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MICROPROBE ANALYSIS OF ELEMENT DISTRIBUTION IN BOVINE EXTRACELLULAR MATRICES AND MUSCLE

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Abstract

The concentrations of some essential elements, Na, K, P, S and Cl were determined by microprobe analysis in bovine extracellular matrices of cartilage, tendon and elastic tissue (ligamentum nuchae) and in muscle cells. The values for the different tissues were compared and related to the blood electrolyte concentrations. Among the connective tissues the highest Na and lowest Cl values were found for cartilage which bears a high negative charge. The lowest concentrations of these elements occurred in elastic tissue which is relatively non-polar. In the three extracellular matrices sodium levels exceeded potassium. In myofibers potassium was the major cation at 30 times the blood value and about 3 times the concentration of sodium. Chlorine values were around 0.4 that of blood. Sulfur and phosphorus are components of the tissue macromolecules. The negative charge on the extracellular matrices is a function of carboxyl and sulfate radicals. In the myofiber this property is largely attributable to carboxyl and phosphate groups. Differences in potassium-sodium distribution in cells and extracellular matrices are attributed partly to the microtrabecular lattice and to the ordered state of cell water. In general the element concentrations and selective distribution can be related to the chemical composition and organization of the tissue, the net immobile charge, the nature of the dispersion medium (water) and changes in its dielectric constant, and to the physico-chemical properties of the individual ions.

KEY WORDS: Extracellular matrix, Muscle, X-ray microprobe analysis, Element distribution.

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Introduction

No unanimity exists regarding the basis of cell and tissue distribution of ions. The concentration of ions in the blood of vertebrates in both sea water and land environments was the topic of Macallum's magisterial study (21). For the land animals it is clear that the absolute values of blood Na, K, Ca, Mg and Cl follow closely similar patterns, and that across the aquatic species as well, ratios of ions with respect to any one of them, e.g., Na, tend to constant values (19).

Cell and tissue values in general differ markedly from those of blood. The present study will attempt to relate these values to: 1) the chemical composition of the tissue, 2) the net immobile charge of the tissue, 3) the nature of the dispersion medium (water) and changes in its dielectric constant, 4) the properties of the individual ions, such as charge, ionic radius and hydration. This treatment is based on work of the present authors and that of N. R. Joseph (6,10,11,16-19).

Morphologic studies of the "microtrabecular lattice", NMR results and recent in vivo measurements of dielectric constants in a number of tissues support the idea that cell water is organized and displays a lower dielectric constant than normal bulk water (5,7,19,20,22-24,27).

Three connective tissues and one type of cell were selected for study by microprobe analysis. Cartilage, tendon, and elastic tissue differ markedly in several of the properties enumerated above. Skeletal muscle used as representative of a cellular tissue likewise has distinctive individualized behavior based on the properties listed. These will be discussed in relation to the results. Microprobe analysis has the advantage over biochemical analysis that cellular and extracellular domains can be individually resolved so that the results are not vitiated by an admixture of the two.

Material and Methods

Tissues were obtained from heifers* aged 18-24

* Beef ligamentum nuchae is a source of elastic tissue.

months at a local packing house within minutes after the animals were slaughtered. The following tissues were collected: costal cartilage, ligamentum nuchae, tendon, and muscle from the leg. To avoid water loss, the tissues were immediately sealed in a plastic envelope and transported to the laboratory for preparation within 30 minutes. Weighed samples of all tissue were dried to constant weight in an oven at 58°C in order to determine the water content.

Small, approximately 1-2 mm, cubes of tissue were placed on aluminum pins and rapidly frozen in isopentane chilled to -150°C in liquid nitrogen. Sections were cut at 8 μm and 18 μm in a cryostat maintained at about -40°C. The sections were then directly preserved by freeze-drying in a chamber mounted in the cryostat.

