BINDING OF AMINOGLYCOSIDE ANTIBIOTICS TO ACIDIC MUCOPOLYSACCHARIDES

Takashi Deguchi, Akio Ishii and Masao Tanaka

Pharmaceuticals Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.
1188 Shimotogari, Nagaizumi-machi, Sunto-gun, Shizuoka, Japan

Binding of aminoglycoside antibiotics to acidic mucopolysaccharides has been studied by means of physicochemical methods. Reactivity was affected markedly by the ionic environment, *e.g.* pH and ionic strength of the medium, the concentrations and the molar ratios of the constituents. The ionic character of binding was further confirmed by gel chromatography. The reduction of metachromasis by an aminoglycoside was also observed. Their affinity is correlated with localization of the aminoglycosides *in vivo*.

According to reactivity, the following descending order of affinity was obtained for each family: neomycin, gentamicin, sagamicin, kanamycin and streptomycin; heparin, chondroitin sulfate and hyaluronic acid. This sequence of aminoglycosides corresponds to the extent of oto-, nephro- and neuro-(acute)toxicity, suggesting that their affinity for acidic mucopolysaccharides contribute to their tissue toxicity.

Aminoglycoside antibiotics interact with other drugs and with the constituents of living matter leading to loss of antibacterial activity. Acidic mucopolysaccharides (AMPS) have been observed to reduce antibacterial activity as the result of interaction with aminoglycosides[1,2], and an interaction of AMPS with aminoglycosides has been suggested to result in accumulation of drug in the kidney[3] or in the inner ear[4,5].

In previous papers[6,7] the authors reported whole body autoradiography of sagamicin-^{14}C in mice and demonstrated that sagamicin was temporarily distributed in cartilaginous tissues other than the cortex of the kidney. The distribution could be caused by the interaction of the aminoglycoside and chondroitin sulfate, an AMPS. Many studies have indicated that biological polyanions interact with various kinds of basic dyes and drugs. In the present investigation, binding of aminoglycosides to AMPS's *in vitro* has been studied quantitatively and their affinity has been correlated with localization or toxicity of aminoglycosides *in vivo*.

Methods

Materials

The following antibiotics (as sulfates) were obtained in pure form of known potency: sagamicin, gentamicin complex, fortimycin A, streptomycin and paromomycin from Kyowa Hakko, kanamycin, dibekacin and ribostamycin from Meiji Seika, and neomycin from Nippon Kayaku. Chondroitin sulfate A and C (ChS A and C), and heparin (Hep) were obtained from Seikagaku Kogyo as sodium salts. Hyaluronic acid (HA) was obtained from the same company as the potassium salt. Acridine orange and methylene blue were purchased from Wako Pure Chemicals.

Experimental

To 5 ml of an AMPS solution, an aqueous solution of an aminoglycoside was added and kept at room temperature, with occasional mixing for 30 minutes. The turbidity of the solution was deter-

mined by a turbidity meter (Nihon Seimitsu Kogaku). Determination of critical salt concentration[8] was carried out by adding an appropriate amount of sodium chloride to an aminoglycoside-AMPS solution. Gel chromatography of the sagamicin complex was carried out on a column (1×25 cm) of Sephadex G-50 (coarse, Pharmacia Fine Chemicals), pre-equilibrated with ChS A (0.5 mM)–NaCl ($I = 0.025$ or 0.25). The aminoglycoside was eluted with the same buffer, fractions of 1 ml collected and assayed by fluorometry (Hitachi MFP-2A) as described previously[9]. Equilibrium dialysis was conducted in seamless cellulose tubing (20/32, Visking Company) containing 5 ml of ChS A solution (1 mM) against aminoglycoside solutions (10 ml) of various concentrations at 37°C for 24 hours. Absorption spectra were obtained with Shimadzu Spectrophotometers QV-50 and MPS-50L.

Results

The relationship between turbidity and the concentrations of aminoglycosides in distilled water is shown in Fig. 1. The molecular weight of the AMPS was calculated as the disaccharide unit. As the concentration of aminoglycoside was increased, the solution showed an opaque colloidal appearance but no precipitate was formed for several hours. The concentrations of aminoglycosides at which turbidity was noted varied with each aminoglycoside. ChS C and other AMPS's (Hep, HA) showed similar patterns of turbidity change. The concentrations at which the turbidity change was observed were affected both by ionic strength and pH, and typical examples are shown in Figs. 2 and 3, respectively. The different water-insoluble complexes were soluble in salt solutions and the salt concentrations (sodium chloride) necessary to solubilize aminoglycoside-ChS A complexes are given in Table 1. These values were obtained from a series of experiments on the effect of ionic strength as shown in Fig. 2. At acidic pH the solutions increased in turbidity as shown in Fig. 3 and the pH dependency of turbidity formation became more distinct as the ionic strength increased.

