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IN VITRO BINDING OF OXIME ACETYLCHOLINESTERASE REACTIVATORS TO PROTEOGLYCANS SYNTHESIZED BY CULTURED CHONDROCYTES AND FIBROBLASTS

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Abstract—The incorporation of the ^{14}C-labelled acetylcholinesterase reactivators 1-(methyl-imidazolium)-3 (4-carbaldoxime-pyridinium) propane dibromide (pyrimidoxime) and *N,N'*-trimethylene bis (pyridinium-4-aldoxime) dibromide (TMB4) into cultured chondrocytes and fibroblasts was measured and their binding to macromolecules synthesized by these cells studied. The results showed that these drugs concentrated slowly and poorly into these cells, but bound firmly to high molecular mass materials in the culture supernatants. The chromatographic properties of these macromolecules on Sepharose CL-2B in non-dissociative or dissociative conditions were similar to those of the proteoglycans synthesized by these cells. Dialysis of the macromolecule-bound drugs against increasing pH buffers showed half-dissociation pH > 8, identical to those for chondroitin sulphate. These results suggest strongly that pyrimidoxime and TMB4 are bound to proteoglycans by ionic interactions, and this together with their poor lipophilicity can explain their high selectivity for the cartilaginous tissues as opposed to other proteoglycan-containing structures such as skin.

1-(Methyl-imidazolium)-3 (4-carbaldoxime-pyridinium) propane dibromide (pyrimidoxime) and *N,N'*-trimethylene bis (pyridinium-4-aldoxime) dibromide (TMB4) (Fig. 1) are two acetylcholinesterase reactivators used widely to treat poisoning by organophosphates [1, 2]. In previous papers [3, 4] we studied the biodisposition and metabolism of these drugs, and found that these molecules rapidly concentrated in the kidney and in proteoglycan-containing tissues such as cartilage and intervertebral discs after i.m. injection. Binding to cartilaginous tissue reached a maximal value 10 min after injection for both compounds and this high level lasted for at least 2 hr after administration, while in the plasma, less than 0.5% of the maximal concentration was present at this time. Skin and other proteoglycan-containing connective tissues concentrated these drugs less. To explain this high specificity for cartilage, we studied the action of pyrimidoxime and TMB4 on cultured chondrocytes and fibroblasts, which are able to synthesize the constituent macromolecules of the cartilaginous and other connective tissues [5, 6]. Previous findings [7–9] showed that several cationic molecules, such as bisquaternary ammoniums and inorganic cations, are able to bind to the anionic components of cartilage and other connective tissue, and this mechanism may be involved in the binding of pyrimidoxime and TMB4. We therefore measured the incorporation of these drugs labelled with ^{14}C into cultured chondrocytes and fibroblasts, and analysed their binding to the proteoglycans synthesized by the cells.

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‡ Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; GuHCl, guanidinium chloride; PMSF, phenylmethylsulphonyl fluoride; TCA, trichloracetic acid; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

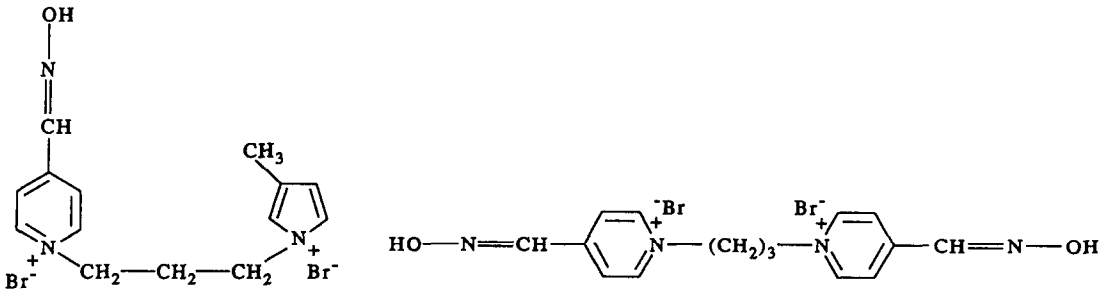
Chemicals

Pyrimidoxime and TMB4 were synthesized and labelled with ^{14}C on the oxime function at INSERM U 71, as described in previous papers [10, 11]. The specific activities were 232 GBq/mol (6.3 Ci/mol) for [^{14}C]pyrimidoxime and 171 GBq/mol (4.6 Ci/mol) for [^{14}C]TMB4. Radiochemical purities were shown by TLC and HPLC to be >98% for both products.

