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Correlation between [Na^+], [Glycosaminoglycan] and Na^+/K^+ Pump Density in the Extracellular Matrix of Bovine Articular Cartilage

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Summary

The plasma membrane abundance of the Na^+/K^+ pump or Na^+,K^+-ATPase depends on the intracellular concentration of Na^+ in almost all animal cells. In cartilage, chondrocytes are surrounded by an extracellular matrix which consists of collagen and proteoglycan, a ground substance composed of glycosaminoglycan (GAG) side chains with a high fixed negative charge density. The polyanionic nature of the GAGs tends to draw monovalent cations into the matrix resulting in high [Na^+] which may exceed 250 mM. As the concentration of GAGs in the tissue increases, so does [Na^+]. In this study, it was found that the density of the Na^+/K^+ pump, measured by ^{3}H-ouabain binding, correlates with the concentration of GAGs in the tissue. This indicates that chondrocytes are sensitive to their ionic environment and respond to local [Na^+] variations by regulating the abundance of the Na^+/K^+ pump.

Key words
Cartilage – Chondrocyte – Glycosaminoglycan – Extracellular [Na^+] – Na^+/K^+ pump density

Introduction

Articular cartilage contains a high fixed negative charge density which results in a high concentration of cations, including Na^+ and K^+, in the extracellular matrix (Gray *et al.* 1988, Lesperance *et al.* 1992). The fixed charge density varies with position and depth in the tissue, and will also vary from joint to joint, and with age and pathology (Maroudas 1979, 1991). The concentration of free Na^+ in cartilage is high compared to other tissues (250–350 mM Na^+) (Maroudas 1979). Therefore, chondrocytes *in situ* are surrounded by an unusual extracellular environment. In most animal cells, including chondrocytes, the Na^+/K^+ pump or Na^+,K^+-ATPase is responsible for maintaining a low intracellular Na^+/K^+ ratio, vital for normal cellular function. The intracellular Na^+ ($[Na^+]_i$) will depend on cell permeability and extracellular Na^+ ($[Na^+]_o$). Since the plasma membrane density of the Na^+/K^+ pump has been shown to be regulated by $[Na^+]_i$ (Wolitzky and Fambrough 1986, Pressley 1988, Brodie and Sampson 1989), Na^+/K^+ pump density may vary with $[Na^+]_o$ and play an important role in the adaptation of chondrocytes to changes in their unusually cationic extracellular environment. In this study, combinations of biochemical approaches were employed to examine the relationship between Na^+/K^+ pump density and the extracellular ionic environment in cartilage. Tissue $[Na^+]_o$ was found to depend on the local [GAG].

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There was also a correlation between Na^+/K^+ pump density and [GAG] *in situ*: the Na^+/K^+ pump density increased with increasing [GAG] (Mobasheri *et al.* 1994, 1995). The results of this study show, for the first time, that the abundance of the Na^+/K^+ pump may vary according extracellular $[Na^+]$ and the polyanionic nature of the extracellular matrix. This suggests a possible feedback mechanism between the cationic environment resulting from the GAGs of the extracellular matrix and the cellular regulation of Na^+/K^+ pump density in chondrocytes.

Materials and Methods

Biochemicals

All chemicals were A.C.S. grade and purchased from Sigma/Aldrich (Poole, Dorset, UK), and Bio-Rad (Hemel Hempstead, UK). Tissue culture media, enzymes and antibiotics were purchased from Sigma/Aldrich. ^{22}Na was obtained from NEN Du Pont Ltd. (Stevenage, Herts., UK), and ^3H-ouabain from Amersham International (Amersham, Bucks., UK).