The 8 μm sections were stained with H & E. The 18 μm sections were prepared for microprobe analysis using methods previously described (14). These sections were mounted on carbon planchets which had been coated with a dried glucose film as an adhesive. The sections were exposed to paraformaldehyde vapor in a sealed vessel for 24 hours to stabilize the tissues against the destructive effects of the electron beam. The specimens were then coated with a carbon film in a vacuum evaporator to facilitate conduction.

The sections were examined in a Cambridge S4-10 scanning electron microscope (SEM) which was equipped with a silicon (Li) energy dispersive X-ray detector. The detector was coupled to an analyzer and computer (EDAX) to record the elemental distribution and element counts. The SEM was operated at 15 kV with the gun current at 150 μA. The specimen current, measured with a sample current meter, was around 10^{-11}A. To standardize the operating conditions the lenses were adjusted to yield 50,000 counts/40 seconds from an aluminum foil mounted on the planchet with the sections. The resolution was calculated at 162 eV using the Kα peak for Mn at 5.3 kV.

The beam size was approximately 100 nm. The resolution laterally and in depth was calculated at around 5 μm. In the case of muscle the analysis was done on individual muscle fibers. In the three connective tissues the field was confined to regions of extracellular matrix which were free of cells. This was confirmed by the virtual absence of a phosphorus peak in the spectra. A limited raster covering around 500 μm^2 was used at a magnification of about 500×. For tendon and ligamentum nuchae sections were taken from 6 animals; for cartilage from proliferating zones of two animals; muscle samples were obtained from 2 animals. X-ray counts were made on 2-3 sections for each animal with a total of 15-20 values for each animal. The counts were converted to milliequivalents (or atom weights for S and P) per kg tissue water using gelatin-element standards according to the method of Roomans (26) and the values for percent water. Tables 1-3 list these values, certain element ratios, and a quantity (Na + K - Cl) which represents a rough estimate of the immobile negative charge (X^-) in the tissue. This is termed "apparent" immobile negative charge and is based on the approximation:

$$Na^+ + K^+ - Cl^- - X^- = 0$$

This approximation does not account for ions which might be bound to macromolecules and it overlooks other mobile charged species such as Ca^{++}, Mg^{++} HCO_3^- which are present in very low concentration or cannot be measured.

Results

Water determinations done on the whole tissue do include cell water which would tend to raise the values assigned to the extracellular matrices in the connective tissue. The value for muscle, 75%, was in any case the highest for the tissues studied. The most highly hydrated extracellular tissue was cartilage with a water concentration of 69%, tendon was intermediate at 61% and a very low value, 51% was found for the elastic tissue (Table 1).

TABLE 1

Percentage of H_2O in Bovine Tissues

(Mean ± S.D.)

Cartilage	69 ± 0.1
Tendon	61 ± 3.0
Lig. Nuchae	51 ± 3.0
Muscle	75 ± 0.3

Microprobe Analysis (Tables 2 & 3, Figs. 1-4)

Connective Tissues: Sulfur concentrations expressed as atom weights per kg H_2O are seven to eight fold higher in cartilage than in tendon or ligamentum nuchae. Virtually no phosphorus was detected in the connective tissue matrices. Sodium levels, 371 meq/kg H_2O, were highest in cartilage, at 2.6 times that of serum. In tendon the concentration was lower, 95 meq/kg H_2O, i.e., two thirds that of serum. The lowest value was recorded for the ligament. Potassium concentrations at 23 meq/ kg H_2O were highest in cartilage, but considerably lower in tendon and ligament, approaching serum levels. Cartilage values for chlorine were lowest. Somewhat higher chlorine concentrations were recorded in the ligament with highest concentations in tendon. The "apparent" immobile charge as represented by the value Na + K - Cl. was high in cartilage and very low in tendon and ligament.

Myofibers: Sulfur concentration was .090 atom weights per kg H_2O. Phosphorus values were .123 atom weighs per kg H_2O. The sodium concentration in myofibers was one-third that in serum while the potassium level was approximately 30 times that of serum and the Na/K ratio, 0.3, was reversed and around two magnitudes lower than that observed in the extracellular matrices. The chlorine level was 0.4 that of the serum concentration. The value for (Na + K - Cl), the "apparent" immobile charge density was relatively high, but lower than that for cartilage.