[^{35}S]Sodium sulphate (carrier-free) in aqueous solution (47 GBq/mmol) was purchased from Amersham (U.K.). Collagenase was from Worthington. Dulbecco's modified Eagle's medium (DMEM‡) and glutamine were purchased from Gibco (Uxbridge, U.K.). Foetal calf serum (FCS) was from IBF (France). Sepharose CL-2B was obtained from Pharmacia (France). *N*-Ethyl maleimide, 6-amino *n*-hexanoic acid, benzamidine hydrochloride, phenylmethylsulphonyl fluoride and chondroitin sulphate were purchased from the Sigma Chemical Co. (Poole, U.K.). EDTA was from Fluka (Buchs, Switzerland) and guanidinium chloride (GuHCl) was purchased from Merck (Darmstadt, Germany).

Cell culture

Chondrocyte culture. Cartilage was taken from the shoulders and the knees of 1-month-old rabbits



Pyrimidoxime TMB 4

Fig. 1. Chemical structures of pyrimidoxime and TMB4.

(Fauve de Bourgogne, IFFA Credo). Chondrocytes were isolated by digestion with 0.2% collagenase overnight at 37° [12] and cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 8 μg/mL gentamycin, at 37° in a 5% CO_2 atmosphere. Cell viability was determined by Trypan blue dye exclusion: 93% of released cells were viable. Experiments were performed on primary confluent cultures, in order to avoid dedifferentiation of the chondrocytes.

Fibroblast culture. Rabbit skin explants were maintained in DMEM containing 20% FCS, 2 mM glutamine, 8 μg/mL gentamycin at 37° in a 5% CO_2 atmosphere, until cell overgrowth. Explants were discarded, and the cells growing out of the explants were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 4 μg/mL gentamycin, as chondrocytes, and were serially passaged by trypsinization. Experiments were carried out at less than four passages.

Protein assay. The cell layers were washed with phosphate-buffered saline (PBS), sonicated and protein concentration was measured by Bradford's method [13] with Coomassie blue G 250 solution, using bovine serum albumin as a standard.

Measurement of drugs uptake by cells. The culture medium (DMEM, 10% FCS) of confluent monolayer cells in 25 cm^3 flasks, was changed 30 min prior to experiments. Radiolabelled drug (1 μmol/mL) was added and the incorporation was performed, up to 48 hr, at 37° in a 5% CO_2 atmosphere. At different times, the cell layer was washed three times with PBS, scraped with a rubber policeman and then centrifuged at 900 *g* for 10 min, and the cell pellet radioactivity determined by scintillation counting, after solubilization with soluene at 37°. In a parallel flask, the number of cells was measured and protein content determined. The results were expressed in nmol incorporated drug per mg cell protein.

Metabolic labelling of proteoglycans with ^{35}S. [^{35}S]-Sodium sulphate (10 μCi) was added to confluent cells in 25 cm^2 flasks and the medium maintained for 24 hr at 37° prior to experiments.

Measurement of drugs binding to trichloroacetic acid (TCA)-precipitable proteins. Monolayer cells were incubated for 24 hr at 37° with 1 μmol/mL radiolabelled drug in culture medium (DMEM, 10% FCS) in a 5% CO_2 atmosphere. The supernatant proteins and cell lysate were precipitated by addition of cold TCA (5% final concentration), washed twice with 5% TCA and then dissolved in soluene, and their radioactivity quantified by liquid scintillation counting.

