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Extraction of Cartilage Protein–Polysaccharides with Inorganic Salt Solutions

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1. Bovine nasal cartilage was extracted with inorganic salt solutions of various ionic strengths. The efficiency of extraction of protein–polysaccharide from the tissue was determined for each extraction. The results confirm and enlarge earlier observations (Sajdera & Hascall, 1969). 2. The chloride salts of lanthanide metals extract high yields of protein–polysaccharide from the tissue at much lower concentrations than was achieved with univalent and bivalent salts. 3. The lanthanum salt of extracted protein–polysaccharide precipitates when the concentration of $LaCl_3$ is decreased. Precipitation is complete in the presence of 0.05M-$LaCl_3$. This finding is relevant to the interpretation of earlier observations on the effect of $LaCl_3$ on elastic recovery of articular cartilage after compression (Sokoloff, 1963). 4. A linear relationship was found between the concentration at which a particular salt is maximally effective in solubilizing protein–polysaccharide from the tissue and the enthalpy of hydration of the cation of the salt. On the basis of this relationship a hypothesis is proposed to explain the characteristic protein–polysaccharide-extraction profiles exhibited by inorganic salt solutions.

Protein–polysaccharide is a major component of the extracellular matrix of hyaline cartilage. Sajdera & Hascall (1969) reported that up to 85% of the protein–polysaccharide of bovine nasal septal cartilage could be solubilized by stirring tissue slices in salt solutions of high ionic strength. Although some investigators had previously used salt solutions of lower concentration for extracting cartilage protein–polysaccharides (e.g. Mathews & Dorfman, 1953; Muir & Jacobs, 1967; Tsiganos & Muir, 1969), this approach solubilized only a relatively small proportion of the macromolecules from the tissue compared with that obtained with concentrated salt solutions.

An alternative method, extraction of the cartilage by high-speed homogenization in water, also solubilizes a high proportion of protein–polysaccharide from the tissue and has been used by many investigators (see, for example, Pal *et al.*, 1966). However, the high shearing forces associated with high-speed homogenization may degrade macromolecules (Harrington & Zimm, 1965). High-speed homogenization of salt-extracted protein–polysaccharide induces a change in the apparent sedimentation coefficient distribution of the polymer (Sajdera & Hascall, 1969), indicating that depolymerization does occur under these conditions. Methods for extracting protein–polysaccharides which solubilize a large amount of the total tissue content of the macromolecules, yet obviate the need for high-speed homogenization, would therefore seem to be advantageous.

The experiments described below were undertaken before a study of the effect of different salt extractions on the composition of a protein–polysaccharide fraction subsequently isolated from the extract (Mayes *et al.*, 1973). They confirm and extend the observations reported by Sajdera & Hascall (1969). A hypothesis is advanced to account for the characteristic profile shown by some concentrated salt solutions in extracting protein–polysaccharide from the tissue.

Experimental

Preparation of cartilage samples

Bovine nasal cartilage was obtained from 2-year-old animals at the slaughterhouse. Cartilages were excised immediately after death and stored at 0°C during transport to the laboratory. The cartilage was freed from the surrounding tissue and the perichondrium and sliced with a Surform blade as described by Sajdera & Hascall (1969). The cartilage slices were stored at −20°C until required.

Extraction of protein–polysaccharide from cartilage slices

Protein–polysaccharide was extracted by the dissociative method of Sajdera & Hascall (1969), but with the following modifications. Cartilage slices

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were suspended in unbuffered inorganic salt solutions (1:20, w/v) and shaken for 20h at room temperature on a flask shaker. The concentrations of salts used are shown in Fig. 1. The suspension was filtered through a glass sinter (porosity no. 1) to give a filtrate and the cartilage residue. The residue was washed with water and the washings were added to the filtrate in a volumetric flask to give a final volume $2\frac{1}{2}$ times the volume of the solution used for the extraction.

When lanthanide salts were used for the extraction, the washings were made with salt solution of the same ionic strength as that used for the extraction.

Measurement of the amount of protein–polysaccharide extracted

The filtrate was assayed directly for hexuronic acid (Bitter & Muir, 1962) with D-glucuronolactone as standard, and for hexose by using the anthrone reaction (Dische, 1962) with D-galactose as standard.

The cartilage residue was digested with papain (EC 3.4.4.10) and ficin (EC 3.4.4.12) as described by Mason & Wusteman (1970). Ethanol (3vol.) was added to the clear digest and the mixture left overnight at 0°C. The precipitate was collected by centrifuging, washed with 80% (v/v) ethanol and dissolved in water. This solution was then assayed for hexuronic acid and hexose as described above.

The efficiency of protein–polysaccharide extraction from the cartilage could then be expressed as a percentage of hexuronate etc. solubilized from the tissue. Glucuronic acid is a component of chondroitin sulphate, which is the major glycosaminoglycan of bovine nasal cartilage protein–polysaccharide (Hascall & Sajdera, 1970). Galactose is a component of the glycosaminoglycan keratan sulphate, which is also present in the protein–polysaccharide.

