[Chem. Pharm. Bull. 24(10)2327—2334(1976)]

UDC 615.31.033.076.9 : 546.26.02.1409

Autoradiographic Studies on the Distribution of Quaternary Ammonium Compounds. IV.[1] Adsorption of Bisonium Ions to Rat Tissues as revealed by *in Vitro* Whole-body Adsorption Autoradiography[2]

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(Received December 27, 1975)

A technique which was designated as "whole-body adsorption autoradiography" was applied to find binding characteristics of bis- and mono-quaternary ammonium ions by rat various tissues. Rat freeze-dried whole-body sections were incubated in an aqueous solution of ^{14}C-labeled deca-, hexa- and dimethonium and tetraethyl- and cetyltrimethylammonium. After rinsing and being freeze-dried, the adsorption of radioactivity was detected by autoradiography. It was found that bisonium ions are highly adsorbed in the cartilage tissues of vertebra, sternum and trachea, wherein a similar high accumulation occurs *in vivo*, and appreciably adsorbed in the thymus, salivary gland and bone marrow. These adsorptions were interpreted as being due to an ionic bonding of the bis-quaternary ammonium cations to the sulfate and phosphate anions of the chondroitin sulfate and cellular desoxyribonucleic acid (DNA), respectively, probably by forming a bridged structure, with an aid of Alcian Blue staining of the rat whole-body sections (whole-body histochemistry). Close relations between the *in vitro* adsorption characteristics and the *in vivo* distribution patterns are pointed out and discussed. It was thus concluded that a physico-chemical binding of a drug molecule to the tissue macromolecules plays an important role in determining the *in vivo* distribution pattern in animal body and that a combined use of this method with conventional whole-body autoradiography provides a useful tool in establishing the drug distribution characteristics and their interpretation.

It was reported in our previous papers[1,4] that bisquaternary ammonium ions have a common characteristic of a rapid and high accumulation in the cartilage tissues following administration to rats or mice. It was further suspected that this must be caused from a binding of bisonium ion to sulfate ions in chondroitin sulfate, possibly by forming a bridged structure. It is generally of great importance to know what factors play roles in determining the distribution pattern of drugs in animal body and it is expected that a physico-chemical binding of a drug molecule to tissue macromolecules is one of the most important factors involved, as well as the transport characteristic.

In the present investigations, a technique which might be called "whole-body adsorption autoradiography" was applied to examine the binding characteristics of bisquaternary ammonium ions to rat various tissues *in vitro*.

Material and Method

Materials——Decamethonium-^{14}C dibromide (specific activity, 20.9 mCi/mmole) and hexamethonium-^{14}C dichloride (specific activity, 1.87 mCi/mmole) were purchased from the Radiochemical Center, Amersham, England. Dimethonium-^{14}C diiodide with specific activity of 3.51 mCi/mmole was prepared by the reaction of N,N′-tetramethyl ethylenediamine with methyl-^{14}C iodide in ethanol, followed by recrystallization from methanol–water. Tetraethylammonium-^{14}C iodide with specific activity of 0.84 mCi/mmole was prepared by refluxing triethylamine with ethyl-^{14}C iodide in ethanol for 2 hr. After cooling, the precipitates were recrystallized

1) Part III: H. Shindo, E. Nakajima, E. Shigehara and N. Miyakoshi, *Chem. Pharm. Bull.* (Tokyo), **22**, 2502 (1974).
2) A part of this work was presented at the 93rd Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1973.
3) Location: *Hiromachi 1-chome, Shinagawa-ku, Tokyo.*
4) H. Shindo, I. Takahashi and E. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **19**, 1976 (1971).

twice from ethanol–water. All the compounds were ascertained to give single radioactive spot on thin-layer chromatogram prior to each experiment. All nonradioactive quaternary ammonium compounds and other chemicals used were of reagent grade and used without further purification.

