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THE BINDING OF INORGANIC AND ORGANIC CATIONS AND H^+ TO CARTILAGE *IN VITRO*

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Abstract—The binding of H^+, Na^+, Ca^{2+}, Ni^{2+}, Ce^{3+}, chlorpromazine, chloroquine, hexamethonium, paraquat and gentamicin to decalcified cartilage from bovine nasal septum has been studied *in vitro*. The results indicated that the chondroitin sulphate is the binding material in the cartilage and that a stoichiometric binding occurs to the carboxyl and ester sulphate groups of the chondroitin sulphate. An analysis of the binding by the method of Scatchard was performed. The H^+ ions were bound to two groups of sites, one representing the carboxyl, the other representing the ester sulphate groups. It was shown that the Scatchard association constants for H^+ may be converted to pK_a values. Such transformation showed that the carboxyl groups of the chondroitin sulphate had a pK_a value of 4.57 and the ester sulphate groups a pK_a value of 2.60. The data for Na^+ indicated a binding to three groups of sites. Two of these may represent the carboxyl and ester sulphate groups. The third, which was a small group with a very strong affinity, may represent a specific localised link with a few groups on the chondroitin sulphate. The experiments with Ca^{2+}, Ni^{2+}, Ce^{3+} and the organic substances indicated that, in contrast to H^+ and Na^+, only one binding class was present, which implies that the binding to the carboxyl and ester sulphate groups for these ligands occurs with a similar strength. The affinity of these cations to the cartilage was related to their positive charge, which is the general characteristic for an electrostatic interaction.

Being a class of anionic polyelectrolytes, the glycosaminoglycans exhibit the property of binding small inorganic cations as well as positively charged organic molecules [1]. Chondroitin sulphate, the glycosaminoglycan most widely distributed among animal tissues and fluids, is present in high concentration in cartilage [1]. Besides a structural role, the chondroitin sulphate, by virtue of its ion binding property, is believed to have an important influence on the maintainance of a steady state in the composition of extracellular fluid [1, 2]. In regard to calcium, the binding may also be of importance for the formation of a matrix suitable for calcification [1, 3]. Knowledge on the mechanism for the binding of inorganic cations to cartilage and chondroitin sulphate is therefore of great interest. The available information in this respect is however surprisingly scanty. For example, the potential binding sites of the chondroitin sulphate are the carboxyl and the ester sulphate groups, but their relative contribution in the binding has not been conclusively established. Therefore in the present investigation the binding of some inorganic cations to cartilage has been investigated. The cations chosen were sodium, calcium, nickel and cerium, which all, by means of radiotracers, have been shown to bind to cartilage *in vivo* [4–7]. It became apparent that knowledge on the interaction of H^+ ions with the cartilage also would be of great interest and an analysis of the H^+ binding was therefore done.

In the present study, we have also analysed the binding of some organic substances to the cartilage. These experiments were performed to get a comparison with the data for the inorganic cations, but also because few reports are available in which the binding of organic substances to cartilage has been examined. Such information is of interest since it has been shown that several organic compounds have an ability to preferentially accumulate in the cartilaginous tissues of the body. Thus, bisquaternary ammonium compounds and aminoglycoside antibiotics have been shown to localise in cartilage *in vivo* and to bind to cartilage and chondroitin sulphate *in vitro* [8–13]. In the present study, the bisquaternary ammonium compounds paraquat and hexamethonium and the aminoglycoside antibiotic gentamicin have been studied. In addition, chlorpromazine and chloroquine were included in the investigation (Fig. 1). An interaction between chlorpromazine and chondroitin sulphate has been demonstrated *in vitro* [14]. Chlorpromazine and chloroquine are also strongly bound to melanin *in vivo* and *in vitro*, via a mechanism which probably involves an electrostatic interaction between the cationic forms of the drugs and anionic sites on the melanin polymer [15, 16], and a comparative determination of the binding parameters for cartilage was considered of interest.

MATERIALS AND METHODS

Chemicals. Non-radioactive compounds were obtained from the following sources. Hexamethonium dibromide from Sigma Chemical Co. (St. Louis, MO), paraquat dichloride from Imperial Chemical Industries Ltd. (Macclesfield, U.K.), chlorproma-

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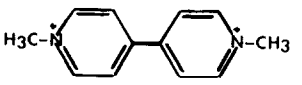
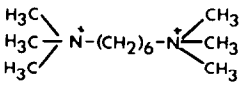
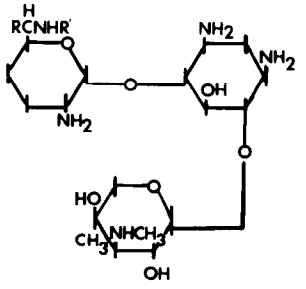