Measurement of the size of the binding macromolecules

Calibration of gel-filtration columns. A Sepharose CL-2B column (0.9 × 55 cm) was calibrated with high relative molecular mass blue dextran for the void volume (V_o) and [^{35}S]sodium sulphate or Bromophenol blue for the total (V_t) volume. V_e is the elution volume of the sample. K_{av}, the partition coefficient, is defined as: $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_e is the elution volume of the sample. Molecular mass was of eluted molecules were determined as described by Ohno *et al.* [14].

Proteoglycan aggregate analysis. Confluent cells in 25 cm^2 flasks were incubated for 24 hr at 37° in a 5% CO_2 atmosphere, with radioactive products: 5 μCi/mL for [^{35}S]sodium sulphate and 1 μmol/mL for pyrimidoxime and TMB4. After incubation, the free drugs were separated from the macromolecule-bound drugs by dialysis for 48 hr at 4° against buffer A (0.5 M sodium acetate, pH 6.8 containing a cocktail of protease inhibitors: EDTA 10 mM, benzaminidium chloride 10 mM, 6-aminohexanoic acid 100 mM, *N*-ethyl maleimide 10 mM, phenylmethylsulphonyl fluoride 1 mM) [15]. The samples were poured onto a Sepharose CL-2B column. Elution was performed with buffer A supplemented with 0.5% Triton X-100 at 6 mL/hr and 0.4 mL samples were collected, dissolved into Packard ultima-gold scintillation cocktail and counted. The cell layer was extracted overnight at 4° by 4 M GuHCl in buffer B (0.05 M sodium acetate, pH 5.8 containing the same cocktail of protease inhibitors). The samples were dialysed for 48 hr at 4° against buffer A and chromatographed on Sepharose CL-2B, as above.

Proteoglycan monomer analysis. Dissociative conditions were obtained by adding 4 M GuHCl to buffer A. This medium is able to dissociate

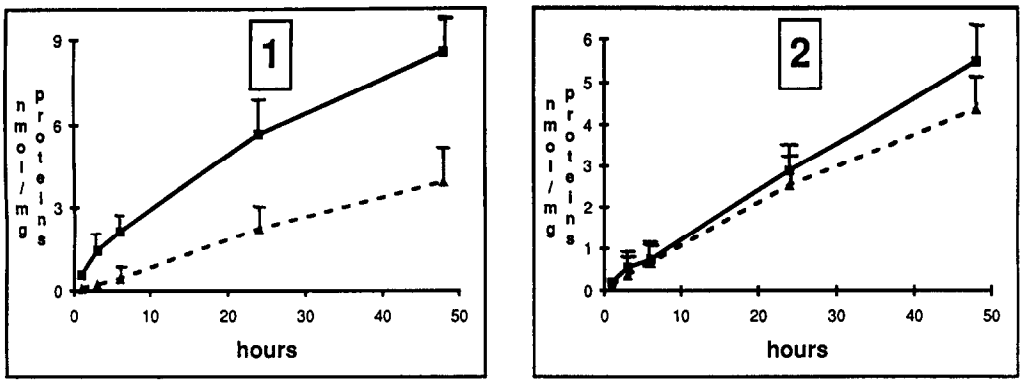


Fig. 2. Incorporation of [^{14}C]pyrimidoxime and TMB4 into cultured chondrocytes and fibroblasts as a function of time following the addition of the labelled drug to the culture medium. (1) Pyrimidoxime; (2) TMB4. (■——■) Chondrocytes; (▲---▲) fibroblasts. Results are the means ± SEM of four experiments.

proteoglycan aggregates into subunits by breaking the bonds between these molecules and hyaluronic acid [15]. Dialysis and gel filtration were conducted as described for proteoglycan aggregates, using this dissociative buffer.

