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BINDING OF POLYMETHYLENE BISQUATERNARY IONS TO CHONDROITIN *IN VITRO**†

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Abstract—Polymethylene bistrimethylammonium ions such as hexamethonium and decamethonium are found to bind strongly to chondroitin, using dialysis methods. The extent of such binding is dependent upon both the concentration of the drug as well as of chondroitin, and is inversely proportional to the ionic strength of the external medium. The monoquaternary choline is bound to a far lesser extent. The binding of hexamethonium to chondroitin is significantly reduced by the presence of pentamethonium or decamethonium in the external medium, and remains unaltered by choline. The binding of various di- and triquaternary ammonium ions, such as hexamethonium, *d*-tubocurare, succinyl choline, gallamine and related compounds, to chondroitin or to acid mucopolysaccharides in general, is postulated to be the major factor in the accumulation of these drugs in cartilage *in vivo*.

HEXAMETHONIUM and decamethonium have been recently reported by Asghar and Roth[1–3] to accumulate preferentially in the cartilaginous tissues of the body. A similar cartilage distribution of hexamethonium has since been observed by Wasserman.[4] Bisquaternary *d*-tubocurare, which is structurally very different from polymethylene bisquaternary ammonium ions, has also been found to accumulate in the cartilage.[5]·§ Bisquaternary succinylcholine is observed to accumulate in the epiphyseal and articular cartilage of the newborn rhesus monkey after umbilical cord injection.‖ Previous studies[1–3] from this laboratory have indicated that the accumulation of hexamethonium in the cartilage is the result of its binding interactions with the acid mucopolysaccharide components, especially chondroitin. The ability of the quaternary ammonium ions to interact with chondroitin may be correlated to the localization *in vivo* of these compounds in joints and cartilage, and may possibly serve as a method to predict the joint–cartilage affinity of a drug *in vivo*. We have, therefore, investigated the effect of some of the physicochemical parameters on the binding *in vitro* of hexamethonium and some other structurally related compounds to chondroitin.

METHODS

Materials. Methyl [^{14}C] labeled hexamethonium dichloride, obtained in alcoholic solution from New England Nuclear Corp., was evaporated to dryness under reduced

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§ E. N. Cohen, personal communication.

‖ E. Van Der Kleijn, personal communication.

pressure and the contents were dissolved in saline. The specific radioactivity of hexamethonium dichloride was 1 mc/146 mg. Radiochemical purity of hexamethonium exceeding 99 per cent was reported by the supplier and was confirmed by descending chromatography using the solvent system of *n*-butanol–HCl (9:1) saturated with water. No radiochemical impurities were detected. Methyl [^{14}C] labeled decamethonium (New England Nuclear Corp.) and methylene [1,2-^{14}C]choline chloride (Schwarz Bioresearch, Inc.) were also used in binding studies. Nonradioactive hexamethonium dichloride, decamethonium dibromide and pentamethonium dibromide were obtained from K & K Laboratories, Inc. Choline chloride was purchased from Sigma Chemical Company.

Experimental. The binding of hexamethonium and decamethonium to chondroitin sulfate (99%+ purity; Nutritional Biochemical Corp.) was studied by the methods of equilibrium and dynamic dialysis. The molecular weight of chondroitin was assumed to be 50,000. Cellulose dialysis sacs (Oxford Laboratories, San Mateo, Calif.) with a pore diameter of 4·8 mμ were used. In the equilibrium dialysis procedure, the sacs were soaked in distilled water for 45 min before use. A 1·5-ml aliquot of 1% chondroitin sulfate solution was dialyzed at 25–26° against 45 ml of 0·04 M sodium phosphate buffer of pH 7·3. Other dialysis media and concentrations of chondroitin sulfate, when used, are specified in the following text. The external media contained 10^{-6} to 10^{-3} M concentration of a [^{14}C]quaternary ammonium compound. The 50-ml test tubes containing the dialysis medium and the suspended dialysis sac were covered with Parafilm during the equilibration period to prevent any errors in drug concentration due to evaporation of water from the media. Preliminary experiments had indicated that the equilibrium was reached at 24 hr; the samples were withdrawn at this time from inside and outside the dialysis sac, and analyzed by liquid scintillation counting. Hexamethonium, decamethonium and choline are known to be chemically stable under the experimental conditions, and their concentrations have been expressed in the text either on the molar basis or as micrograms per milliliter of the quaternary ammonium salt.