Whole-body Adsorption Autoradiography——Male rats of Wistar-Imamichi strain weighing about 100 g were used. The animals were lightly anesthetized with ether and frozen by immersion in a mixture of hexane and solid carbon dioxide at about −70°. After a frozen animal was embedded on a microtome stage with aqueous carboxymethyl cellulose gel, the sagittal 50 μ sections through the whole animal were cut with a heavy microtome (Yamato type 1111) according to Ullberg's method[5] and dried at −15°. Several sections including the vertebral bone were thus obtained from each animal. The dried section on adhesive tape was then incubated in 50 to 100 ml of 2 to 3 mM aqueous solution of the test labeled compound (*e.g.*, about 0.1%, 0.05 μCi/ml solution in the case of decamethonium-^{14}C dibromide) at 5° or room temperature for 20 min. In some cases, the sections were fixed in 10% formaldehyde solution at 20° for 30 min prior to the incubation, but no appreciable change was observed in the adsorption pattern depending on the fixation. After the incubation, the sections were rinsed several times in cold distilled water and frozen rapidly on a surface of a block of solid carbon dioxide. The frozen sections were then dried overnight at −15° and brought to contact with Sakura Type N X-ray film and exposed for a period of 1 to 3 days.

In order to see the competitive effect of different quaternary ammonium ions on the adsorption, the sections were incubated in 0.1% (2.4 mM) aqueous solution of decamethonium-^{14}C dibromide added with 10 times molar non-radioactive dimethonium, hexamethonium, tetraethyl ammonium and cetyltrimethylammonium ions.

Staining of Whole-body Sections with Alcian Blue——The method[6] of histochemical staining with Alcian Blue established for tissue section was applied to rat whole-body sections. The freeze-dried whole-body sections on the tape were fixed in 10% formaldehyde solution at room temperature for 30 min. After rinsing them in running water and blotting with paper towel, the sections were stained in 1% solution of Alcian Blue 8GX (Imperial Chemical Industry, England) in 0.1 N HCl at pH 1.0 or in 3% acetic acid at pH 2.5 at room temperature for 30 min. After rinsing in running water, the stained sections were dried at room temperature.

Differential staining of mucopolysaccharides on the whole-body section was performed by applying the histochemical method of Scott, *et al.*[7] The rat whole-body sections fixed in 10% formaldehyde were stained in 0.05% solution of Alcian Blue 8GX in 0.05 M acetate buffer at pH 5.7 or in 3% acetic acid solution at pH 2.4 containing different concentration of $MgCl_2$: 0, 0.05, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, and 1.0 M, at room temperature for 1.5 hr. The sections were rinsed and dried as described above.

Binding Experiment of Quaternary Ammonium Ions with Chondroitin Sulfate——Each 10 ml aqueous solution containing 40 mg sodium chondroitin sulfate and 1.2 μmoles (0.2 μCi) of ^{14}C-labeled deca-, hexa- and dimethonium and tetraethyl- and cetyltrimethylammonium was dialyzed in a Visking cellulose tube against 350 ml of distilled water at room temperature overnight. The radioactivity was then determined for the fluid inside and outside the tube by liquid scintillation spectrometer (Beckman LS-250) to calculate % dialyzed. In a further experiment to compare the strength of binding, each 1 ml of the test solutions were dialyzed progressively against 40 ml of aqueous solution of KCl of increasing concentrations: 30, 75, 150, and 300 mM.

Result

Adsorption of ^{14}C-Labeled Decamethonium, Hexamethonium and Dimethonium Ions by Rat Whole-body Sections

Autoradiograms obtained after *in vitro* incubation of rat whole-body sections with ^{14}C-labeled deca-, hexa- and dimethonium ions are represented in Fig. 1. These showed that the highest radioactivity is located specifically in the cartilage tissues such as that of the vertebra, sternum, trachea and bronchi in all the bis-quaternary ammonium ions tested, indicating a high affinity of these ions to some component of the cartilage tissues. A very high selectivity of this adsorption can be seen clearly from an enlarged autoradiogram shown in Fig. 2. In addition to the cartilage tissues, a high adsorption of radioactivity was shown in the thymus and an appreciable radioactivity in the salivary gland and bone marrow, as can be seen from Fig. 2. Only a faint, nonuniform radioactivity was observed in some parts of the skeletal muscle, which might be due to mainly a nonspecific adsorption. No substantial difference

5) S. Ullberg, *Acta Radiol. Suppl.*, **1954**, 118.
6) A.G.E. Pearse, "Histochemistry—Theoretical and Applied", Vol. 1, 3rd Ed., J. & A. Churchill Ltd., London, 1968, p. 672.
7) J.E. Scott and J. Dorling, *Histochemie*, **5**, 221 (1965).

was noted in the adsorption patterns among three different bis-quaternary ammonium ions tested: di-, hexa- and decamethonium. The adsorption characteristic of bis-onium ions by the cartilage tissues corresponds well to the distribution characteristics observed after *in vivo* administration[4]; however, no appreciable radioactivity was detected in the liver wherein a very high accumulation occurred after administration of deca- and hexamethonium *in vivo*. When the whole-body sections were incubated with decamethonium-^{14}C in the solution containing 3% sodium chloride, almost no adsorption of radioactivity was observed in any tissue on the autoradiogram, including the cartilage tissues, which indicated that the adsorption is inhibited by a high ionic strength of the medium and thus the binding is due to ionic forces.