TABLE 2

Concentration (Meq./kg H_2O) of Selected Elements in Bovine Tissues (Mean ± S.E.)

Tissue	P*	S*	Na	K	Cl	Na+K-Cl
Blood**			140	5	105	
Cartilage	-	357 ± 11	371 ± 12	23 ± 1	59 ± 2	335
Tendon	-	44 ± 3	95 ± 10	3 ± 0.4	93 ± 4	4
Lig. Nuchae	-	55 ± 2	63 ± 8	4 ± 1	63 ± 3	4
Muscle	128 ± 12	90 ± 2	46 ± 7	154 ± 7	46 ± 3	154

*Values for P & S are atom weight per kg H_2O
**Reference (19)

Comparison of Results with Other Studies

Connective Tissues: Comparative values for tendon and elastic tissues by microprobe analysis are not available. The electrolyte figures for tendon are consistent with an extracellular tissue of low apparent colloidal charge previously measured by an independent electrometric method (16). The low sodium content of elastic tissue is to be discussed.

Some difficulties arise in attempting to relate our cartilage matrix results with the considerable SEM literature on both matrix and chondrocytes, in part because of a different focus of our work. The literature tends to concentrate on the calcifying process per se (1-4,14,29,30). Some reasons for the discrepancies where they exist are: 1) tissue source with respect to species and site, 2) differing fixation and freezing procedures, 3) use of embedding techniques (which we have avoided), 4) variation in section thickness and differing programs of analysis, 5) expression of results, e.g. tissue water values are not always available.

Point (1) above is serious for cartilage because of the varying composition of the matrix as it progresses from resting to a mineralized state, and of the changing composition of the chondrocyte cytoplasm which is responsible for the elaboration and secretion of this matrix. For the comparative purposes of this study, we attempted to use a relatively "stable" cartilage matrix, which is not in a mineralizing mode, from an adult animal. Our preparations were taken from proliferating areas, confirmed by the virtual absence of phosphorus and the very low Ca peak.

There are individual areas of agreement but because of the previously cited factors it is not always possible to make direct quantitative comparisons with our data. All results show high overall cation levels and high levels of sulfur. The cation levels far exceed chlorine values and the latter are in all instances lower than those for blood. Sodium is the dominant cation and the value given by Wroblewski and Makower (30) is comparable to ours as is that for sulfur.

The value for cartilage potassium appears to be unresolved, that reported by Wroblewski and Makower (30) being much higher than that found by us, which nevertheless is still 4-5 times higher than that in blood. In a previous report employing an electrometric method we stated that the negative colloidal charge density of cartilage is high and that sodium and potassium levels were strongly elevated compared with blood (9). We agree that sodium and potassium are neutralizing the negative charge on the proteoglycan matrix (14,29,30).

Myofibers: The SEM results here reported for bovine leg muscle generally agree with those of Wroblewski and Edstrom (28) for control rat soleus muscle (assuming the water content of the latter to be the same as ours) for phosphorus, sulfur, sodium and potassium. Our chlorine values are considerably higher

TABLE 3

Some Significant Element Relations (Equiv.) in Bovine Tissues

	Na/K	Na/Cl
Blood	28.0	1.3
Cartilage	16.1	6.3
Tendon	31.7	1.0
Lig. Nuchae	15.7	1.0
Muscle	0.3	1.0

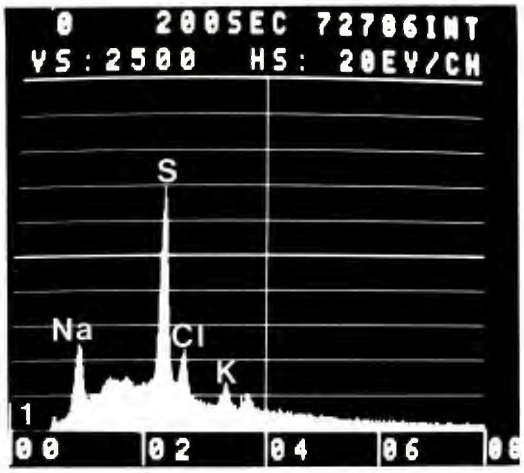

Fig. 1. Spectrum from bovine cartilage.