Binding of [^{14}C]hexamethonium to the dialysis sacs was determined by suspending the sacs in the 0·04 M phosphate buffer containing 10^{-4} M hexamethonium. Although the concentration of hexamethonium inside and outside the sac remained the same, the total concentration of hexamethonium in solution decreased by approximately 4 per cent during the 24-hr equilibration period and was assumed to be bound to the sac. In dynamic dialysis, a negligible fraction of the total hexamethonium was bound to the dialysis sac during the 4-hr period. The extent of binding to chondroitin has been described in the terms of $(C_i - C_e)/C_i$ and $(C_i - C_e)/C_e$ where C_i is the drug concentration inside the dialysis sac and C_e is the drug concentration in the external medium. The former term expresses the bound drug as a fraction of the total drug inside the dialysis sac, while the latter term describes the amount bound as a fraction of the free drug in the external medium.

The dynamic dialysis method used was basically that of Meyer and Guttman.[6–8] Twenty μl containing 58 μg hexamethonium ($5{\cdot}9 \times 10^5$ counts/min) was added to 2 ml of 0·04 M phosphate buffer solution either in the absence or presence of chondroitin sulfate at 25–26°. This solution was transferred to a dialysis sac which was suspended in an Oxford dialyzer containing 300 or 600 ml of 0·04 M buffer medium. The external medium was wholly replaced every 40 min, thereby maintaining almost sink conditions.

The radioactivity from the [^{14}C]labeled bisquaternary ions was assayed in media samples from inside and outside the dialysis sac. These aqueous samples were incorporated in 15 ml of a phosphor solution of the following composition: 2,5-diphenyloxazole, 4 g; 1,4-bis[2-(5-phenyloxazolyl)] benzene, 400 mg; naphthalene, 75 g in 1 l. dioxane. All reagents were of scintillation or analytical grade. The samples were counted on a Packard Tri-Carb spectrometer three times for 10 min at an instrumental setting of 050–1000, 12 per cent. The counting efficiency under these conditions was found to be about 75 per cent by the internal standardization method. No samples with less than four times the background counts were used in the computation of our data.

RESULTS

Influence of chondroitin concentration on the extent of binding. Binding of the bisquaternary ammonium compounds was determined by equilibrium dialysis, using four different concentrations of chondroitin sulfate: 0·1, 0·25, 0·5 and 1·0%. The influence of the concentration of chondroitin sulfate on the extent of binding of [^{14}C]hexamethonium at a constant concentration of 10 μg/ml is shown in Fig. 1. The fraction of ^{14}C-hexamethonium bound $[(C_i - C_e)/C_i]$ in these experiments increases in a sigmoid fashion as a function of the concentration of chondroitin sulfate in the dialysis sac. However, the increase in $[(C_i - C_e)/C_e]$ appears to be nearly linear over the range of chondroitin concentration studied. Similar curves were obtained when these experiments were conducted in a medium of higher ionic strength, i.e. 0·04 M phosphate

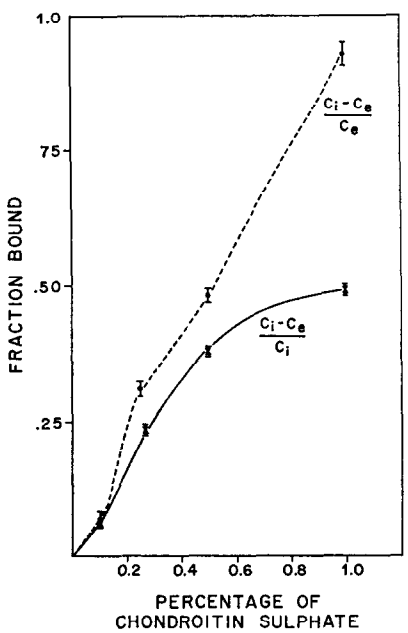

FIG. 1. Effect of the concentration of chondroitin sulfate on the fraction of hexamethonium bound. The terms $(C_i - C_e)/C_e$ and $(C_i - C_e)/C_i$ express the bound fraction with respect to the drug concentration in the external medium and in the dialysis sac respectively. These experiments were carried out at an external medium hexamethonium dichloride concentration of 10 μg/ml, and the above values represent the mean of at least 3 experiments $\pm$ standard error.