In contrast to the bis-onium ions, when the whole-body sections were incubated with tetraethylammonium-^{14}C, a typical mono-onium ion, no such a prominent adsorption of radioactivity was detected in the cartilage tissues on the autoradiogram, indicating that the high adsorption at the cartilage tissues is specific for bis-onium type structure. Cetyltrimethylammonium-^{14}C, another mono-onium ion with a long alkyl chain, revealed a completely different pattern of adsorption and the whole sections showed a very high, uniform radioactivity in all tissues, suggesting a non-specific adsorption of the ion to the tissue protein and/or lipid probably because of its surfactive or hydrophobic properties.

Effects of Different Quaternary Ammonium Ions on the Adsorption of Decamethonium-^{14}C

The rat whole-body sections were incubated with decamethonium-^{14}C in the presence of 10 fold quantity of di- and hexamethonium, tetraethylammonium and cetyltrimethylammo-

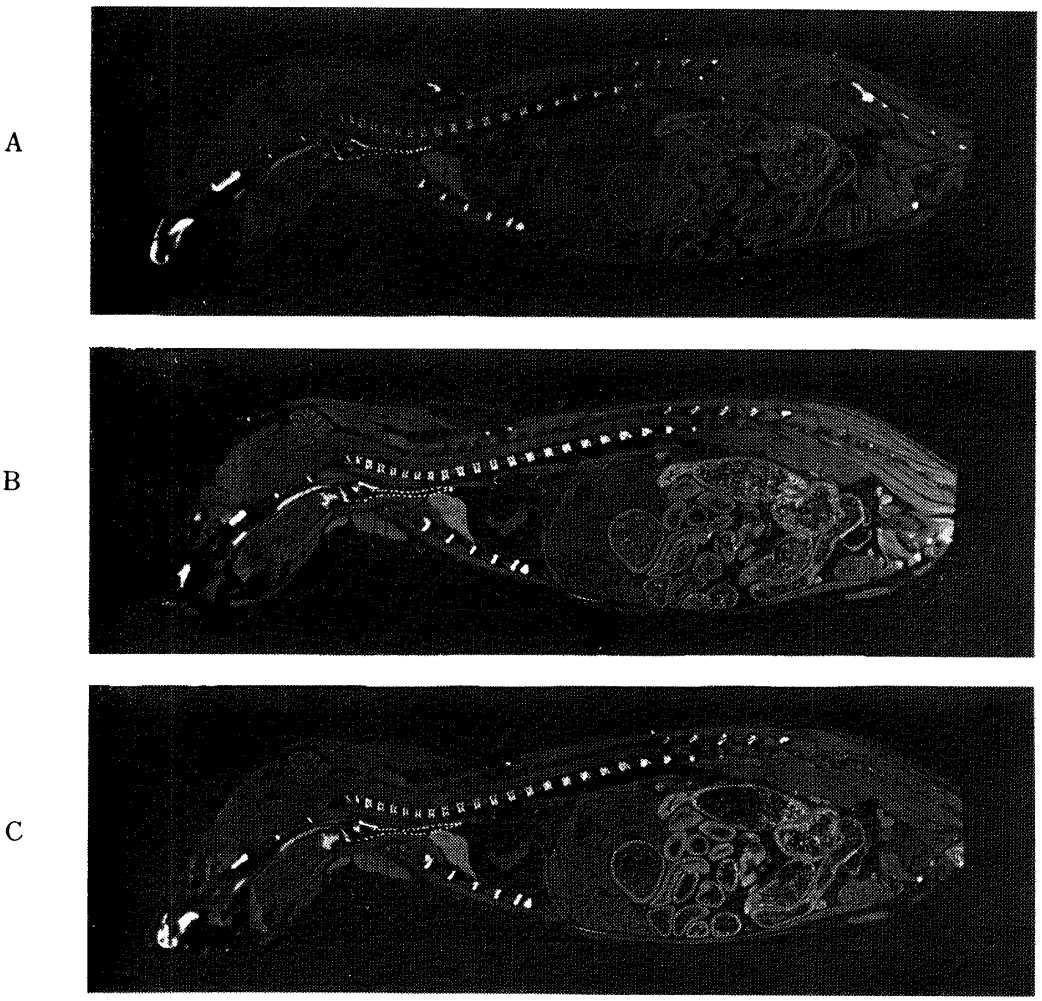

Fig. 1. Autoradiograms of Rat Whole-body Sections after Incubation with ^{14}C-Labeled Decamethonium (A), Hexamethonium (B) and Dimethonium (C)

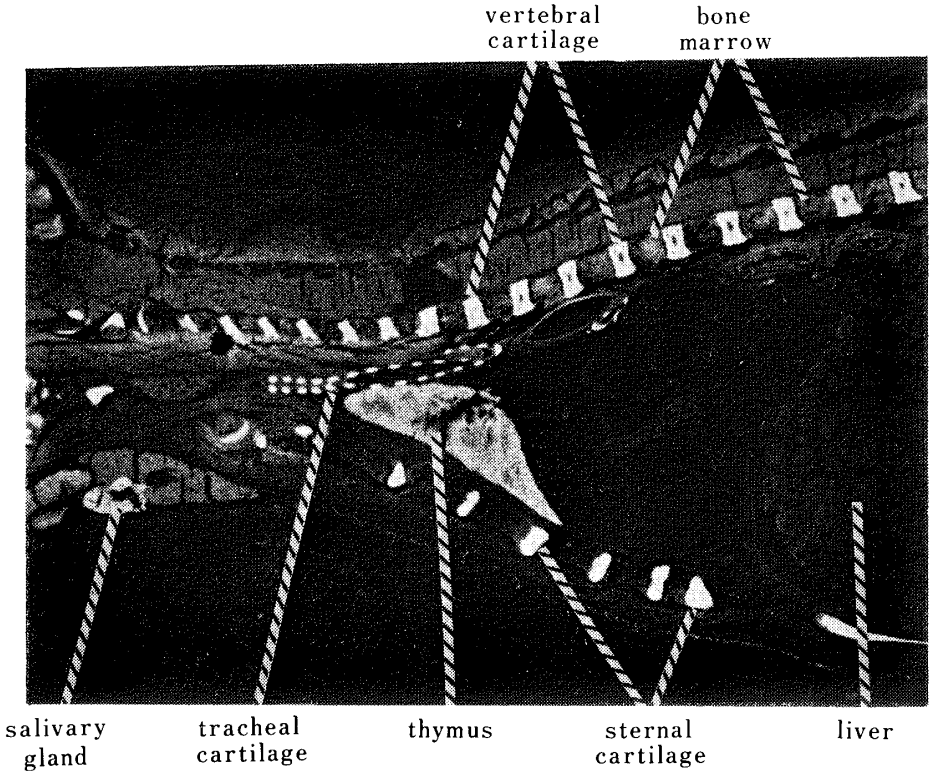

Fig. 2. Enlargement of the Autoradiogram obtained after *in Vitro* Incubation of Rat Whole-body Section with Decamethonium-^{14}C Dibromide