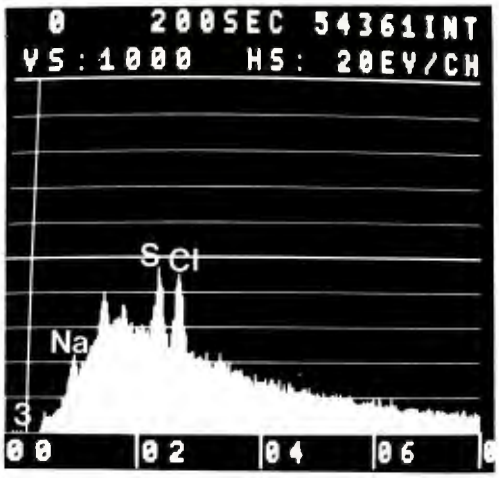

Fig. 3. Spectrum from bovine ligamentum nuchae.

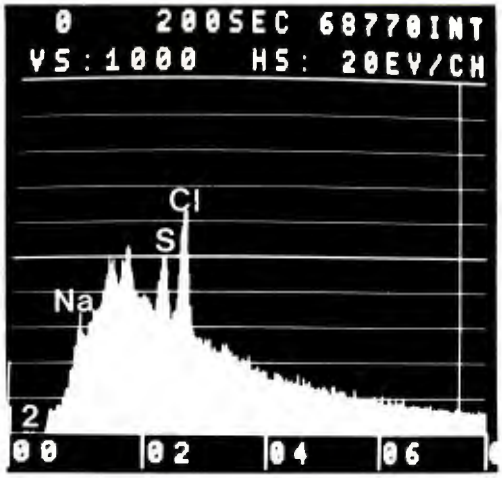

Fig. 2. Spectrum from bovine tendon.

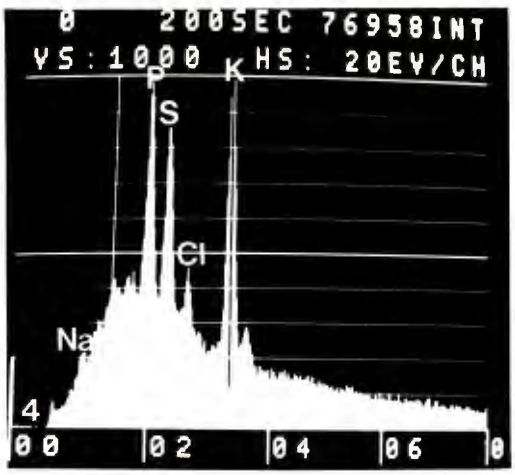

Fig. 4. Spectrum from bovine skeletal myofiber.

than Wroblewski and Edstrom and somewhat higher than results for rabbit masseter previously reported (11). The electrolyte values agree with the composition of many mammalian cells which are high in potassium, low in sodium, and relatively low in chlorine with respect to blood, as long reported in the literature based on analytical measurements (17).

Discussion

Ion distributions in tissues can be adequately explained by the physico-chemical properties of the ions, the immobile negative charge density on the macromolecules and the dielectric constant of the aqueous dispersion medium. The negative charge density is determined by the composition, state of aggregation and the interactions of charged macromolecules (6,10,16-19).

The organization of tissues into cells and extracellular matrices reflects differences in morphology, function and physical and chemical composition. This is illustrated by a comparison of cells of muscle, the myofibers, and a group of connective tissue matrices in cartilage, tendon and ligamentum nuchae. The distribution of elements, especially the most abundant electrolytes Na, K, and Cl, is unique for each of these tissues and depends on the composition and state of aggregation of the macromolecules. Also within the group of connective tissues there are distinct and specific characteristics of the ionic distribution.