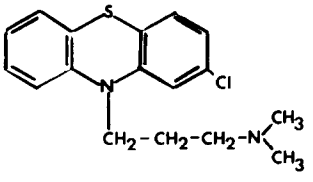
PARAQUAT



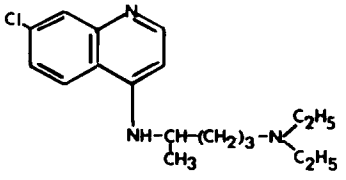
HEXAMETHONIUM



GENTAMICIN	R	R'
C_1	CH_3	CH_3
C_2	CH_3	H
C_{1a}	H	H



CHLORPROMAZINE



CHLOROQUINE

Fig. 1. Structural formulae of the studied organic substances.

zine chloride from AB Leo (Helsingborg, Sweden), chloroquine diphosphate from Bayer AG (Leverkusen, West Germany), and gentamicin complex sulphates from Schering Corporation (Bloomfield, NJ). Labelled compounds: [^{3}H]hexamethonium dichloride ([^{3}H]methyl, sp. act. 62.5 μCi/mg), and [^{14}C]chloroquine diphosphate (ring-3-^{14}C, sp. act. 58.8 μCi/mg) from New England Nuclear (Boston, MA); [^{14}C]paraquat dichloride ([^{14}C]methyl, sp. act. 125 μCi/mg), [^{35}S]chlorpromazine chloride (sp. act. 20.3 μCi/mg), ^{22}NaCl (carrier free, sp. act. > 100 mCi/mg Na^+), $^{45}CaCl_2$ (sp. act. 11.7 mCi/mg Ca^{2+}), $^{63}NiCl_2$ (sp. act. 10.4 mCi/mg Ni^{2+}), and $^{139}CeCl_3$ (carrier free, sp. act. > 20 mCi/mg Ce^{3+}), from the Radiochemical Centre (Amersham, U.K.); [^{14}C]gentamicin complex sulphates ([^{14}C]methyl, sp. act. 0.83 μCi/mg) from Schering Corporation, Bloomfield, NJ. Other chemicals used in the study were of analytical grade and purchased from regular commercial sources.

Cartilage. Decalcified cartilage was prepared from bovine nasal septum as described by Dunstone [17]. The sulphate content of the cartilage was determined gravimetrically by refluxing the samples in 2 M HCl for 4 hr and then precipitating the barium sulphate by the addition of barium chloride [15]. Protein was determined according to Lowry *et al.* [19].

Binding experiments. Samples (20 mg) of the finely ground cartilage were shaken at room temperature in 10 ml of the incubation medium. Usually equilibrium was reached after about 8 hr, but to ensure complete equilibrium in all instances, an incubation period of 24 hr was used. The medium was distilled water or distilled water supplemented with various concentrations of HCl. In the medium there were also present various concentrations of the non-labelled inorganic cation or organic substance which was studied together with a fixed tracer amount of the same material in labelled form. No attempts were made to buffer the solutions, due to the possible competitive effects that other cations might exert on the binding. Determination of the pH of the incubation solutions showed that in no instance (except in the experiments with H^+) was the pH lower than 6.0. After the incubation period, the cartilage was sedimented by centrifugation for 10 min at 500 *g* in a Wifug centrifuge. Aliquots of the supernatants were then taken and analysed for radioactivity by gamma scintillation counting ($^{22}Na^+$ and $^{139}Ce^{3+}$) or liquid scintillation counting (all other substances) and the amount of substance bound to cartilage was then calculated.

Analysis of the binding. In order to determine if more than one class of binding sites were present,

to estimate the total binding capacity and to derive association constants, the binding of the substances was analysed by the method developed by Scatchard and his associates [20–22]. In our investigation, the value n for the number of binding sites cannot be considered to be an integer, since the molecular weight of the binding materials cannot be stated. Furthermore, the molar ratio $\bar{v}$ is used for the ratio between the number of μmoles of bound ions or molecules and the dry weight in milligrams of the cartilage.

Analysis of the H^+ binding to the carboxyl- and ester sulphate-groups in the cartilage and determination of their pK_a values. Since knowledge on the interaction of H^+ ions with the cartilage appeared to be of great interest for the interpretation of our data, an analysis of the H^+ binding was performed. We chose to study the binding parameters for H^+ by performing a Scatchard analysis. Thus, 0.85 M HCl was added in portions of 0.05 ml to 400 mg samples of the cartilage present in 5 ml 'boiled-out' distilled water. The pH was determined after each addition, using a PHM62 Standard pH-meter with a glass electrode (Radiometer, Copenhagen, Denmark). An interval of 2 hr was found to be sufficient to obtain equilibrium.