nium ions. The obtained autoradiograms are presented in Fig. 3. Adsorption of radioactive decamethonium ion by the cartilage and other tissues was shown to be significantly and uniformly reduced in the presence of both di- and hexamethonium (Fig. 3-A), while completely no effect was observed with tetraethylammonium (Fig. 3-B). The results suggest that different bis-quaternary ammonium ions compete for a common binding site and confirm further that the adsorption is specific for the bis-quaternary ammonium structure. It was of interest to note that cetyltrimethylammonium ion inhibited almost completely the adsorption of decamethonium in the thymus, salivary gland and skeletal muscle, while no significant inhibition was observed in the cartilage tissues, as can be seen from Fig. 3-C.

Identification of Site of Adsorption by Alcian Blue Staining of Rat Whole-body Sections

Alcian Blue staining of the rat whole-body sections at pH 1.0 and 2.5 are reproduced in Fig. 4. At pH 1.0, only the cartilage tissues such as that of the vertebra, sternum and trachea were stained in a deep blue very selectively. At pH 2.5, in addition to the cartilage tissues, the thymus, salivary gland and bone marrow were stained strongly and the cerebellar glandular layer, epidermis and intestinal mucosa moderately. From microscopic histochemistry,[6] it is known that chondroitin sulfate is stained with Alcian Blue at pH's of both 1.0 and 2.5, while the nuclear desoxyribonucleic acid (DNA) can be stained only at pH 2.5.

