of the sera were different between Co-BLM and $CoCl_2$. These facts strongly indicate that Co-BLM was fairly

19) H. Suzuki, K. Nagai, E. Akutsu, H. Yamaki, N. Tanaka, and H. Umezawa, *J. Antibiotics*, **23**, 473 (1970).
20) H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka, and H. Umezawa, *J. Antibiotics*, **22**, 446 (1969).
21) J.O. Edwards, "Inorganic Reaction Mechanisms," W.A. Benjamin Inc., New York, 1964, Chapter 6, p. 90.
22) R. Tsuchida and Y. Shimura, *Bull. Chem. Soc. Japan*, **28**, 572 (1955).
23) R. Carbin, "Transition Metal Chemistry," Vol. 1, Marcel Dekker Inc., New York, 1965, p. 3.

stable in a mammalian body. It is worth noting that Co-BLM was accumulated in the tumor tissue much more than BLM or $CoCl_2$. For this reason Co-BLM is superior to BLM as a tumor scanning agent.

The affinity of an anticancer antibiotic to the nuclei of tumor cells was particularly interesting in correlation to its biological activity.[24] Unique property of Co-BLM is the excellent affinity to tumor cell nuclei which was not observed in BLM, $CoCl_2$ and other BLM metal chelates. Moreover, in fractionation of tumor tissue of ^{57}Co-BLM injected mice, radioactivity seemed to be closely associated with DNA content. This suggests Co-BLM may be bound strongly to nuclear components or a nuclear membrane. This may be correlated to the affinity of Co-BLM in active parts of tumor tissue.

The further studies will be described in the succeeding papers.

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24) I.H. Goldberg and P.R. Friedman, *Annu. Rev. Biochem.*, **40**, 775 (1971).