buffer + 0·1 M NaCl. Maximum binding of hexamethonium was observed at a chondroitin concentration of 1%, and all further studies were conducted with this concentration of chondroitin sulfate, unless otherwise specified.

Influence of concentration of quaternary ammonium ions on binding. The extent of binding of the bisquaternary [^{14}C]hexamethonium and [^{14}C]decamethonium and monoquaternary [^{14}C]choline to chondroitin sulfate was determined separately at various concentrations of these compounds. An increase in the concentration of the quaternary ammonium ions in the external medium leads to a decrease in the percentage of drug bound $[(C_i - C_e)/(C_e) \times 100]$. However, the moles of drug bound per mole of chondroitin, v, increase linearly with the increase in the concentration of free quaternary ammonium ion, D_f (Fig. 2). This increase in v, however, is biphasic for the diquaternary ammonium ions and monophasic for choline. It appears that each of the bisquaternary ammonium ions is bound to one class of receptor sites on chondroitin, until its concentration in the external medium, C_e, reaches approximately

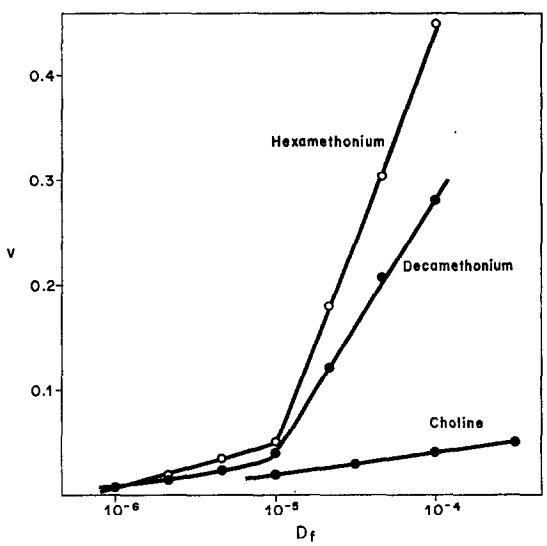

FIG. 2. Effect of increasing drug concentration in the external medium (D_f) on the moles of the quaternary ammonium ion bound per mole of chondroitin (v).

10^{-5} M, and then another class of sites binds these drugs. It should be noted that, at equivalent molar concentrations, higher amounts of hexamethonium were bound to chondroitin than decamethonium. Both the diquaternary ammonium compounds were bound to chondroitin to a much greater extent than was the monoquaternary choline.

The binding data are further analyzed in terms of Scatchard plot[9] (Fig. 3) where v/D_f is plotted against v. Both the bisquaternary ammonium compounds yield biphasic curves, indicating more than one class of binding sites, while choline yields a straight line plot, indicating only a single class of binding sites. Extrapolation of the curves to the ordinate and abscissa, as shown in Fig. 3, estimates $\Sigma n_i k_i$ and Σn_i, respectively, where n_i is the number of binding sites in the ith class and k_i is the binding constant of the drug to the ith receptor site on the chondroitin molecule. No correc-

tions were made for activity coefficients, ionic potentials on either side of the membrane, or for possible interactions between various classes of binding sites on chondroitin, which may help or hinder subsequent binding of hexamethonium. For hexamethonium, Σn_i was graphically estimated to be equal to 3·0 and $\Sigma n_i k_i = 7800$; for decamethonium, $\Sigma n_i = 2$, $\Sigma n_i k_i = 8200$. For choline, the total number of binding sites was found to be less than 0·1, and the binding affinity was relatively very low under the conditions of the experiments. Thus, it appears that hexamethonium and decamethonium are bound strongly to chondroitin *in vitro*.