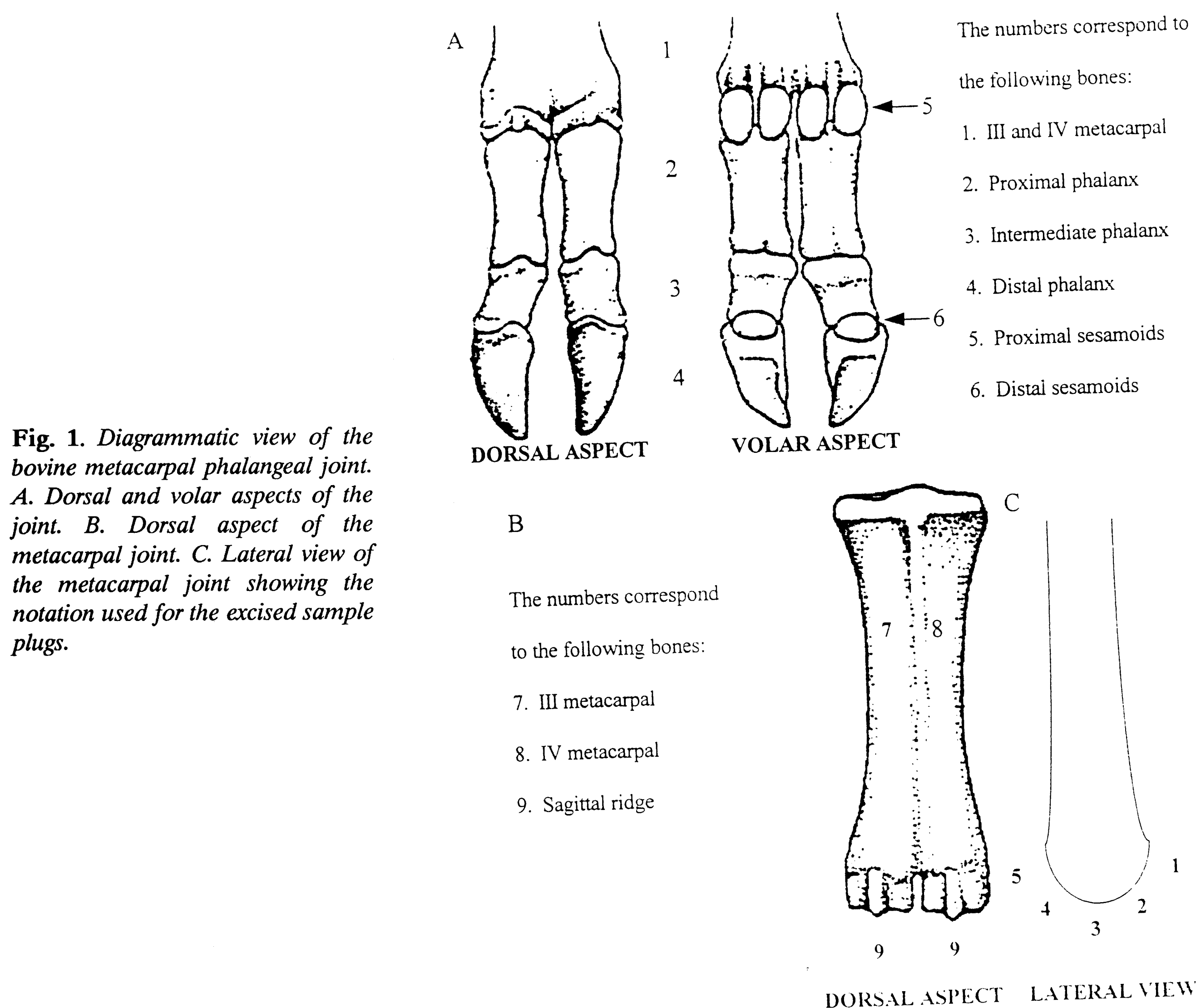

Fig. 1. *Diagrammatic view of the bovine metacarpal phalangeal joint. A. Dorsal and volar aspects of the joint. B. Dorsal aspect of the metacarpal joint. C. Lateral view of the metacarpal joint showing the notation used for the excised sample plugs.*

Tissue sampling

Bovine metacarpal-phalangeal joints from the front feet of 18 to 24-month-old steers were obtained fresh from a local abattoir. The joints were washed, skinned, sprayed with 70 % ethanol (v:v with water) and opened under sterile conditions in a tissue culture hood. Full depth cartilage plugs were excised from delineated regions (see Fig. 1) of the articular surface of the joint avoiding the bone, using a ×6 cork borer and used for measurements of tissue composition. In some experiments, plugs were incubated in DMEM containing 120 and 220 mM Na^+ for 18 h before determining Na^+/K^+ pump density. Cartilage plugs were weighed immediately to obtain fresh wet weight and placed in DMEM (Dulbecco's Modified Eagle's Medium, with L-glutamine and 1000 mg/l glucose,

without sodium bicarbonate, buffered with HEPES, pH 7.4, 280 mOsm). An antibiotic/antimycotic solution was added to final concentrations of 160 units penicillin, 0.16 mg streptomycin and 0.5 μg amphotericin B per ml DMEM. The swelled wet weight was also determined because of the tendency of this tissue to swell. All measurements were calculated back to dry weight which was determined following overnight drying (at 67 °C) to constant weight.