After each addition of HCl, the amount of H^+ ions bound to the cartilage at equilibrium was calculated by subtracting the unbound fraction (pH) from the total amount of added HCl. A Scatchard curve was then drawn in the usual way by plotting $\bar{v}/c$ versus $\bar{v}$, where $\bar{v}$ is the amount (μmoles) of H^+ ions bound per mg cartilage and c is the concentration (M) of the unbound H^+ ions. From the law of mass action, Scatchard has deduced the following relationship,

$$\bar{v}/c = K_s(n - \bar{v}) \qquad (1)$$

which may be rearranged to

$$K_s = \frac{\bar{v}}{c(n - \bar{v})} \qquad (2)$$

In our case, n is used for the total amount of available binding sites on the cartilage expressed in μmoles per mg cartilage. K_s is the association constant. If equation (1) is linear, this means that there is only one class of binding sites, i.e. all ligands are bound with the same strength. The values for n and K_s may then be derived, since n is the abscissa intercept and $K_s n$ is the ordinate intercept. If the relationship is curvilinear (with an upward concavity), there is more than one class of binding sites. The curve may then be analysed by a process of approximations designed to obtain the rectilinear curves from which the curvilinear relationship may have been derived, and in this way obtain additional n and K_s values [20–22]. When the ionization constant for an acid is determined, it is usual to designate the free H^+ concentration for $|H^+|$, the free anion concentration for $|A^-|$ and the concentration of the reacted molecules for $|HA|$. Using these designations, we can exchange c for $|H^+|$, $(n - \bar{v})$ for $|A^-|$ and $\bar{v}$ for $|HA|$. Equation (2) then becomes

$$K_s = \frac{|HA|}{|H^+|\,|A^-|}.$$

The acid ionization constant (K_a) used for acid–base equilibria is defined as

$$K_a = \frac{|H^+|\,|A^-|}{|HA|}$$

Thus, the Scatchard association constant $K_s = \frac{1}{K_a}$.

This means that $K_a = \frac{1}{K_s}$ and thus $pK_a = -\log\frac{1}{K_s}$.

Statistic calculations. In the Scatchard analysis the estimates of errors and test variables were based on linear regression analysis [23]. The standard deviation (S_K) of the association constant (K) was calculated according to

$$S_K = \left\{S_E^2/[\Sigma\,\bar{v}^2 - (\Sigma\bar{v})^2/p]\right\}^{1/2}$$

where $\bar{v}$ is the bound fraction and p designates the number of observations. S_E^2 is the residual mean square and may be obtained from the expression

$$S_E^2 = \Sigma\,(\bar{v}/c - \widehat{\bar{v}/c})^2/(p - 2)$$

where c is the unbound fraction and $\widehat{\bar{v}/c}$ is the expected value of $\bar{v}/c$ estimated from the regression equation.

To test whether the association constants differ significantly, the following expression was used,

$$t = \frac{K_1 - K_2}{(S_{K_1}^2 + S_{K_2}^2)^{1/2}}$$

where t is the test variable, K_1 and K_2 are the association constants and S_{K_1} and S_{K_2} are the standard deviations of the association constants. The number of degrees of freedom is $(p_1 + p_2 - 4)$.

RESULTS

The sulphate determinations showed that the content of ester sulphate groups was 0.61 ± 0.05 μmoles/mg cartilage (mean ± S.D., $n = 5$). It was also found that the sulphate was not removed at any of the incubation procedures. Assuming a mean molecular weight of the disaccharide-repeating unit of the chondroitin sulphate of 559.5 (Na-form and one ester sulphate group per unit), the total content of chondroitin sulphate can be estimated to be about 0.34 mg/mg cartilage, i.e. about 34% of the cartilage dry weight.

The protein determinations showed that there was no release of protein into the media during the incubations.

Scatchard plots for the binding of H^+, the inorganic cations and the organic substances are shown in Figs. 2–4 and the calculated binding parameters are given in Table 1. For the binding of H^+ and Na^+, curvilinear Scatchard plots were observed, which shows that more than one binding class must be implicated. In the other instances, the Scatchard plots gave straight lines (except in the low concentration area, see below) indicating a binding to only one class of sites.