Binding studies as a function of pH. Chondrocyte culture supernatant (1 mL), incubated for 24 hr with [^{14}C]pyrimidoxime or [^{14}C]TMB4, was poured into a dialysis bag and dialysed for 48 hr at 4° against 200 mL 0.1 M glycine-NaOH buffer at pH 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. After this time, radioactivity was measured after the dissolution of an aliquot in Packard ultima-gold scintillation cocktail. Binding to proteoglycans was checked by Sepharose CL-2B chromatography as described above. Similar experiments were conducted with purified chondroitin sulphate: 0.2 μmol of labelled drugs were incubated for 1 hr with 10 mg chondroitin sulphate dissolved in PBS. The bound radioactivity was measured as described above. Bound radioactivity was plotted as a function of pH and the half-dissociation pH determined from the curve.

RESULTS

In Fig. 2 the amounts of pyrimidoxime and TMB4 incorporated into chondrocytes and fibroblasts for up to 48 hr after addition of labelled compounds to the culture medium are shown. For pyrimidoxime, less radioactive drug was incorporated into fibroblasts than into chondrocytes. For TMB4 no significant difference was found between the two cell cultures.

Table 1. Binding of pyrimidoxime and TMB4 to macromolecules synthesized by chondrocytes and fibroblasts

	Molecule	Chondrocytes	Fibroblasts
Cell uptake	Pyrimidoxime	1.22 ± 0.18*	0.53 ± 0.30*
	TMB4	0.60 ± 0.17	0.54 ± 0.06
Cell TCA-precipitable macromolecules	Pyrimidoxime	0.019 ± 0.009	0.024 ± 0.011
	TMB4	0.012 ± 0.005	0.020 ± 0.003
Extra-cellular TCA-precipitable macromolecules	Pyrimidoxime	0.84 ± 0.18	0.35 ± 0.002
	TMB4	0.98 ± 0.19	0.51 ± 0.005
Cell macromolecule subunits	Pyrimidoxime	1.20 ± 0.38*	0.45 ± 0.29*
	TMB4	0.76 ± 0.30	0.65 ± 0.30
Extra-cellular macromolecular aggregates	Pyrimidoxime	13.51 ± 4.54	7.61 ± 2.09
	TMB4	10.08 ± 2.85	6.14 ± 1.21
Extra-cellular proteoglycan subunits	Pyrimidoxime	12.16 ± 5.25	7.612 ± 2.94
	TMB4	9.43 ± 2.30	5.70 ± 1.58

Results, given in nmol of labelled drugs per 10^6 cells, are the means ± SEM of four experiments.

Binding to macromolecules was obtained by measuring the eluted radioactivity from Sepharose CL2B columns under associative (aggregates) and dissociative conditions (subunits).

* Significant difference between chondrocytes and fibroblasts ($P < 0.005$).

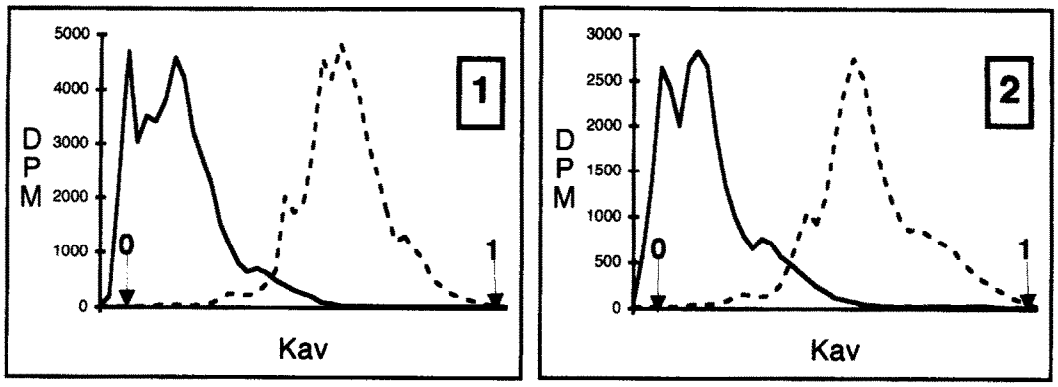


Fig. 3. Chromatographic analysis on Sepharose CL2B of the radioactivity bound to macromolecules synthesized by cultured chondrocytes and of the ^{35}S-labelled proteoglycans. (1) Pyrimidoxime: supernatant. (——) Non-dissociative conditions; (----) dissociative conditions. (2) ^{35}S-Labelled proteoglycans: supernatant. (——) Non-dissociative conditions; (----) dissociative conditions.