In order to further differentiate these substances, the sections were stained with Alcian Blue at pH 2.4 and 5.7 in media containing different concentrations of $MgCl_2$. The results are summarized in Table I, in terms of the relative grade of staining. It was shown that the cartilage tissues were stained most intensely at $MgCl_2$ concentrations of 0.05 to 0.2 M and 0.05 to 0.4 M at pH 5.7 and 2.4, respectively, slightly more intensely in the latter pH. This pattern of staining is in good accordance with the characteristic for chondroitin sulfate described by Scott, *et al.*[7] The thymus and bone marrow, on the contrary, were stained intensely only at the $MgCl_2$ concentration of 0.05 M at both pH's, while in the concentration range from 0.1 to

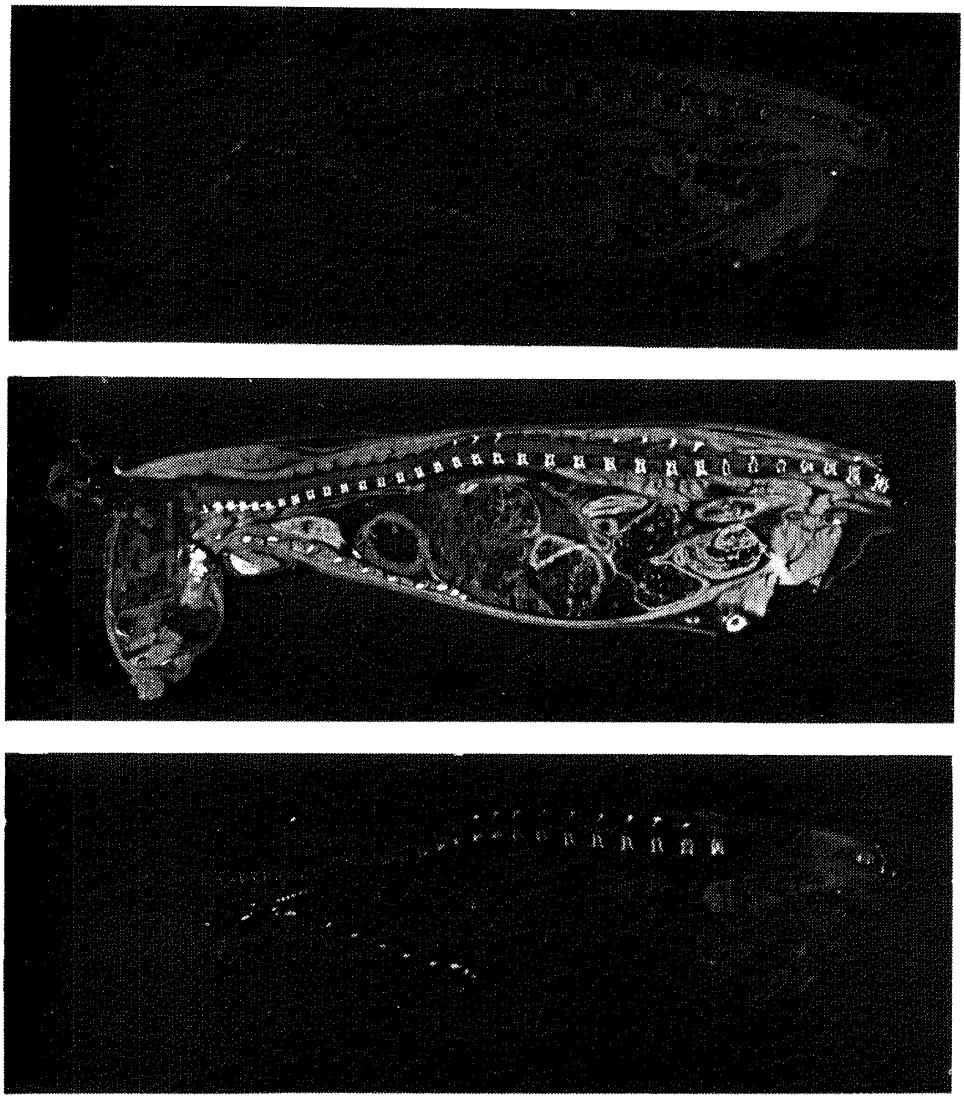

Fig. 3. Autoradiograms after Incubation of Rat Whole-body Sections with Decamethonium-^{14}C in the Presence of 10 fold Quantity of Dimethonium (A), Tetraethylammonium (B) and Cetyltrimethylammonium (C)

0.5 M they were appreciably stained only at pH 2.4. These characteristics are in good accordance with those of the nuclear DNA.