The data for the H^+ could be fitted by the assumption of two classes of binding sites (Fig. 2, Table 1). Table 1 shows that the number of binding sites

Table 1. Binding parameters for the interaction of H^+, Na^+, Ce^{3+}, Ca^{2+}, Ni^{2+}, gentamicin, paraquat, hexamethonium, chloroquine and chlorpromazine with cartilage. Apparent association constants (K, M^{-1}) with standard deviations (±S.D.) and number of binding sites (n, μmoles per mg cartilage) analysed by the method of Scatchard

H^+	$n_1 = 0.61$	$K_1 = 3.72 \times 10^4 \pm 0.001 \times 10^4$	$pK_a = 4.57$*
	$n_2 = 0.61$	$K_2 = 3.98 \times 10^2 \pm 0.33 \times 10^2$	$pK_a = 2.60$*
	$\Sigma\ n_i = 1.22$		
Na^+	$n_1 = 0.04$	$K_1 = 21.9 \times 10^4 \pm 0.1 \times 10^4$	
	$n_2 = 0.54$	$K_2 = 4.4 \times 10^3 \pm 0.3 \times 10^3$	
	$n_3 = 0.71$	$K_3 = 1.5 \times 10^2 \pm 0.2 \times 10^2$	
	$\Sigma\ n_i = 1.29$		
Ce^{3+}	$n = 0.46$	$K = 13.4 \times 10^4 \pm 1.2 \times 10^4$	
Ca^{2+}	$n = 0.61$	$K = 10.2 \times 10^4 \pm 0.7 \times 10^4$	
Ni^{2+}	$n = 0.58$	$K = 9.4 \times 10^4 \pm 0.6 \times 10^4$	
Gentamicin	$n = 0.32$	$K = 12.0 \times 10^4 \pm 1.5 \times 10^4$	
Paraquat	$n = 0.59$	$K = 6.5 \times 10^4 \pm 0.2 \times 10^4$	
Hexamethonium	$n = 0.62$	$K = 4.9 \times 10^4 \pm 0.2 \times 10^4$	
Chloroquine	$n = 0.61$	$K = 4.3 \times 10^4 \pm 0.1 \times 10^4$	
Chlorpromazine	$n = 1.38$	$K = 2.6 \times 10^4 \pm 0.3 \times 10^4$	

* The pK_a values for the two groups of H^+ binding sites were obtained from the equation

$$pK_a = -\log \frac{1}{K_{\text{Scatchard}}}.$$

(For derivation: see Materials and Methods).

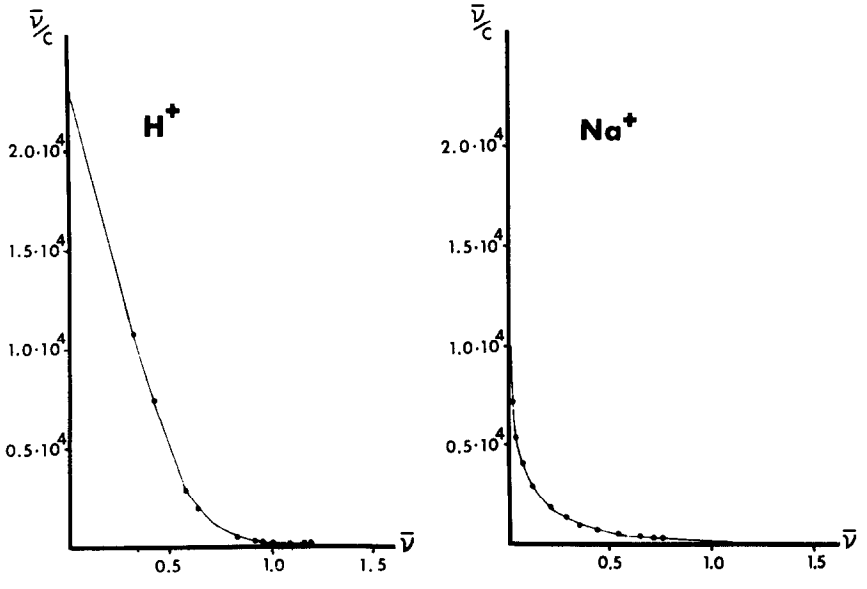


Fig. 2. Scatchard plots for the cartilage binding of H^+ and Na^+. $\bar{\nu}$, μmoles ion bound per mg cartilage; c concentration (M) of the unbound fraction of ion.

(n, μmoles per mg cartilage) for both the H^+ binding classes was 0.61. This is the same value which was obtained for the number of ester sulphate groups, determined by estimating the sulphate content of the cartilage (see above). This must imply that one binding site for the H^+ is the ester sulphate groups of the chondroitin sulphate. Since the number of ester sulphate groups on the chondroitin sulphate equals the number of carboxyl groups, the latter consequently includes the other H^+ binding sites. As discussed in Materials and Methods, the Scatchard association constant for the H^+ ions can be transformed to pK_a values. Such transformation of K_1 gives a pK of 4.57, and K_2 a pK_a of 2.60 (Table 1). It is thus apparent that the n_1 and K_1 values represent the data for the carboxyl groups and the n_2 and K_2 values represent the data for the ester sulphate groups on the chondroitin sulphate.