Determination of fixed charge density (FCD), tissue [Na+], GAG and DNA content of cartilage explants

(a) The fixed charge density (FCD) was measured by determining tissue [Na^+] using radio isotope exchange with $^{22}NaCl$ (Maroudas and Urban 1983). Briefly, full-depth articular cartilage explants were allowed to equilibrate in a suitable volume of a 0.015 M NaCl solution containing trace amounts of ^{22}Na (about 0.1 μCi/ml, 3.7 kBq/ml) for FCD. A 0.15 M NaCl solution was used for measuring tissue [Na^+] (Maroudas and Urban 1983). The incubation was performed at 4 °C to minimize autolysis and the incubation time varied between 4 h to overnight depending on the thickness of the plugs. Plugs were then removed from the tracer salt solution, blotted dry and transferred to a γ-counter for the determination of radioactivity. Known volumes of the standards were counted, the background counts measured simultaneously, and the FCD determined using the formula below:

Tissue [Na^+] = Specimen CPM x concentration of incubating solution (mmol/g)/wet/dry weight of the specimen x CPM per g radioactive solution.

For tissue equilibrated in 0.015 M NaCl, it was shown that the tissue [Na^+] is equal to the FCD (units in mEq/g).

(b) Glycosaminoglycan content of tissue explants was quantified by a modified colorimetric assay employing dimethylmethylene blue (Farndale *et al.* 1986).

(c) The DNA content of cartilage explants and measurements of cell density were performed using a simple two-step fluorometric method utilising the bisbenzimidazole dye Hoechst 33258 (Kim *et al.* 1988). Cartilage explants were initially digested with 300 μg/ml of two times crystallized papain in a sodium phosphate buffer (20 mM Na_2PO_4, 1 mM EDTA, 1 mM cysteine-HCl, 2 mM dithiothreitol, pH 6.8).

Ouabain binding

The Na^+/K^+ pump number was measured by ^3H-ouabain binding according to Erdmann (1982), in a K^+ free artificial medium (to accelerate the rate of binding and because of the competition between K^+ and ouabain) containing all the chemical components present in DMEM, except for amino acids and vitamins. The cartilage was cut as described above into DMEM, incubated for 18–20 h, and then washed for one hour (in a K^+-free medium) to reduce the [K^+] within the tissue. The determination of Na^+/K^+ pump density was measured in this solution containing 4–8 kBq/ml of the specific inhibitor ^3H-ouabain. Non-specific ouabain binding was determined by the addition of excess non-radioactive ouabain (10^{-4} M). Explants were then washed with ice-cold, non-radioactive K^+-free medium (3 times over one hour). The explants were then digested with papain as described above. Half of the aliquot was used for the determination of radioactivity by scintillation counting, the other half was used for the GAG and DNA assays. By taking a value 7.7 pg DNA/chondrocyte (Kim *et al.* 1988) these data yielded specific ouabain bound/chondrocyte or Na^+/K^+ pump density/chondrocyte in the explant.