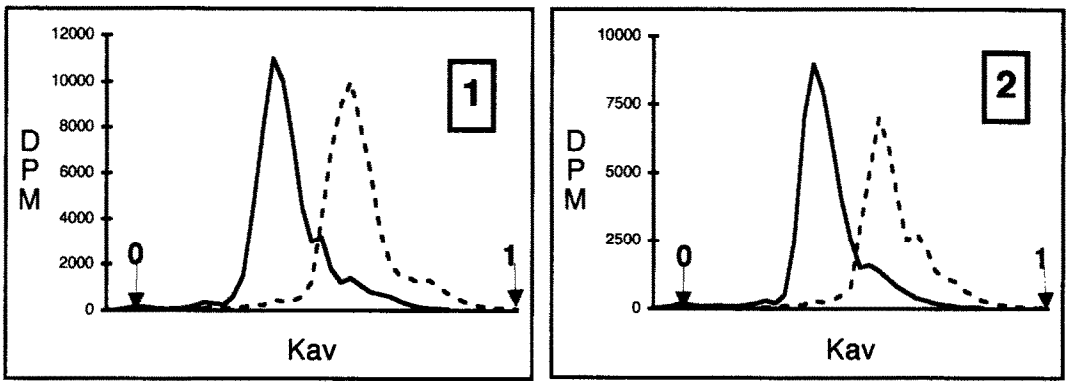


Fig. 4. Chromatographic analysis on Sepharose CL2B of the radioactivity bound to macromolecules synthesized by cultured fibroblasts and of the ^{35}S-labelled proteoglycans. (1) Pyrimidoxime: supernatant. (——) Non-dissociative conditions; (----) dissociative conditions. (2) ^{35}S-Labelled proteoglycans: supernatant. (——) Non-dissociative conditions; (----) dissociative conditions.

Table 1 shows the amount of oximes bound to macromolecules after 24 hr incubation. The main part of the radioactivity appeared to be in the extracellular medium, for both oximes and for both chondrocytes and fibroblasts, but the amount of bound labelled molecules was greater for chondrocyte cultures. Measurement of the radioactivity in TCA-precipitable proteins shows that there was very weak binding to these molecules, the main part remaining in the supernatant that contains the non-precipitable proteoglycans.

In Figs 3–5 are plotted the results of the chromatographic analysis on Sepharose CL-2B of the macromolecule-bound radioactivity after 24 hr reaction with pyrimidoxime, and the labelled proteoglycans after 24 hr [^{35}S]sulphate incorporation (results obtained with TMB4, identical to those with pyrimidoxime, are not shown in the Figs 3–5). The chromatographic profiles are similar for the macromolecules bound to oximes and proteoglycans labelled with [^{35}S]sulphate. Proteoglycans from chondrocyte culture medium, under non-dissociative conditions, were present as aggregates, eluted in the void volume, with a high molecular mass $>10^4$ kDa. An important shift of the K_{av} (average value = 0.55) was observed under dissociative conditions, which correspond to proteoglycan subunits, with a molecular mass between 5×10^2 and 1.5×10^3 kDa. Proteoglycans from fibroblast culture medium were eluted with a K_{av} of about 0.35 (2×10^3 kDa) under