It might be concluded, from the foregoing results, that the adsorption of bis-quaternary ammonium ions in the cartilage tissues is due to their binding to the sulfate anions of chondroitin sulfate, while that in the thymus and bone marrow to their binding to the phosphate anions of cellular DNA and, possibly, ribonucleic acid (RNA).

In Vitro Binding of Quaternary Ammonium Ions to Chondroitin Sulfate

When a mixed solution of ^{14}C-labeled quaternary ammonium ions, di-, hexa- and decamethonium and tetraethyl- and cetyltrimethylammonium, with chondroitin sulfate was dialyzed against water overnight, it was found that only 0.5 to 5.0% of the total radioactivity was dialyzed into the outer medium in all cases, indicating that all the quaternary ammonium ions tested bind strongly to chondroitin sulfate.

In order to compare the relative binding strength, the mixed solution was dialyzed successively against water containing an increasing concentration of KCl. As shown in Table II, tetraethylammonium ion was found to be easily dialyzed at the lowest KCl concentration of 0.03M, indicating that the binding of a simple mono-onium ion is the weakest. All the

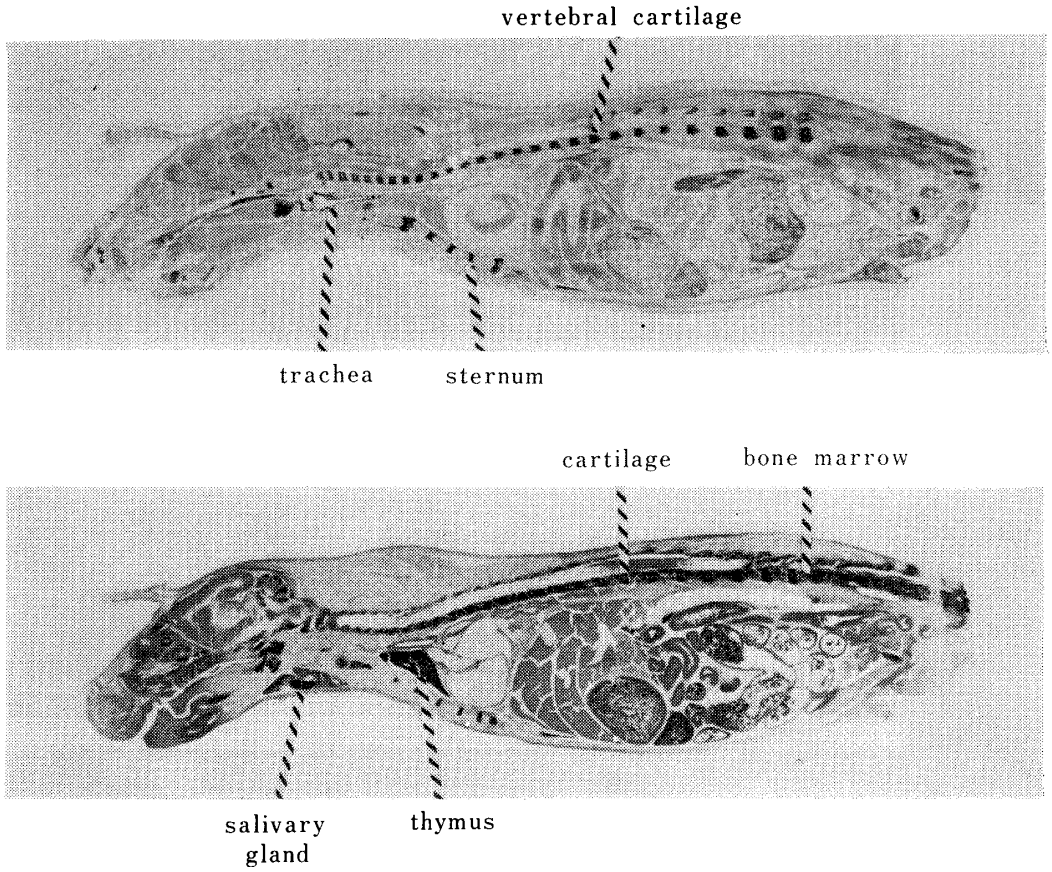

Fig. 4. Alcian Blue Staining of Rat Whole-body Sections at pH 1.0 (A) and 2.5 (B)