The elements P and S are generally components of the macromolecules. Intracellular phosphorous exists as part of the charged phosphate radicals, essential components of nucleoproteins, nucleic acids and phosphoproteins, and smaller amounts of adenosine phosphates. Sulfur is present in both cellular and extracellar phases, but there is an important distinction in its role. Extracellular sulfur exists mainly as part of the charged sulfate radical of glycosaminoglycans; the intracellular form occurs mainly in cysteine and cystine of proteins, with the

exception of the secretory cells of connective tissue where variable amounts of proteoglycans may occur in fibroblasts, osteoblasts and chondroblasts (13,29,30).

In both extracellular matrices and the intracellular phases fixed negative charges are mainly neutralized by the physiologic cations of blood viz., Na^+ and K^+ and to a smaller degree by Ca^{++} and Mg^{++}. The mobile anion, Cl^- would tend to be repelled and excluded, its concentration being always less than in blood.

Connective tissue matrices are composed of collagen, varying amounts of elastin, glycosaminoglycans, and carbohydrate-protein complexes. The matrices carry a net negative charge which is related to the concentrations and interactions of these components. In cartilage the relatively large amounts of glycosaminoglycans result in a high net negative charge (6,10,16,18). By virtue of its numerous sulfonated groups it is also relatively hydrophilic. In tendon, collagen forms a major component and glycosaminoglycans comprise a much smaller moiety. Intermolecular cross-linking of the collagen and collagen-glycosaminoglycan interactions result in a lowering of the net negative charge. Ligamentum nuchae contains as a major component around 60% of the protein elastin (8) which is relatively hydrophobic, containing a high concentration of non-polar amino acids. It is extensively cross-linked (12) and appears to have the lowest negative charge density of the connective tissues studied.

The cation distribution follows the charge gradient. Therefore sodium concentration is highest in cartilage, 2.6 times its concentration in blood, and is much lower in tendon and ligament. The potassium level is considerably higher in cartilage than in the tendon and ligament where it nears blood concentrations. On the other hand, the lowest concentration of chlorine occurs in cartilage and reflects the effects of the high negative charge density of this tissue. This would almost be consistent with a Donnan distribution with respect to blood. It is noteworthy that in the elastic tissue matrix where the charge density is lowest, accompanied by the lowest concentration of water, the total concentration of electrolyte elements is also lowest.

Using the relations between ion concentrations in blood and extracellular matrices, Joseph calculated dielectric constants for some tissues (18,19). Applied to the present results an "apparent" dielectric constant for cartilage is 91 approaching that of normal water, 80. Calculated dielectric constants of tendon and ligament are lower, namely 60 and 48, respectively. Experimental evaluation of the effective dielectric constant of some proteins yields values of around 50 (25).

Intracellular colloids are distinctly different from those of the extracellular matrices. Lipids, lipoproteins, phosphoproteins, nucleoproteins, and histones are among the major macromolecules. While the water content is high, several of the cell components would suggest selective hydrophobicity and a lowered dielectric constant. The phosphate and carboxyl groups of the polyelectrolytes would be expected to yield a significant net negative charge. The principal cations of the cell available for neutralization of the fairly high negative charge are sodium and potassium. Parenthetically, the concentrations of free calcium and magnesium in the cell are extremely low.

When the distribution of electrolytes in cells, as represented by the myofiber is compared to the connective tissue matrices, the striking difference is that the immobile negative charge is largely neutralized by potassium. The intracellular potassium concentration is around 30 times the blood value and the K/Na ratio is 3.3. An available explanation of this inversion is that the calculated dielectric constant of cell water is from 30 to 50, as opposed to that for blood and connective tissue matrices of cartilage and tendon which range from 60 to 90. This implies a low affinity of this ordered cell water and the hydrophobic colloids for the highly hydrated sodium ion and a high affinity for the relatively unhydrated potassium ion (17).