The relationship between turbidity and the molar ratios (aminoglycoside/ChS A) is shown in

Fig. 1. Turbidity change of the chondroitin sulfate A complex with the concentration of aminoglycoside antibiotics. The concentration of ChS A was 0.5 mM.

a, Neomycin; b, gentamicin; c, sagamicin; d, paromomycin; e, fortimycin A; f, dibekacin; g, kanamycin; h, sagamine; i, ribostamycin; j, streptomycin; k, deoxystreptamine.
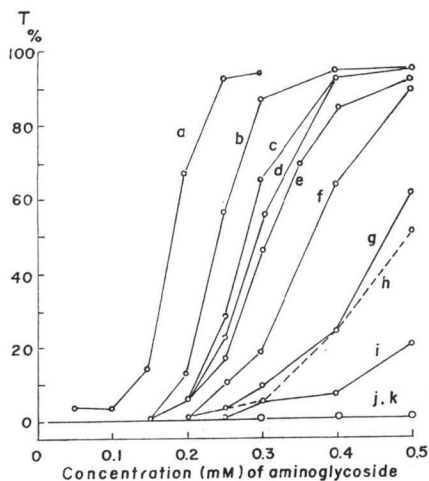
Fig. 2. Effect of ionic strength on binding of sagamicin and kanamycin to chondroitin sulfate A (0.5 mM).

Sagamicin; curve A, at ionic strength 0.025; curve B, 0.10; curve C, 0.15.
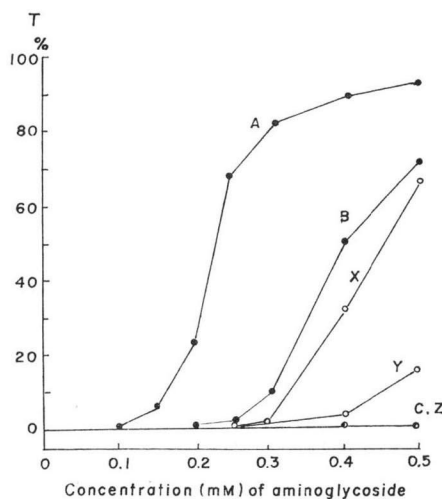Kanamycin; curve X, 0.025; curve Y, 0.035; curve Z, 0.05.

Fig. 3. Turbidity of sagamicin-chondroitin sulfate A at various pH ranges.

The concentrations of both components were 0.5 mM. Curve A: in 0.5 mM citrate phosphate buffer; B: in 5 mM the same buffer.

Table 1. Critical salt concentrations in sodium chloride solution of aminoglycoside-chondroitin sulfate A complexes (at room temperature). For details, see text.

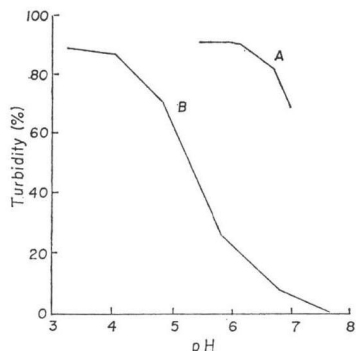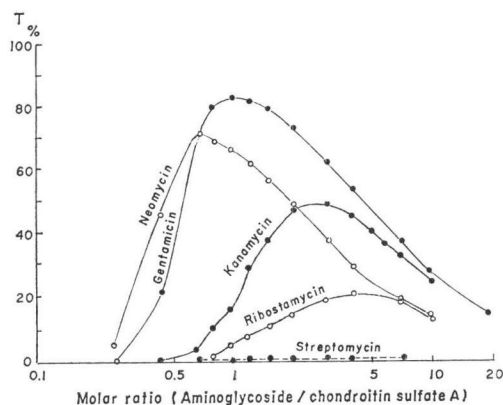| Aminoglycoside	Ionic strength
Neomycin	0.25
Gentamicin	0.15
Sagamicin	0.15
Paromomycin	0.15
Fortimycin A	0.075
Dibekacin	0.075
Kanamycin	0.05
Ribostamycin	0.035
Streptomycin	0.0

Fig. 4. Turbidity change of aminoglycoside-chondroitin sulfate A complex at various molar ratios. Total molar concentration adjusted to 0.5 mM other than in the case of neomycin (0.25 mM).