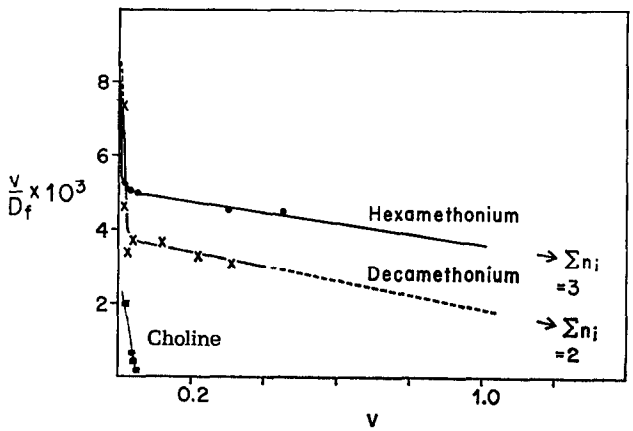

FIG. 3. Scatchard plot for the binding of hexamethonium and decamethonium to chondroitin. For details, see text.

Effect of ionic environment on binding of hexamethonium. The binding of hexamethonium and decamethonium is markedly altered by the presence of other ions in the medium. The highest degree of binding seems to take place in deionized water, but the extent of binding $[(C_t - C_e)/C_t \simeq 99\%]$ could not be determined accurately because of osmotic imbalance of chondroitin sulfate solution against water on the external side of the dialysis sac. However, the influence of ionic strength on the binding of hexamethonium ($C_e = 10$ μg/ml) to chondroitin was investigated at various ionic strengths of NaCl. The binding of hexamethonium decreases linearly with the increase in the ionic strength of NaCl in the external medium (Fig. 4). The bound fraction of the total hexamethonium in the dialysis sac $[(C_t - C_e)/C_t]$ is plotted against the ionic strength, μ, in Fig. 4, where $\mu = 1/2\ \Sigma c_i z_i^2$. The term c_i is the concentration in moles per liter of any of the ions in the external medium and z_i is its valence. The external medium in these experiments consisted entirely of a solution of sodium chloride in deionized water. Increasing the ionic strength of the 0·04 M phosphate buffer by the addition of NaCl similarly reduces the bound fraction.

The extent of binding of hexamethonium (10 μg/ml, external concentration) at an ionic strength of 0·154 of potassium chloride was found to be similar to that of sodium chloride (Table 1). In Table 1, the data show the percentage binding $[(C_t - C_e)/(C_t) \times 100]$ of [^{14}C]hexamethonium ($C_e = 10$ μg/ml) in various electrolyte solutions of equivalent ionic strength, i.e. 0·154. Different fractions of hexamethonium

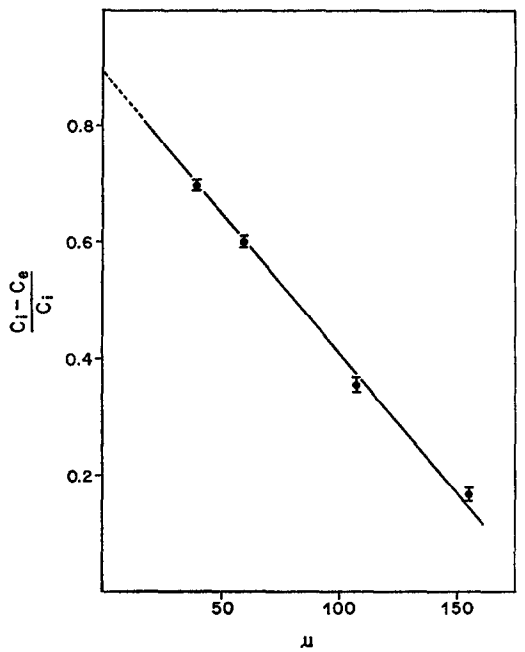

FIG. 4. Linear decrease in the binding of hexamethonium to chondroitin with increasing ionic strength (μ) of sodium chloride medium. $(C_i - C_e)/C_i$ represents the fraction of the total drug bound in 3 or 4 experiments at an external medium concentration of 10 μg/ml. The vertical bars represent the standard error of the mean.

were bound to chondroitin in an external medium of either calcium chloride or magnesium chloride as compared to sodium or potassium chloride. The binding of hexamethonium to chondroitin is increased significantly in the presence of calcium ions. A moderate increase in binding was observed in $MgCl_2$ medium.