The analysis of the Scatchard data for Na^+ showed that three classes of binding sites were discernible (Fig. 2, Table 1): one small class with a strong binding and two larger classes with lower binding strength. In comparison with H^+, the intermediate class for Na^+ showed a lower binding than the 'carboxyl-class' for H^+ and the lowest affinity group for Na^+ a lower binding than the 'ester-sulphate-class' for H^+. However, the number of binding sites (n_2 and n_3) for these groups were about equal to the number of binding sites in the H^+ groups.

The Scatchard plots for Ce^{3+}, Ca^{2+} and Ni^{2+} gave straight lines, except in the low concentration area (Fig. 3). The straight lines indicate a binding to only one class of sites. The upward convexity at the low concentrations of the cations is probably due to a positive cooperativity between the binding-sites and the cations in this concentration area. Table 1 shows that the number of binding sites, which also illustrates the total binding capacity of the cartilage (n, μmoles per mg cartilage) is higher for Ca^{2+} and Ni^{2+} (0.61 and 0.58 respectively) than for Ce^{3+} (0.46). This is also shown in Fig. 5 in which the amounts of

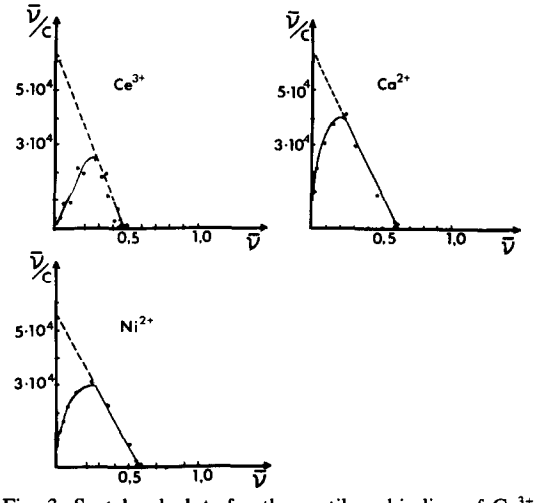


Fig. 3. Scatchard plots for the cartilage binding of Ce^{3+}, Ca^{2+}, and Ni^{2+}. $\bar{\nu}$, μmoles ion bound per mg cartilage; c, concentration (M) of the unbound fraction of ion. The dotted parts of the curves are extrapolations which partly underly the calculation of binding parameters (Table 1).

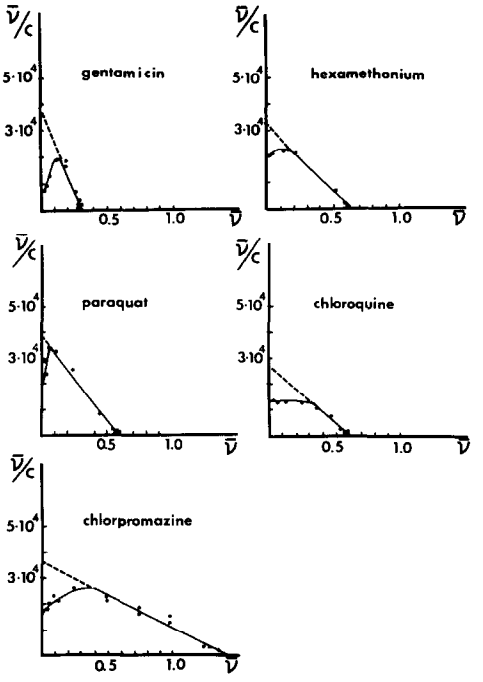


Fig. 4. Scatchard plots for the cartilage binding of gentamicin, hexamethonium, paraquat, chloroquine and chlorpromazine. $\bar{\nu}$, μmoles substance bound per mg cartilage; c, concentration (M) of the unbound fraction of substance. The dotted parts of the curves are extrapolations which partly underly the calculation of binding parameters (Table 1).

the cations which were bound are plotted against the concentration of the cations present in the incubation media. It can be observed that the total binding capacity of the cartilage for the divalent cations equals half of the total binding capacity for H^+, whereas for the trivalent cerium the total binding capacity equals one third of the total binding capacity for H^+. The analysis of the association constants (K), (Table 1), shows a stronger binding for Ce^{3+} than for Ca^{2+} and Ni^{2+}. The statistic calculations showed that the Ce^{3+}-association constant differed significantly from the Ca^{2+}- and Ni^{2+}-association constants at the 5% level, whereas between the association constants for Ca^{2+} and Ni^{2+} there was no statistically significant difference.