Fig. 5. Turbidity change of sagamicin complexes with various acidic mucopolysaccharides.
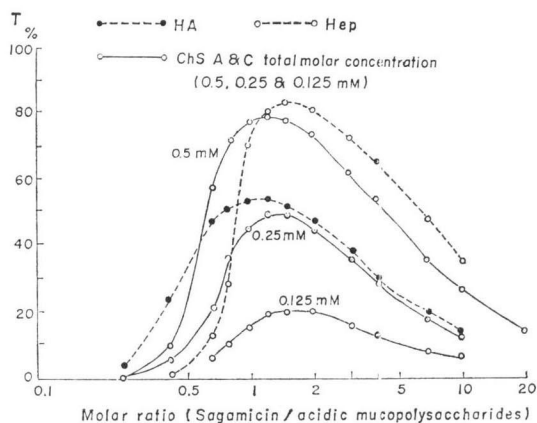

Table 2. Influence of concentration of aminoglycosides on binding to chondroitin sulfate A in equilibrium dialysis. For details, see Methods section.

Concentration of aminoglycoside (μg/ml)		309	154.5	126.8	92.7	61.8	30.9
Molar ratio (aminoglycoside/ChS A)		2	1	0.8	0.6	0.4	0.2
Bound (%)	Sagamicin	21.3	54.0	66.8	86.7	97.6	92.0
	Gentamicin	—	50.7	60.8	82.5	98.0	98.7

Fig. 4, where the total molar concentration was kept constant[10]. Similar experiments were carried out on the other AMPS's with a typical aminoglycoside (Fig. 5). The maximum turbidity was obtained at a characteristic molar ratio for each aminoglycoside-AMPS complex. At 0.5 mM total molar concentration, sagamicin bound to HA, ChS and Hep in the molar ratios of 1.0, 1.2 and 1.5, respec-

Fig. 6. Gel chromatograms of sagamicin-chondroi-
tin sulfate A complex at altered ionic strength.
Chromatogram A: at ionic strength 0.025; B: 0.25
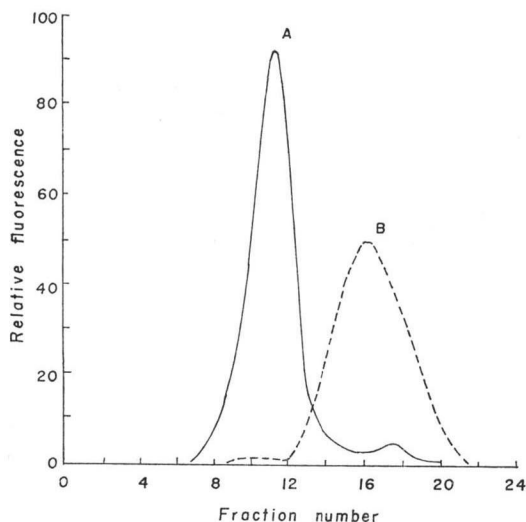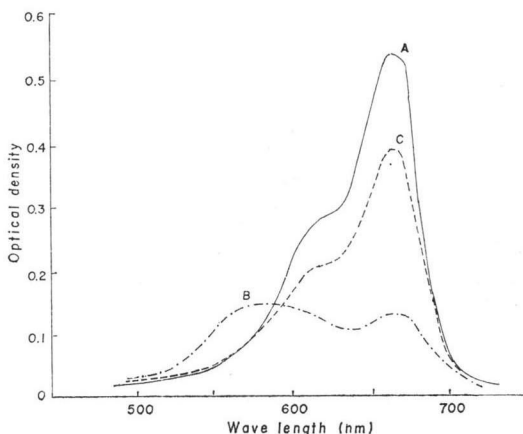(see Methods section).

Fig. 7. Influence of sagamicin on the absorption
spectrum of methylene blue-chondroitin sulfate A
complex.
Curve A: methylene blue; B: methylene blue-ChS
A; C: methylene blue-ChS A in the presence of
sagamicin (6.25 μM). The concentrations of
methylene blue and ChS A were 6.25 μM and 25
μM, respectively.

tively. A similar relationship was shown in the case of sagamicin-ChS A complex (Fig. 5) where the
total molar concentration was changed; these values were reduced slightly as the total concentration
increased. Equilibrium dialysis revealed that sagamicin or gentamicin was quantitatively bound to
ChS A in the molar ratio range less than 0.4 (Table 2).