Table I. Staining of Rat Whole-body Sections with Alcian Blue in the Presence of Different Concentrations of $MgCl_2$ at pH 2.4 and 5.7

Tissue	pH	Relative grade of staining[a]								
		0	0.05	0.1	0.2	0.4	0.5	0.6	0.8	1.0[b]
Cartilage	5.7	++	+++	+++	+++	++	++	+	±	±
	2.4	+	+++	+++	+++	+++	++	++	+	±
Thymus	5.7	+	++	+	−	−	−	−	−	−
	2.4	+	+++	+	+	±	±	−	−	−
Salivary gland	5.7	++	++	+	±	−	−	−	−	−
	2.4	++	+	+	±	±	±	±	±	±
Skeletal muscle	5.7	+	+	±	±	±	±	−	−	−
	2.4	−	+	±	±	±	±	−	−	−
Bone marrow	5.7	+	++	+	−	−	−	−	−	−
	2.4	++	++	+	+	+	+	+	±	±

a) Relative strength of the staining was evaluated visually on the whole-body sections and expressed as six grades from the strongest (+++) to the weakest (+) and undetectable (−).
b) $MgCl_2$ concentration (M)

bisquaternary ammonium ions tested showed a much stronger binding and were dialyzed at a higher KCl concentration of 0.15 M. There was no significant difference among three different ions. Cetyltrimethylammonium ion showed the strongest binding and was not dialyzed even at a high KCl concentration of 0.3 M. It might be possible, therefore, that the binding of cetyltrimethylammonium is rather hydrophobic than ionic in nature and that this is the reason why it did not compete for the adsorption of decamethonium to the cartilage tissues (Fig. 3-C).

Table II. Dialysis of ^{14}C-Labeled Quaternary Ammonium Ions in a Mixture with Chondroitin Sulfate against Water containing an Increased Concentration of KCl

Quaternary ammonium ion	% dialyzed[a]					Residue (%)
	0	30	75	150	300[b]	
Decamethonium	2.8	18.1	23.1	**51.9**	6.9	0.7
Hexamethonium	0.6	10.3	14.6	**68.8**	3.5	2.8
Dimethonium	2.9	8.6	15.1	**66.0**	9.3	0.9
Tetraethylammonium	4.9	**52.0**	28.1	17.6	0	2.3
Cetyltrimethylammonium	1.1	24.8	0.6	10.5	6.2	**62.4**

a) A mixed solution of ^{14}C-labeled quaternary ammonium ion and sodium chondroitin sulfate was dialyzed successively against aqueous solution of KCl with an increased concentration.
b) KCl concentration (m M)

Discussion

In the previous papers, it was reported that bis-quaternary ammonium ions such as di-, hexa- and decamethonium[4] and pancuronium and d-tubocurarine[1] have a characteristic distribution pattern of being commonly accumulated in the cartilage tissues after administration to rats or mice. From the present results, it is evident that the distribution in the cartilage tissues is caused from a physico-chemical ionic binding of the bisquaternary ammonium cations to the sulfate anions of chondroitin sulfate in the cartilage tissues, possibly by forming a bridged structure. Although the binding nature of the bisquaternary ammonium ions to chondroitin sulfate has been pointed out *in vitro* by Asghar, *et al.*,[8] a very selective adsorption of the ions by the cartilage tissues could be demonstrated by autoradiographic technique visually on the rat whole body sections.

In Table III, the distribution of radioactivity in some tissues is compared for bis- and mono-quaternary ammonium ions between the autoradiographic results obtained after *in vivo* administration[4] and *in vitro* incubation. *In vivo*, mono-onium ions do not show any appreciable distribution in the cartilage tissues, while they show a high distribution in the salivary gland. Bis-onium ions, on the contrary, show *in vivo* a high distribution in the cartilage tissues, while they show almost no distribution in the salivary gland. In the *in vitro* adsorption study, bis-onium ions showed a strong adsorption in the cartilage tissues almost specifically, while mono-onium ions showed no appreciable adsorption, in good agreement with the *in vivo* distribution patterns. In the salivary gland, however, only bis-onium ions showed an appreciable adsorption in contrast to the *in vivo* distribution.