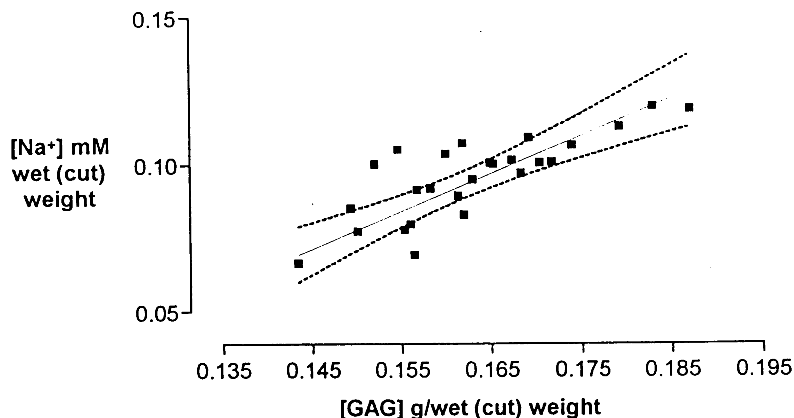

Fig. 2. *The correlation between tissue [Na+] (measured by radio-isotope exchange) and [GAG] (assayed colorimetrically using dimethylmethylene blue dye binding). For 26 sample plugs taken from various sites of one bovine metacarpo-phalangeal joint, there was a good correlation between tissue [Na+] and [GAG] (correlation coefficient r^2=0.546, P<0.0001; df=24;). As tissue [GAG] increases, the concentration of [Na+] counteracting the fixed negative charges (FCD) of tissue GAGs also increases resulting in a nearly linear relationship.*

Results

Relationship between tissue [Na^+] and [GAG]

A significant correlation was found between tissue [Na^+] and [GAG] (Fig. 2). As the concentration of GAGs in the matrix increased so did the [Na^+]. Good agreement existed between tissue [Na^+] and [GAG]; as the [GAG] increased in the tissue from 0.179 to 0.352 g/g dry weight so did [Na^+] from 0.182 to 0.266 g/g dry weight. This correlation was observed in several bovine joints examined, although the data presented are from a single joint. This relation was linear over the range of GAGs found in the tissue.

Correlation between Na^+/K^+ pump density and [GAG]

The correlation found between tissue [Na^+] and [GAG] (Fig. 3A) extended to the abundance of the Na^+/K^+ pump in chondrocytes (Fig. 4B). As the [GAG] increased in the tissue, so did the Na^+/K^+ pump density in chondrocytes.

Variation of Na^+/K^+ pump density and GAG content with position across the joint surface

There was a clear link between Na^+/K^+ pump density and [GAG]. As [GAG] varied across the joint so did Na^+/K^+ pump density (Fig. 4B); the Na^+/K^+ pump density of cartilage explants closely mirrored variable tissue [GAG] across the joint. There was also a dependence between tissue [Na^+] and [GAG]. Tissue [Na^+] and [GAG] closely mirrored each other across the articular joint (Fig. 3A). Chondrocytes *in situ* expressed a range of pump densities depending on the position and local concentration of GAGs in the joint. The range was 85 000–160 000 sites/cell and varied with [GAG].

Variation of Na^+/K^+ pump density with increased extracellular [Na^+]

Na^+/K^+ pump density was upregulated in response to a long-term increase in $[Na^+]_o$ (Fig. 4A). Chondrocytes *in situ* upregulated Na^+/K^+ pump density after 18 h in elevated $[Na^+]_o$ (220 mM).