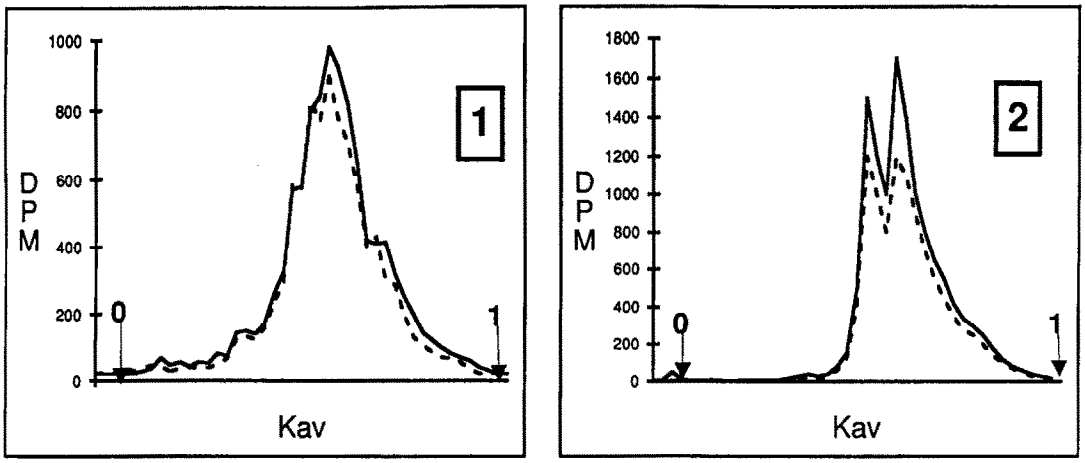


Fig. 5. Chromatographic analysis on Sepharose CL-2B of the intracellular radioactivity bound to macromolecules synthesized by cultured chondrocytes and fibroblasts and of the ^{35}S-labelled proteoglycans. (1) Chondrocytes; (2) Fibroblasts. (——) Pyrimidoxime; (– – – –) ^{35}S-labelled proteoglycans.

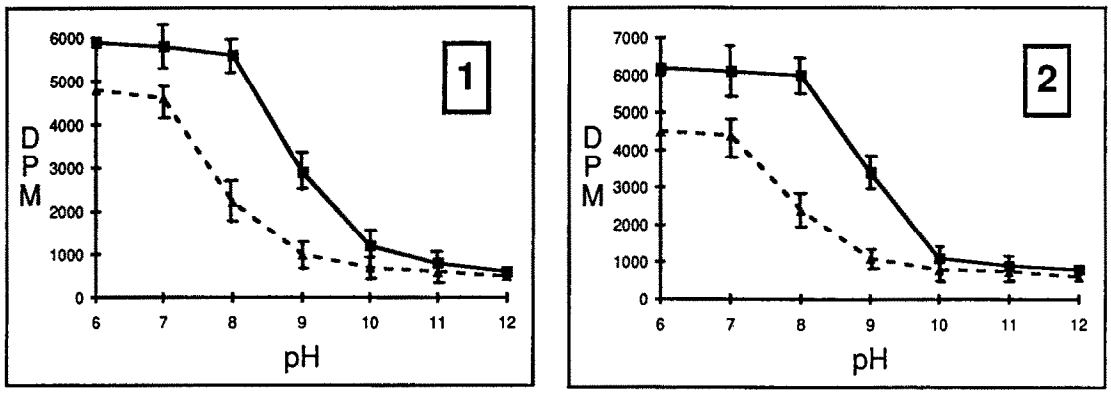


Fig. 6. Dialysis of macromolecule-bound radioactivity against increasing buffer pH. (1) Cultured chondrocytes: supernatant; (2) chondroitin sulphate. (■——■) Pyrimidoxime; (▲– – –▲) TMB4. Results are the means ± SEM of four experiments.

non-dissociative conditions, and with a K_{av} of 0.55 corresponding to a molecular mass of about 0.5×10^3 kDa, under dissociative conditions.

The intracellular macromolecule-bound radioactivity analysis shows a broad peak with a K_{av} of about 0.45–0.80 identical for the two cell types. No measurable K_{av} shift was observed between dissociative and non-dissociative media for either chondrocytes or fibroblasts.