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Discussion with Reviewers

G.M. Roomans: As example of a living cell to be compared with the extracellular matrix, the authors use striated muscle. It must be questioned whether this cell type is really relevant to the comparison. Fibroblasts and chondrocytes would have been a much better choice. Unfortunately, these cells may be too small to be investigated with the technique used by the authors. However, the authors could refer to, e.g., Wroblewski and Makower (30) and Appleton (31) and the literature cited in these papers to obtain data on the elemental composition of chondrocytes. This point is particularly serious since the Na/K ratio in chondrocytes appears considerably higher than in muscle cells, indicating that already the intracellular matrix (i.e., the extracellular matrix during its production in the cell) binds Na.

Authors: We did not, in fact, consider using a connective tissue cell for comparison since:1) primarily the tissue of reference was to be the blood and 2) these mesenchymal cells are changing morphologically and chemically, especially in an area of calcification. The muscle cell, with which we were quite familiar, is a mature cell with a relatively fixed, and already fairly well known composition. The point of our experiment was to compare structures having different water contents, different chemical compositions and (inferred) different dielectric properties compared with each other and with blood. Cartilage matrix versus cell comparisons have their own difficulties, because of widely differing stages of cell activity. We feel that all statements about "binding" are subjudice. Our view is that extracellular "binding" is present for Ca and Mg but not for the monovalent ions. For cells we are in a snakes' nest of "binding", "association", "activity" and the like. In our thinking we have stuck to the concept of an "apparent" standard chemical potential for each ion which is calculable for the observed values for each tissue (17).

G.M. Roomans: I do not agree with the assumptions underlying the definitions of the "apparent immobile negative charge". The concentration of calcium in the cartilage matrix is, according to Wroblewski and Makower (30) between 50 and 100 mmol/kg, which certainly is not negligible. According to Appleton (31) the Mg concentration in cartilage matrix is not much less than that of calcium. Bicarbonate is not the only negative charge that cannot be measured: in muscle, beta- and gamma- carboxyl groups are assumed to be the main carriers of negative charge (see Edelmann, 1988, Scanning Microscopy 2:851-865). Also, the approximation accounts for the Na, K, and Cl also when these ions are bound to macromolecules.

Authors: The concentrations of calcium and magnesium in cartilage are indeed significant. Analytic studies of Eichelberger, et al. (35) and microprobe results (Wroblewski and Makower (30), Appleton (3,31), Hargest, et al. (14)) establish this fact. We did not prepare Ca and Mg standards for our work so we were unable to determine the concentrations. The counts were significant. In all of the other tissues

the Ca and Mg count levels were too low to be considered reliable. The bivalent cations, Ca^{++} and Mg^{++}, appear to interact more strongly with the tissue colloids, forming bound complexes. These relations and their implications in calcification were discussed in previous publications (9,32). The "apparent immobile negative charge" is a first approximation of the immobile charge, exclusive of that which is bound. In extracellular matrices Na, K and Cl do not appear to be bound to any significant degree and tend to follow the Donnan distribution (6,9,16-19).

I.L. Cameron: Although I am sympathetic to the idea that net immobile charge in the different tissues play a key role in determining Na^+, Cl^-, and K^+ distributions between the tissues measured (i.e. tendon, cartilage, elastic tissue and muscle), I am nevertheless concerned if the techniques used in your element analysis are really adequate to convincingly warrant the conclusions drawn from the data. For example, would not there be major extracellular to intracellular redistribution of ions from the 30 minute time delay between muscle collection and cryofreezing: If so this might have a profound effect on the calculated element content data. Likewise, the use of "thick sections", paraformaldehyde vapor fixation, placement of sections on solid carbon planchets for microprobe analysis (which involve questions of background counts under the x-ray microanalysis conditions you used) all lead to concern about the element quantification. Is it your contention that the measured differences are so different between tissues or the elements so immobile that careful quantification is not required?