In order to investigate the character of the binding, gel chromatography was carried out and the
elution pattern of the sagamicin-ChS A complex is illustrated in Fig. 6. At an ionic strength of 0.025,
sagamicin was eluted as the bound form and at 0.25 it was present as free antibiotic. Another evidence
of aminoglycoside-AMPS interaction was shown by the absorption spectral change of dye complexes
in the presence of an aminoglycoside. In Fig. 7 is shown the influence of sagamicin on the spectrum
of the methylene blue-ChS A complex. In the presence of sagamicin (Fig. 7 curve C), the absorption
spectrum of the complex changed to that of free methylene blue (curve A) from the complex (curve B),
which exhibited a maximum at 575 nm. Other AMPS's and methylene blue gave the same results.
In the case of acridine orange (23.5 μM) the metachromatically bound dye (λ_{max} 455 nm) to ChS A
(25 μM) was liberated to show the absorption maximum at 495 nm in the presence of sagamicin (12.5 μM).

Discussion

The affinity of aminoglycosides for AMPS *in vitro* has been demonstrated by turbidimetric analysis.
Under physiological conditions sagamicin was preferentially retained by cartilaginous tissues, such as
epiphyseal cartilage, auris externa, nasal cavity, trachea, larynx, dental pulps, fetal bones and also in
fetal membranes other than the kidney cortex.[6,7] Retention of sagamicin in these tissues may be at-
tributed to its binding to AMPS, since ChS and HA are major constituents of cartilage and umbilical
cord, respectively. In the living system sagamicin bound to tissues probably exchanged with calcium
ion and disappeared from cartilaginous tissues in 4 hours. The binding is relatively weak compared
with hexamethonium which is accumulated in cartilage for as long as 30 hours[11].

The stability of aminoglycoside-AMPS complexes was affected markedly by ionic strength (Figs. 2

and 6), indicating the ionic character of their binding. At low ionic strength the complex was stable, whereas under elevated ionic strength the aminoglycoside existed as free form. The dissociation of aminoglycosides was necessary for complex formation, since the complex was stable only at acidic range in the solution of high ionic strength (Fig. 3). The optimum molar ratios for binding have been obtained for some combinations of aminoglycosides and AMPS. Although the binding of sagamicin or gentamicin to ChS A changed with molar ratios (Table 2), the optimum ratio or the amount of sagamicin bound changed little with variation in total molar concentrations. The values for HA, ChS and Hep (1.0, 1.2 and 1.5, respectively) correlated well with the number of functional groups in the corresponding disaccharide unit. The effect of an aminoglycoside on the metachromatic reaction of a basic dye with an AMPS provides additional evidence for the character of binding. The affinity of aminoglycosides for AMPS was a function of the critical salt concentrations and aminoglycosides could be divided into several families on this basis. Furthermore, according to the minimum concentrations of aminoglycosides at which turbidity was noted (Fig. 1), the following descending order of affinity was obtained: neomycin, gentamicin, sagamicin, paromomycin, fortimycin A, dibekacin, kanamycin, ribostamycin, streptomycin. The same sequence was observed in the order of the molar ratios of aminoglycosides bound to AMPS (Fig. 4). Binding was most marked with the greatest number of amino groups. However, lesser affinity was observed for sagamine, a subunit of sagamicin[9], and 2-deoxystreptamine did not bind to ChS A. These facts implied that basic amine character was not a predominant factor producing affinity for AMPS, as pointed out in the case of hexamethonium[11].

Binding of aminoglycosides to AMPS's *in vitro* generally correlates with the extent of ototoxicity[12], nephrotoxicity[3,13] and acute toxicity[14], with the exception that sagamicin has not been evaluated as a more toxic drug than dibekacin. Retention of aminoglycosides in the inner ear has been discussed in regard to interaction with AMPS. According to histochemical investigation, toluidine blue-positive reaction, indicating the presence of AMPS's, was observed to decrease in the case of hearing disorder caused by physical (sonic) or chemical (kanamycin) treatment[4,5]. Thus the polycationic character of aminoglycosides has been suggested to contribute to their tissue affinity and toxicity through binding to biological polyanions. Recently, the acute toxicity of aminoglycosides was found to correlate with their concentrations in the kidney, and ionic interactions have been proposed to be their mechanism of action on the end-plate[15]. In addition, nephrotoxicity or neurotoxicity due to gentamicin has been attributed to lysosomal abnormality in the proximal tubules or nerves induced by the aminoglycoside[16,17], which as polycations could interact with acid lipoproteins in the lysosomes of these tissues.

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