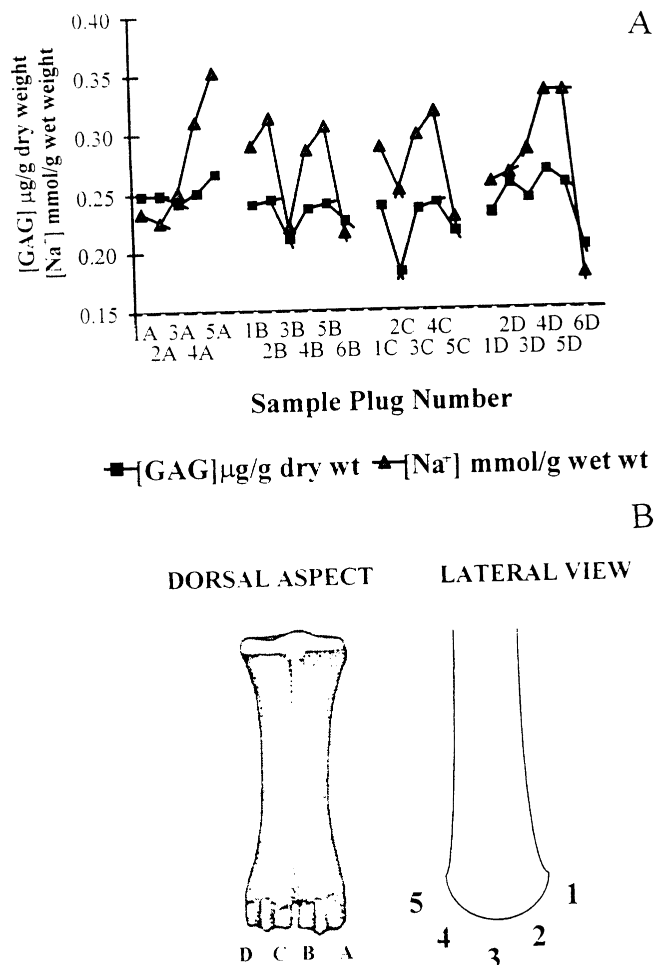

Fig. 3. *The relationship between tissue [GAG] and tissue [Na^+] along the bovine joint. [GAG] mg/g dry weight and [Na^+] mmol/g wet weightwere plotted against sample cartilage plug numbers taken from the end of the metacarpal bones III and IV (excluding the sagittal ridge) (B) to show the relationship between [GAG] and [Na^+] along the joint surface (A).*

Discussion

In view of the high concentration of cations in the extracellular ionic environment of chondrocytes, it was of interest to examine the relationship between the Na^+/K^+ pump and the extracellular matrix. Intracellular [Na^+] has been shown to be the key regulator of Na^+/K^+ pump abundance (Brodie and Sampson 1989). The Na^+/K^+ pump density in the tissue was studied by ^{3}H-ouabain binding and the results related to the concentration of matrix GAGs. The results presented indicate that the Na^+/K^+ pump density of chondrocytes in articular cartilage depends on the local [GAG]. Furthermore, there appears to be a site dependent variability in Na^+/K^+ pump density *in situ*: chondrocytes in different areas of the joint exhibit different Na^+/K^+ pump densities (Fig. 4B). The results of specific ^{3}H-ouabain binding experiments clearly indicated that Na^+/K^+ pump density/chondrocyte of cartilage explants closely mirrored [GAG] across the joint (Fig. 3). The manner in which tissue $[Na^+]_o$ and [GAG] varied across the joint was also very similar. The results from the tissue measurements suggest a possible feedback mechanism between $[Na^+]_o$ (resulting directly from the fixed negative charges on GAG side chains in the extracellular matrix) and Na^+/K^+ pump density. Increasing $[Na^+]_o$ resulted in the upregulation of

Na^+/K^+ pump sites in the cartilage (Fig. 4A). This study suggests for the first time that the Na^+/K^+ pump density of articular chondrocytes is sensitive to the external ionic environment and may vary according to changes in extracellular $[Na^+]$. Thus, changes to the expression and turnover of the Na^+/K^+ pump may be required to maintain the optimal intracellular Na^+/K^+ ratio for matrix turnover (Urban *et al.* 1993). This may be achieved by transcriptional and translational regulation at the gene and protein levels, respectively, in normal cartilage (Mobasheri *et al.* 1997).