TABLE 1. COMPARATIVE BINDING OF [^{14}C]HEXAMETHONIUM TO CHONDROITIN IN SOLUTIONS OF VARIOUS ELECTROLYTES AT EQUIVALENT IONIC STRENGTH*

Electrolyte ($\mu = 0{\cdot}154$)	$(C_i - C_e)/(C_i) \times 100$	N
Sodium chloride	16·8 ± 1·1	3
Potassium chloride	18·9 ± 3·9	3
Calcium chloride	29·8 ± 1·5†	4
Magnesium chloride	23·5 ± 1·4	4

* The extent of binding was determined with 1% chondroitin solution. The $(C_i - C_e)/C_i$ values indicate the mean percentage of the total drug bound ± the standard error of the mean. N indicates the number of experiments.

† P value $< 0{\cdot}05$ when compared by t-test to percentage of hexamethonium bound in sodium chloride solution.

The change in the concentration of hydrogen ions in the external medium was produced by the addition of hydrochloric acid to the sodium chloride medium ($\mu = 0{\cdot}154$). A variation of pH from 6·0 to 5·0 was found not to cause significant alteration of the bound fraction. The percentage binding in these experiments also did not differ from that of experiments conducted at pH 7·3.

Effect of structurally related compounds on the binding of hexamethonium to chondroitin. To study the effect of congeners on the binding of hexamethonium (10 μg/ml) to chondroitin sulfate, other quaternary ammonium compounds were added to the external medium in a 100-fold higher molar concentration than hexamethonium and binding was studied in the normal way. The control dialysis experiments were run at the same time under identical conditions. In control experiments, 48·9 per cent of the total hexamethonium was bound $[(C_i - C_e)/(C_i) \times 100]$ to 1% chondroitin sulfate solution. This extent was reduced to 43·0 per cent in the presence of pentamethonium and

TABLE 2. INFLUENCE OF THE STRUCTURALLY RELATED COMPOUNDS ON THE BINDING OF [^{14}C]HEXAMETHONIUM TO CHONDROITIN SULFATE*

Compounds and their concentrations in the external medium(M)	$(C_i - C_e)/C_i \times 100$	N
Hexamethonium, $3{\cdot}66 \times 10^{-5}$	$48{\cdot}9 \pm 0{\cdot}7$	4
Hexamethonium, $3{\cdot}66 \times 10^{-5}$ + pentamethonium, $3{\cdot}66 \times 10^{-3}$	$43{\cdot}0 \pm 1{\cdot}1$†	4
Hexamethonium, $3{\cdot}66 \times 10^{-5}$ + decamethonium, $3{\cdot}66 \times 10^{-3}$	$44{\cdot}8 \pm 0{\cdot}4$†	4
Hexamethonium, $3{\cdot}66 \times 10^{-5}$ + choline, $3{\cdot}66 \times 10^{-3}$	$49{\cdot}7 \pm 0{\cdot}65$	3

* The $(C_i - C_e)/(C_i) \times 100$ values indicate the mean percentage of the total drug bound ± the standard error of the mean. N indicates the number of experiments. All experiments were carried out in 0·04 M phosphate buffer, pH 7·3.

† Statistically significant inhibition of binding of hexamethonium.

44·8 per cent in the presence of decamethonium (Table 2). The magnitude of the effects is greater when values calculated by the term $[(C_i - C_e)/(C_e) \times 100]$ are compared. Although the inhibitions of binding $[(C_i - C_e)/C_i]$ were of the order of 8–13 per cent, they were statistically significant ($P < 0{\cdot}05$), since the variance of the repeated binding experiments was small.