Since there is a transport barrier at the cell membranes *in vivo*, it is reasonable that there are discrepancies between *in vivo* distribution pattern and *in vitro* binding characteristics. It is of interest to note here that a strong adsorption was found for bis-onium ions in the thymus and an appreciable adsorption in the bone marrow *in vitro*. It has been observed, in fact, that decamethonium accumulates very slowly in the thymus after administration to mice,[4] while pancuronium gradually accumulates in the bone marrow.[1] It is highly probable, therefore, that the binding of bis-onium ions to cellular DNA and/or RNA also plays an important role in determining their *in vivo* accumulation in the thymus and bone marrow and some difference in the transport characteristic through the cell membranes between decamethonium and pancuronium ions might determine the observed difference in the *in vivo* distribution patterns. Since these adsorptions are caused from an ionic bonding, it is reasonable that the ions are finally eliminated from the tissues *in vivo*.

8) K. Asghar and L.J. Roth, *Biochem. Pharmacol.*, **20**, 3151 (1971).

Another one of the present results that no adsorption phenomenon was detected in the liver in both mono- and bis-onium ions *in vitro* might indicate that the *in vivo* accumulation and a long retention of radioactive decamethonium[4] and pancuronium[1] ions was not caused by any adsorption or binding of the ions to the tissue macromolecules of the liver. The *in vivo* accumulation might be, therefore, most probably explained by an active transport mechanism involved in the penetration of the ions through the liver cell membranes,[9] although a specific binding of quaternary ammonium ions to the liver lysosomes has been reported.[10]

TABLE III. Comparison between *in Vivo* Distribution and *in Vitro* Adsorption Patterns of Some Bis- and Mono-quaternary Ammonium Ions

	Decamethonium	Hexamethonium	Dimethonium	TEA[a]	CTA[b]
In vivo distribution[c]					
Cartilage tissue	++	+++	+++	−	−
Thymus	+	−	−	−	−
Salivary gland	−	−	−	+++	+++
Skeletal muscle	+	−	−	−	+
Liver	+++	++	±	+	++
In vitro adsorption					
Cartilage tissue	+++	+++	+++	−	++(?)
Thymus	++	++	++	−	+++
Salivary gland	+	+	+	−	+++
Skeletal muscle	−	−	−	−	+++
Liver	−	−	−	−	++

a) TEA=tetraethylammonium
b) CTA=cetyltrimethylammonium
c) Whole-body autoradiograms 60 min after intraperitoneal administration of ^{14}C-labeled compounds to mice

The present results provided a good example which demonstrates that a physico-chemical bonding of a drug molecule to tissue macromolecules plays an important role in determining the distribution pattern of the drug in animal body. The method of *in vitro* incubation of whole animal sections with a radioactive substrate followed by detection by autoradiography might be designated as "whole-body adsorption autoradiography" and must be quite useful for detecting drug binding to tissue components at macroscopic levels and for interpretation of *in vivo* whole-body autoradiography. The method of staining the whole animal sections to differentiate the tissue components as examplified by Alcian Blue staining might be designated as "whole-body histochemistry," as well as the method of enzyme detection on the whole-body sections reported previously.[11] A combined use of these methods with conventional *in vivo* whole-body autoradiography might provide a useful tool in establishing the drug distribution characteristics and their interpretation.

Acknowledgement The authors express their deep gratitude to Dr. H. Watanabe of the Tokyo Medical College for his helpful advice and discussions in the histochemical experiments and to Prof. Dr. S. Tsurufuji of Tohoku University for his valuable discussions. Thanks are also due to Dr. K. Arima, director of this laboratories, and to Dr. Y. Kishida of this laboratories for their kind encouragement.

9) C.B. Christensen, *Acta Pharmacol. Toxicol.*, **28**, 215 (1970).
10) Y. Echigoya, Y. Matsumoto, Y. Nakagawa, T. Suga and S. Niinobe, *Biochem. Pharmacol.*, **21**, 477 (1972).
11) K. Fukuda and H. Shindo, *Acta Histochem. Cytochem.*, **7**, 181 (1974).