The Scatchard plots for the organic substances were similar to those for Ce^{3+}, Ni^{2+} and Ca^{2+}, with straight lines at the high concentrations and an upward convexity at the low concentrations (Fig. 4). These data indicate a single class of binding sites and a positive cooperativity between the drugs and the binding sites at the low concentrations. It can be seen in Table 1 that gentamicin shows the strongest binding, chlorpromazine the weakest binding, and paraquat, hexamethonium and chloroquine intermediate binding strengths. The statistical calculations showed that in all instances the association constants for the organic substances differed significantly from each other at the 5% level. The total binding capacity of the cartilage (μmoles per mg cartilage) was 0.32 for gentamicin, about double that for paraquat, hexamethonium and chloroquine (0.59, 0.62 and 0.61 respectively) and again about

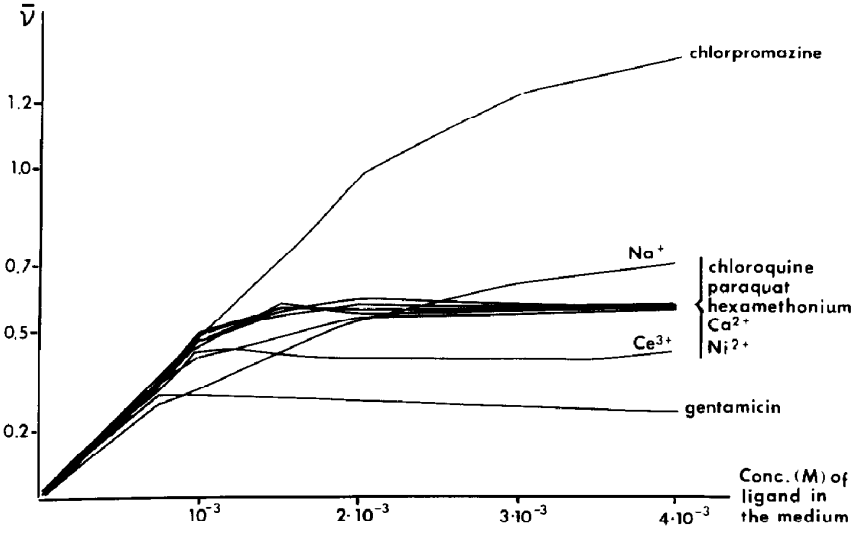


Fig. 5. Binding to cartilage of Ce^{4+}, Ca^{2+}, Ni^{2+}, Na^+, gentamicin, paraquat, hexamethonium, chloroquine and chlorpromazine at increasing concentrations of the ligands. $\bar{\nu}$, μmoles ion (substance) bound per mg cartilage. Note that Na^+ has not reached its maximal binding within the concentration interval shown in the figure.

double that of the latter values for chlorpromazine (1.38). Compared with the total H^+ binding, the binding capacity of the cartilage is about one fourth for gentamicin, about half for paraquat, hexamethonium and chloroquine, and about equal for chlorpromazine. The different total-binding of the organic substances is also illustrated in Fig. 5, together with the data for the inorganic cations. Note that Na^+ has not reached its maximal binding within the concentration interval which is shown in Fig. 5.