With 0·25% of chondroitin sulfate solution in the dialysis sac, a higher magnitude of the effect of the congeners was observed. For example, a 100-fold higher concentration of decamethonium in the external medium produced about 9 per cent inhibition of $[(C_i - C_e)/C_i]$ when 1% chondroitin sulfate was used. However, about 23 per cent inhibition was obtained when 0·25% chondroitin sulfate was used in the dialysis sac. This is to be expected, since the total binding capacity for the bisquaternary ammonium ions was decreased at the lower concentration of chondroitin. Choline was

not found to alter the extent of binding of hexamethonium to either 1% or 0·25% chondroitin sulfate. This, it appears that pentamethonium, hexamethonium and decamethonium compete with each other for the same binding site on the chondroitin molecule, while monoquaternary choline may bind to a different site.

Dynamic dialysis. The rate of release of free hexamethonium from inside the dialysis sac in the presence and absence of 1% chondroitin sulfate in 0·04 M phosphate buffer of pH 7·3 is illustrated in Fig. 5. Since almost sink conditions were maintained in the external medium (dynamic dialysis), the rate of escape of hexamethonium from the dialysis sac, dD_t/dt, was a first-order process, depending upon the concentration of the unbound hexamethonium (D_f): $dD_t/dt = KD_f$, where K = first-order rate constant characterizing the diffusion process and the area and thickness of the membrane.

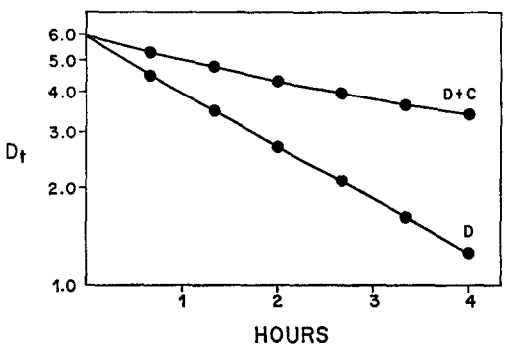

FIG. 5. Rate of release of free hexamethonium from inside the dialysis sac in the absence (D) and presence of 1% chondroitin sulfate ($D + C$). D_t represents the total radioactivity inside the dialysis sac expressed as millions of counts per 10 min.

Once the value of K is calculated in the absence of chondroitin, the instantaneous rate at a particular value of D_t in the presence of chondroitin can be estimated from the plot and the D_f calculated. Curvature in the plot in the presence of chondroitin (Fig. 5) is due to the fact that as the concentration of hexamethonium in the dialysis sac decreases, the bound fraction increases. At a concentration of 20–29 μg/ml in the dialysis sac, 68–56 per cent of the total drug was bound to chondroitin under the conditions of the experiment. Since the instantaneous rate can only be approximated at any particular value of D_t by the graphic method, the bound fraction $[(C_i - C_e)/C_i]$ estimated by equilibrium dialysis, which was found to be 50–47 per cent, was considered to be relatively accurate.

DISCUSSION

Our results indicate that the polymethylene bisquaternary ammonium compounds, hexamethonium and decamethonium, are strongly bound to chondroitin under the experimental conditions of the dialysis procedures. The binding of hexamethonium to chondroitin is influenced by its own concentration, the concentration of chondroitin in the dialysis sac, and the ionic strength of the external medium. The presence of Ca^{2+} or Mg^{2+} in an equi-ionic strength increases the binding of hexamethonium to chondroitin as compared to NaCl or KCl media. We do not have an explanation for

the effect of these inorganic cations on the hexamethonium binding. However, it is plausible that these ions may be somehow unfolding more anionic binding sites on the chondroitin molecule. A change of pH of the sodium chloride solution was not found to produce significant alteration of the bound hexamethonium fraction, indicating that the ability of chondroitin binding groups is not reduced by the addition of H^+ (pH 5).