For the macromolecule-bound drugs, identical radioactivity was found after elution under non-dissociative and dissociative conditions, for both chondrocytes and fibroblasts. No free drug was found to elute in the total volume of the column ($K_{av} = 1$), indicating that after dialysis all the radioactivity was bound to the macromolecules.

Figure 6 shows the macromolecule-bound radioactivity in the chondrocyte culture medium as a function of the pH of the dialysis buffer. For pyrimidoxime, the half-dissociation pH was found at pH 9.1 and for TMB4 at pH 7.9. The same experiments conducted on purified chondroitin sulphate gave a half-dissociation pH of 9.1 for pyrimidoxime and 8.2 for TMB4.

DISCUSSION

The results described show that ^{14}C-labelled pyrimidoxime and TMB4 bind firmly to the macromolecules synthesized by cultured chondrocytes and fibroblasts. Gel filtration analysis on Sepharose CL-2B of the bound radioactivity shows binding of more than 90% to a very high molecular mass macromolecules. In a dissociative medium, this size is markedly decreased. These properties agree with those described previously for proteoglycans

synthesized by chondrocytes and fibroblasts [6, 16]. Moreover, the binding molecules are smaller in the intracellular cytosol than in the extracellular culture medium. This result agrees with the scheme of proteoglycan biosynthesis: subunit synthesis in the cells and polymerization with hyaluronic acid in the extracellular matrix [16, 17]. After incubation with [^{35}S]sulphate, chondrocytes and fibroblasts synthesized proteoglycans exhibiting the same chromatographic behaviour. These findings suggest strongly that pyrimidoxime and TMB4 are bound to proteoglycans. These large polyanionic molecules contain sulphate and carboxyl residues that are probably responsible for the fixation of the drugs by ionic interaction between their quaternary ammonium and the anionic residues of the polymers. This is supported by the results of the dialysis of the macromolecule-bound radioactivity in increasing pH buffers. For both molecules, dissociation occurred at pH > 8. The same experiments, conducted with oximes bound to purified chondroitin sulphate, the main anionic component of proteoglycans, showed the same half-dissociation pH as those observed with macromolecules synthesized by the cells. This result shows that pyrimidoxime and TMB4 are bound to proteoglycans by ionic interactions between their quaternary ammonium and the anionic functions of these macromolecules. These properties can explain the high affinity of these drugs for proteoglycan-containing tissues. The low amounts of these molecules found in the intracellular cytosol shows poor penetration through the cellular membrane. Ionic binding to proteoglycans in the extracellular matrix is probably the main reason for their high affinity for the cartilaginous tissue. Our results confirm the findings of Ashgar and Roth [7] and Olsen *et al.* [8] on bisquaternary ammonium compounds *in vivo* and on purified chondroitin sulphate. Larsson *et al.* [9] have shown that organic and inorganic cations are able to bind chondroitin sulphate by ionic interactions with their sulphate and carboxyl functions. Our results suggest that similar mechanisms are undergone for pyrimidoxime and TMB4. Our previous pharmacokinetic studies [3, 4] showed high drug concentrations in the cartilage, but not in the other proteoglycan-containing structures such as the skin. This selectivity can be explained by the high hydrophilicity of these molecules. Indeed, according to the findings of Maroudas *et al.* [18], the ionic concentration in the cartilage matrix is higher than that in the external medium, and this difference induces a Donnan osmotic pressure that is responsible for the viscoelastic properties of this tissue. When the cartilage is submitted to compressive loading, the internal hydrostatic pressure increases with subsequent loss of water from the tissue to reach a new equilibrium where the osmotic pressure balances the applied load. When this external load is removed, water from the external medium, containing exogenous cationic substances such as quaternary ammoniums, impregnate the matrix to restore the preloading equilibrium [16]. On the other hand, other connective tissues, such as the skin, contain lipids and are less hydrophilic than cartilage. Drug hydrophilicity is unfavourable for its penetration into these structures, but furthers its diffusion into the cartilage matrix.

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