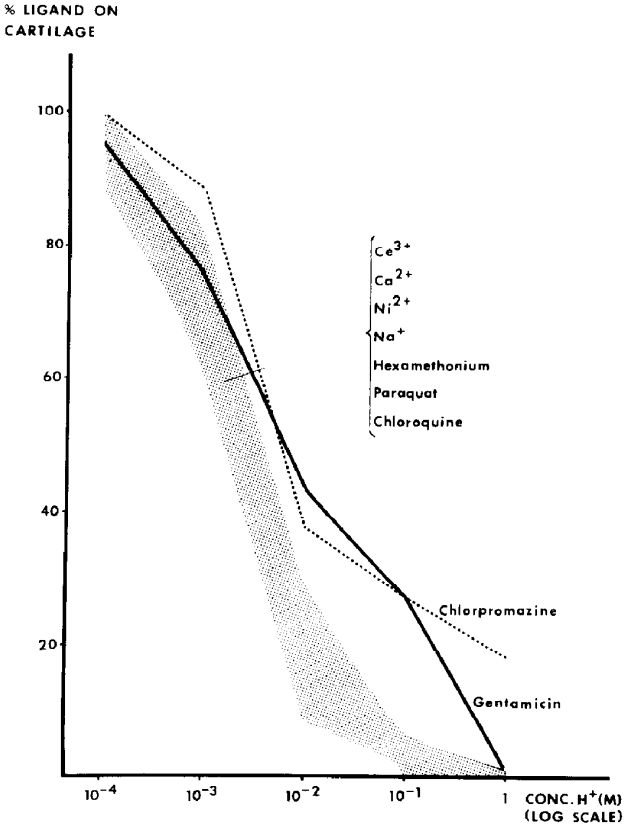


Fig. 6. Effect of H^+ on the cartilage binding of Ce^{3+}, Ca^{2+}, Ni^{2+}, Na^+, gentamicin, paraquat, hexamethonium, chloroquine and chlorpromazine. Twenty micromoles (corresponding to 2.0×10^{-3} M) of each ion or substance were added to 20 mg samples of cartilage in 10 ml distilled water containing various amounts of HCl. After 24 hr incubation, the binding to the cartilage was determined. The amount of the ligand bound to the cartilage in distilled water is assigned the value 100%, and the binding obtained in the HCl-containing solutions is then expressed as a percentage of this value.

The addition of HCl to the incubation media inhibited the binding of Ce^{3+}, Ca^{2+}, Ni^{2+} and Na^+ as well as the binding of the organic substances to the cartilage (Fig. 6). The pH-sensitivity for chlorpromazine and gentamicin was less expressed than for the other substances. However, at the highest H^+ concentration, chlorpromazine was the only compound of which a small amount remained attached to the cartilage. At this H^+ concentration, a red reaction product of chlorpromazine appeared. This has previously been shown to be a positive ion radical of chlorpromazine formed as the first oxidation product of the drug [24].

DISCUSSION

The Scatchard analysis of the H^+ binding to the cartilage showed a binding to two classes of sites. The number of sulphate groups estimated from the sulphate determinations were identical with the number of binding sites in each of the H^+ binding groups determined by the Scatchard analysis. Since the sulphate is present as ester sulphate, each of these groups carry only one negative charge. In the chondroitin sulphate of the cartilage, the number of ester sulphate groups equals the number of carboxyl groups. This implies that the H^+ binding occurs at both the carboxyl and the ester sulphate group of the chondroitin sulphate. A further implication of these data is that the chondroitin sulphate is entirely responsible for the H^+ binding to the cartilage. As discussed in the Materials and Methods, the Scatchard association constants for H^+ can be transformed to pK_a values. Such transformation gives a pK_a value for the group of strongest binding sites of 4.57 and for the group of weakest binding sites of 2.60. This implies that the strongest binding sites must be the carboxyl groups, and that the weakest binding sites must be the ester sulphate groups. The pK_a values for the carboxyl groups of chondroitin sulphate reported in the literature are of the same magnitude as found in the present study [1]. The pK_a value of the ester sulphate groups has also been proposed to be of the same magnitude as that we have found in the present study [1], but, probably due to the technical difficulties involved in the determination of this pK_a value, there are no exact data available. The definition of a weak acid on the basis of our association constants is that in a weak acid the H^+ ions have a strong affinity to the anionic groups and, consequently, in a strong acid, the H^+ ions have a low affinity to the anionic groups. We think that this is a good way to define the strength of acids, and this may also be a practicable approach to the determination of pK_a values, especially if more than one ionizable H^+ binding group is present—since the Scatchard plot offers a convenient way to accurately analyse the titration data. This will be developed in more detail in a separate paper.

The analysis of the data for the other inorganic cations and the organic substances fits with the assumption that there is a stoichiometric binding to the carboxyl and the ester sulphate groups of the chondroitin sulphate. Thus, Ce^{3+} may occupy three negative groups—the total amount bound (μmoles) being about one third of the total amount (μmoles) of carboxyl and ester sulphate groups. Ca^{2+} and Ni^{2+} may occupy two negative groups—the total amount bound being about one half of the sum of the carboxyl and ester sulphate groups. Chlorpromazine is present as a monovalent cation at physiological pH and may thus occupy only one group—the total amount bound being about the same as the total amount of the carboxyl and the ester sulphate groups. Paraquat, hexamethonium and chloroquine are divalent organic cations, and thus able to occupy two negative groups. Gentamicin has five basic nitrogen atoms per molecule. Its ionization equilibrium is very complex and has not been quantitatively described. Our present observation that the total binding capacity of the cartilage is about one fourth of the number of carboxyl and ester sulphate groups indicates that at a pH of 6.0, which was the pH of the gentamicin-containing solutions, the molecule has four protonated amino groups. The total amount of Na^+ which was bound, was also about equal to the total amount of carboxyl and ester sulphate groups available, indicating a stoichiometric binding to these groups. The observation that—like H^+, but in contrast to the other inorganic cations and the organic substances—more than one binding class must be implicated in the binding of Na^+, will be discussed later.