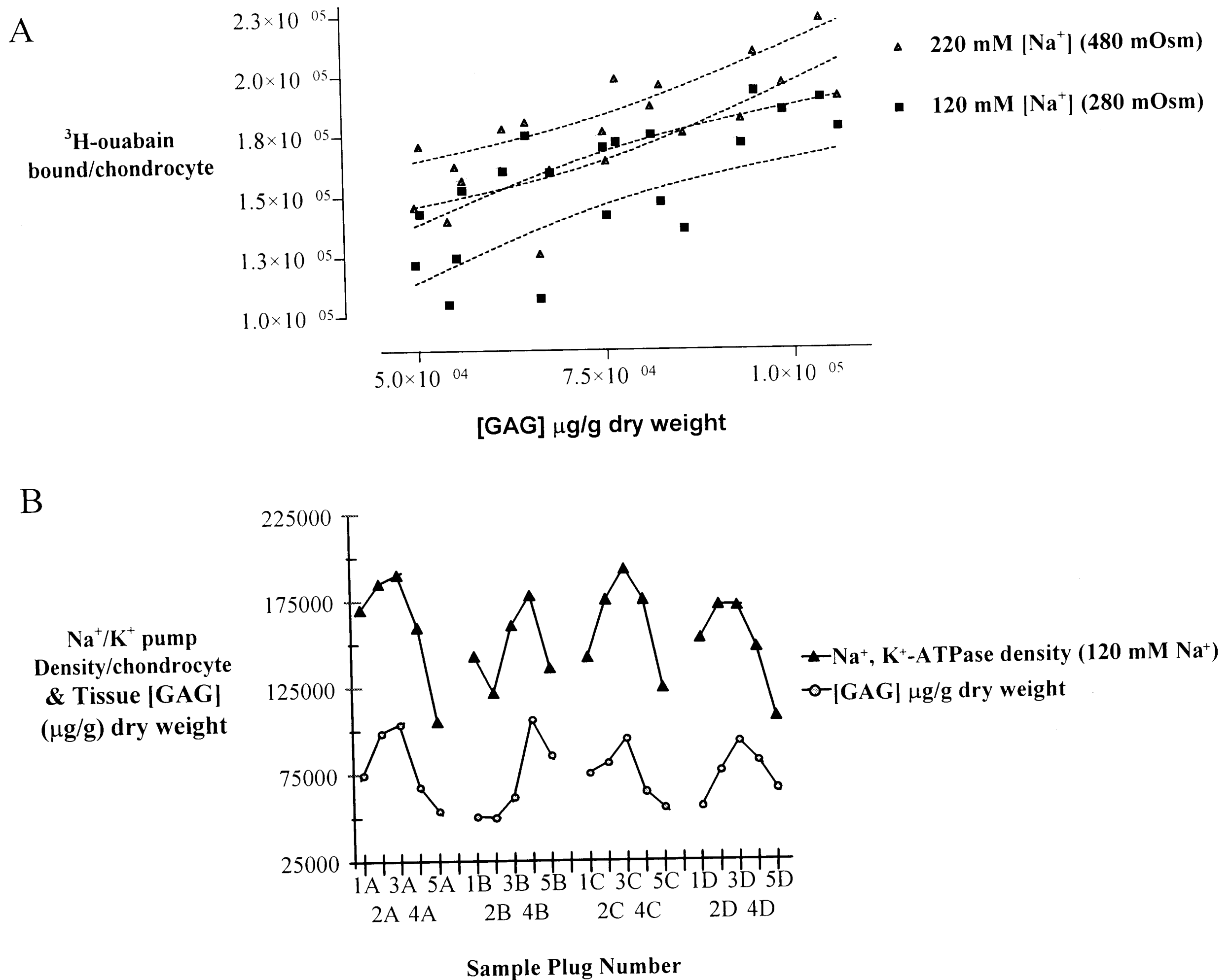

Fig. 4. *The relationship between Na^+/K^+ pump density measured by ^3H-ouabain binding in K^+-free conditions and tissue [GAG] assayed by dimethylmethylene blue dye binding. The first graph (A) shows ^3H-ouabain bound/cell DNA (cell DNA assayed fluorometrically) plotted against [GAG] mg/g dry weight in two ionic conditions; 120 mM Na^+ and 220 mM Na^+. The straight lines indicate linear regressions and the dotted lines are the 95 % confidence intervals. In the second graph (B) the 120 mM Na^+ data are re-plotted to show the variation with [GAG] across the joint (sample plug numbers 1–5). Sample plug #3 (the site experiencing the highest loads) appears to have a higher Na^+/K^+ pump density than plugs 1–2 and 4–5. Correlation coefficients: 120 mM $[Na^+]$ $r^2=0.488$, .220 mM $[Na^+]=r^2=0.558$. (df=18, $P<0.0005$).*

However, in degenerate cartilage this regulation may be perturbed by circulating endogenous digitalis-like factors (for a review see Schreiber 1991) that have penetrated the matrix and inhibited the Na^+/K^+ pump of chondrocytes thus disrupting the low Na^+/K^+ ratio essential for extracellular matrix synthesis. Normal cartilage extracellular matrix tends to sterically exclude large solutes, hormones, peptides and growth factors. The endogenous digitalis-like factor would normally be excluded from healthy cartilage and thus would not affect or inhibit the chondrocyte Na^+/K^+ pump. In contrast, in

degenerated cartilage where the extracellular matrix has been compromised (and has become significantly more permeable), the endogenous digitalis-like factor may gain increased access to chondrocytes inhibiting the Na^+/K^+ pump and hence disrupting the Na^+/K^+ ratio. This scenario would disrupt the delicate balance between extracellular matrix biosynthesis and degradation and have a detrimental effect on cellular repair mechanisms that may be initiated during the early stages of cartilage pathology.

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