Authors: We chose to work with bovine tissues because the best source of elastic tissue is bovine ligamentum nuchae. Conditions at the abattoir did not permit the immediate quenching which we would have preferred. Roomans et al (41) have reported some redistribution of some elements in rat quadriceps femoris muscle at the end of two hours. Our own unpublished experiments on the rat gastrocnemius left in a moist chamber at room temperature for one hour showed no significant change in element distribution at the microscopic level. We have discussed the methods of tissue preparation and analysis in detail in earlier publications (11, 15, 36). Anhydrous paraformaldehyde powder is used in the vapor fixation. The method of bulk analysis described by Russ (42) allows the use of thick sections. With the parameters we employ the beam does not penetrate the sections and the lateral and depth resolution are approximately 5 μm. The computer program (EDIT EM) subtracts background and strips the spectral peaks compensating for overlaps and separating the elements according to considerations detailed by Russ. Individual element counts are converted to concentrations using standards according to the method of Roomans (26). We do not believe that the elements are displaced at the microscopic level. If one takes into account the various limitations of current microprobe analysis our results could be regarded as semi-quantitative. Of course, "careful quantitation" should be the ultimate goal. The measured differences in element distribution among the tissues studied is authentic and highly significant.

I.L. Cameron: Why have you ignored discussion or any mention of what most current biologists think to be the main explanation of ionic gradients between cells (muscle) and the extracellular environment (blood serum). Specifically I refer to the cell membrane bound energy requiring pump or pumps?

Authors: The sodium pump hypothesis or theory (Dean, 34), from which all other ionic pumps derive, was based on the concept that the cell cytoplasm is a solution (of proteins, enzymes, etc.) and on the observation that the sodium ion did not conform to the Donnan ratio for potassium. Potassium obeyed the Nernst equation; sodium did not. To explain this apparent discrepancy, sodium had to be pumped out of the cell by an energy-consuming mechanism. Thus, compare Hodgkin (38): "It is necessary to suppose that sodium is continuously pumped out of the cell by a process depending on metabolism." If the cell cytoplasm is not a solution, as many now believe, then cell ions need not be supposed to conform to the Donnan or Nernst equations, but rather to the generalized Gibbs relation: $E = \Delta\mu^\circ + RT \ln C_2/C_1$ where E = emf, C_2 = ion concentration in the cell, C_1 = ion concentration in the blood and $\Delta\mu^\circ$ = change of standard chemical potential of a given ion between blood and cell which can be calculated from the data (17,19). This eliminates the "necessity" of a sodium pump. Active transport (the "sodium pump") is based squarely on the failure of sodium to obey the wrong law! Cells do not conform to the Donnan or Nernst membrane equilibria. They conform to the Gibbs phase equilibrium. Joseph (39) further showed that no part of ionic distribution in the sense of energy-requiring "active" transport is valid, since the efficiency of all ionic pumps is zero or less. As to the "energy" argument made by some opponents of energized ionic transport, namely that too little energy is available to "power" all the currently postulated pumps, it was pointed out that energy is neither required, nor is it available, for processes of ionic distribution (38); i.e., ionic pumps violate both the first and second laws of thermodynamics (33,40).

J. Wroblewski and R. Wroblewski: Why did you choose to use paraformaldehyde fixation of the sections? Did you perform any comparative studies on the unfixed and fixed sections?

Authors: We used powdered paraformaldehyde which yields an anhydrous vapor in order to cross link tissue proteins, stabilizing them against destructive effects of the electron beam. The theoretical basis for vapor fixation has been discussed by Gersh et al. (37). In our work the procedure is entirely empirical since we have not done experimental studies comparing treated with untreated sections.

J. Wroblewski and R. Wroblewski: The value for K in the cartilage matrix is rather low. Don't you think that it may be due to the postmortem changes that take place during the 30 minutes it takes before the samples are frozen?

Authors: There is considerable variation in the reported values for cartilage matrix K with the highest values reported in your publications (29,30). Our value is low, as is that to be inferred from the spectrum

shown by Appleton (3). As expressed in the reply to Cameron, we do not believe that the 30 minute delay has significantly affected our results.

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