Our observations of a stoichiometric binding of inorganic cations to cartilage agree with the results obtained by Boyd and Neuman [25] and Dunstone [17, 26]. Our results also fit with those made by Ehrenpreis and Fishman [11]: studies by these authors indicated a stoichiometric binding of cetyl pyridinium chloride and D-tubocurarine to chondroitin sulphate.

The Scatchard analysis showed that for Ce^{3+}, Ca^{2+}, Ni^{2+} and the organic substances, rectilinear plots were obtained at the high concentrations, which implies the presence of only one binding class. Since both the carboxyl and the ester sulphate groups are involved in the binding, this result is unexpected. It appears that the binding to these groups occurs with such similar strength that straight lines are obtained in the Scatchard plots. The analysis of the association constants for the binding showed an increased attraction to the cartilage at increasing charges, which is a general characteristic of cation exchange reactions and is due to the electric fields of the cations [27]. However, structural factors also appear to be of importance. Thus the divalent inorganic cations Ca^{2+} and Ni^{2+} were more strongly bound than the organic divalent cations paraquat, hexamethonium and chloroquine. Steric hindrances may prevent the latter compounds from getting into as close contact with the negative sites as the inorganic cations, resulting in a weaker binding. Steric factors may also explain the observation that the association constants for the divalent organic compounds differed significantly from each other, whereas this was not the case for the association constants for Ca^{2+} and Ni^{2+}.

The binding of Na^+ differed from that of the other cations. As with H^+, more than one binding class must be implicated. However, whereas two groups were present for H^+, three groups were present for Na^+. The small group with the strongest binding had the highest affinity of all the studied cations. The association constant for the second Na^+ group was

lower than the association constant for the H^+ binding to the carboxyl groups and the association constant for the binding of Na^+ to the lowest affinity group was lower than the H^+ binding to the ester sulphate groups. However, the number of binding sites were about equal for the H^+ binding and the Na^+ binding to these groups. It appears possible therefore that the binding of Na^+ to these groups represents a binding to the carboxyl and the ester sulphate groups. An electrostatic interaction between these groups and Na^+ should be weaker than their interaction with H^+. The small Na^+ group with the strongest affinity may suggest the presence of a specific localised link with a few of the anionic groups, as for example an 'ion pair' or a binding induced through a specific stereochemical complex formation. It is possible that such a specific binding is of physiological importance.

Additional studies are required to establish why the carboxyl and ester sulphate groups may bind the H^+ and Na^+ ions with different strengths, whereas in the other cases there is no differentiation between the binding strength to the carboxyl and ester sulphate groups. Table 1 shows that the association constant for the H^+ binding to the carboxyl groups is lower than the association constants for the inorganic and organic cations with two or more charges, whereas for the monovalent chlorpromazine and the binding sites for Na^+, which may represent the carboxyl groups (n_2, K_2), the association constants are lower than the H^+ value. This indicates that the H^+ binding follows the ordinary electrostatic laws, which are dependent primarily on the electrostatic fields —in turn determined by the charges—of the cations. To this additional factors must be considered, such as for inorganic cations atomic radii and weights and for organic compounds steric factors and the presence of non-electrostatic bonds (e.g. van der Waals bonds). When added in excess, H^+ was found to displace the binding of the other cations to the cartilage. This confirms that the binding occurs at the same sites and shows that in spite of the weak attraction, also the ester sulphate groups may be blocked when high concentrations of H^+ ions are present. The relative insensitivity of the binding of gentamicin towards the H^+ ions may be due to the strong electrostatic attraction of this polyvalent organic cation to the cartilage. In contrast to the other ligands, the binding of chlorpromazine to the cartilage was not completely depressed by the highest concentration of H^+. At this H^+ concentration, the positive ion radical of the compound, which is the first oxidation step of the drug [24], was formed. The ion radical of chlorpromazine formed at high H^+ concentrations has previously been shown to be strongly bound to melanin [15]. The mechanism for this strong binding is not known, but conceivably the formation of a radical cation may imply that bonds other than electrostatic may develop.

The Scatchard curves for the cations with only one binding class showed an upward convexity at the low concentrations. This type of curve is obtained when a positively cooperative binding occurs between a substance and the binding sites on a macromolecule. The molecular mechanism underlying the cooperativity is not known.

We have previously analysed the binding of chlorpromazine, chloroquine, paraquat and gentamicin to melanin and shown an important role of electrostatic forces for the complex formation [13, 15]. However, several differences are apparent in a comparison with the binding to cartilage. These differences are probably related to the different molecular structures of the building-stones of these polyanions. Melanin is a polymer with an indole nucleus as the main monomer [28] and additional binding-forces may occur at the conjunction of the aromatic rings of the substances and the aromatic rings in the melanin polymer.

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