Decamethonium and hexamethonium appear to have about the same affinity to bind ($\Sigma n_i k_i$) to chondroitin, while hexamethonium is bound to a relatively greater extent. The monoquaternary choline binds to a far lesser extent to chondroitin sulfate *in vitro*. The observation of a biphasic curve by the Scatchard method indicates the existence of more than one class of binding sites on the chondroitin molecule for both hexamethonium and decamethonium. It is probable that the bisquaternary ammonium compounds are bound to the polyanionic chondroitin by ionic interactions; however, further studies have not been done to test this hypothesis. Significant reduction of hexamethonium binding by pentamethonium or decamethonium indicates that the bisquaternary ammonium compounds may be competing for the same sites on the chondroitin molecule.

The polyanionic chondroitin sulfates, being the most abundant of the acid mucopolysaccharide components, have been implicated in the exclusion to some extent of some anionic dyes from the cartilage.[10] The cartilage components have been shown to bind benzhydrylpiperazine compounds such as norchlorcyclizine *in vitro*,[11] while demineralized cartilage has been reported to exhibit the properties of cation exchange.[12] Several of the biological polyanions of the connective tissue, such as heparin, hyaluronate and DNA, bind monoquaternary cetyl pyridinium, *n*-dodecyl trimethyl ammonium ions and their analogs *in vitro*.[13] Preparations of acid polysaccharides from the aqueous extracts of electric organ of the electric eel have been shown to bind bisquaternary dimethyl *d*-tubocurare, *d*-tubocurare, succinylcholine and triquaternary gallamine.[14–17] Chondroitin sulfate A also has been found to bind *in vitro* various mono- and diquaternary ammonium ions, including pyridine 2-aldoxime, methyliodide, curare, cetyl pyridinium and polymethylene bispyridinium ions.[18] However, our data could not be compared to the latter studies, since high concentrations of quaternary ions were used in dialysis experiments and the concentration of chondroitin was not indicated.

Binding of hexamethonium and decamethonium to chondroitin *in vitro* correlates well with the accumulation of these drugs in cartilage *in vivo*. The binding of hexamethonium to chondroitin is significantly inhibited by pentamethonium *in vitro*, whereas, *in vivo*, prior administration of pentamethonium significantly reduces the accumulation of hexamethonium in cartilage.[3] Further histochemical evidence has already been presented[3] which indicates that the accumulation of hexamethonium and related compounds in the cartilage may be the result of binding of these drugs to acid mucopolysaccharide components, although it is possible that polyanionic acid mucopolysaccharide components other than chondroitin may also be binding the bisquaternary ammonium ions *in vivo*. Since all the above di- or triquaternary ammonium ions have been known to localize preferentially in the cartilage, it may be postulated that all those bis- or triquaternary ammonium ions which bind to chondroitin *in vitro* or to acid polysaccharides in general, preferentially localize in the cartilage *in vivo*.

The monoquaternary choline is bound to chondroitin to a far lesser extent *in vitro*

and shows an appreciably lower accumulation in cartilage *in vivo* as compared to hexamethonium and decamethonium, by autoradiographic and biochemical techniques.[3] The strongly binding hexamethonium accumulates in the distal femoral epiphyses of the mouse to a greater extent than does the weakly binding choline. *In vivo*, hexamethonium attains a C_e/C_d (ratio of the concentration of the drug in the epiphyses to that in the diaphyses) of 6·4 at 20 min after an i.p. administration (20 mg/kg), while in the case of choline, a C_e/C_d ratio of 2·7 was attained at 10 min after an i.v. administration (10 mg/kg). Similarly, the monoquaternary compounds, Aprobit,[19] Cetiprin[20] and *N*-methyl-3-(1-hydroxyethyl) pyridinium iodide,[21] do not show the hexamethonium type of marked localization in the cartilage; however, no binding data are available for these drugs.

Thus, it may be possible to predict the cartilage and joint accumulation property of various compounds by testing their binding ability *in vitro* to chondroitin. Understanding the relationship between the chemical structure and cartilage–joint accumulation of drugs may be of significance in the chemical design of drugs for the treatment of lesions of the articular cartilage and of joints. Chemical design of those drugs which combine both the antiarthritic property and the cartilage–joint accumulation property is likely to enhance their potency and diminish undesirable side effects.

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