Results and Discussion

Solubilization of cartilage protein–polysaccharide with different salt solutions

Table 1 shows the concentrations of various inorganic salt solutions that were found to be maximally effective in solubilizing protein–polysaccharide from bovine nasal cartilage. The values for extracts made with LiCl, $MgCl_2$ or $CaCl_2$ are in close agreement with those found by Sajdera & Hascall (1969).

With the exception of LiCl, the chloride salts of the Group I metals solubilize between 10 and 20% only of the protein–polysaccharide at concentrations between 1.0 and 5.0M. Unlike the other salts tested, they do not show a peak of maximal extracting activity at a particular ionic strength in the concentration range examined, the results for KCl being typical for the group as a whole (see Fig. 1). LiCl may exhibit different properties in this respect, because its greater solubility allowed more concentrated solutions to be tested.

Group II metal chlorides are particularly effective in solubilizing a high percentage of protein–polysaccharide at high ionic strength (Table 1). Each of these salts shows a characteristic optimum concentration for maximal extraction of protein–polysaccharide from the tissue (Fig. 1).

Table 1. *Concentrations of various inorganic salt solutions found to be maximally effective for solubilizing protein–polysaccharide from bovine nasal cartilage*

The amount of protein–polysaccharide solubilized is expressed in terms of the percentage of the total hexuronic acid extracted from the tissue. The percentage of the total hexose extracted from the tissue at the same salt concentration is also given. Extracts made with $CaCl_2$ or with $BaCl_2$ could not be assayed by using the anthrone reaction, since a precipitate formed during the latter reaction.

Salt	Concn. (M)	Percentage of total hexuronate extracted	Percentage of total hexose extracted
LiCl	6.00	74.4	62.7
NaCl	1.00	16.5	22.3
KCl	1.00	18.4	22.4
CsCl	1.00	18.3	32.6
$MgCl_2$	3.00	82.5	76.1
$CaCl_2$	2.00	83.9	—
$BaCl_2$	1.80	66.3	—
$LaCl_3$	0.50	83.2	79.3
$CeCl_3$	0.50	62.8	48.8

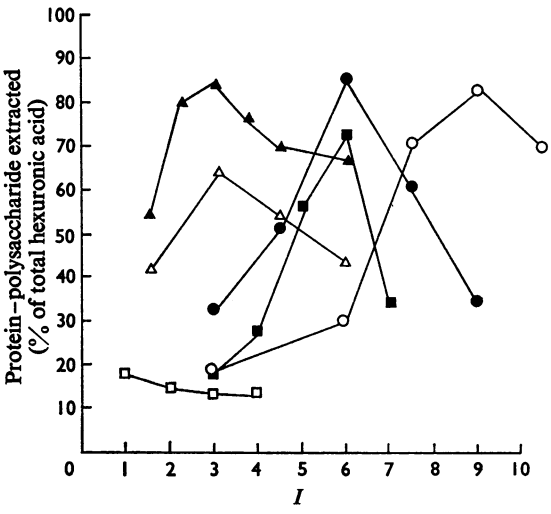

Fig. 1. *Extraction of protein–polysaccharide from bovine nasal cartilage with salt solutions of various ionic strengths*

The amount of protein–polysaccharide solubilized is expressed as the percentage of total hexuronic acid extracted from the tissue. ■, LiCl; □, KCl; ○, $MgCl_2$; ●, $CaCl_2$, ▲, $LaCl_3$; △, $CeCl_3$.

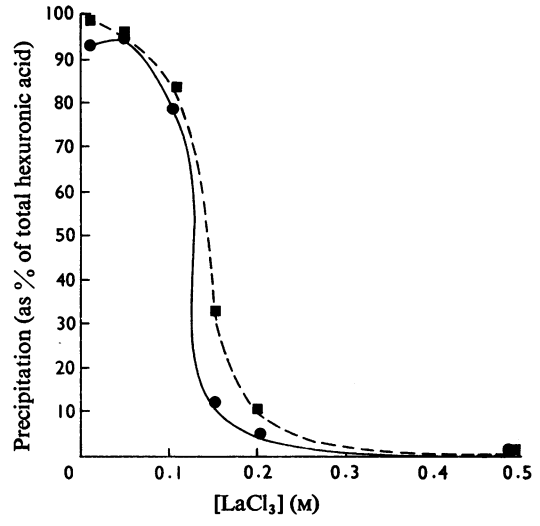

Fig. 2. *Precipitation of protein–polysaccharide from a cartilage extract prepared with* 0.5M-*$LaCl_3$*

Precipitation occurs when the $LaCl_3$ concentration of the extract is lowered. The amount of protein–polysaccharide precipitated is expressed as a percentage of the total hexuronic acid present in the extract. ■, Decrease in $LaCl_3$ concentration by dialysis; ●, decrease in $LaCl_3$ concentration by dilution.

Salts of the lanthanide metals extract a large proportion of the protein–polysaccharide from the tissue at much lower ionic strengths than Group II metal salts extracting similar amounts (Table 1). As with Group II salts, lanthanide chlorides exhibit a typical peak extracting concentration (Fig. 1).

Precipitation of protein–polysaccharide extracted with lanthanide chlorides

When the concentration of $LaCl_3$ in a cartilage extract is decreased from 0.5M to lower concentrations, the extracted protein–polysaccharide precipitates. For example, precipitation is complete at 0.05M-$LaCl_3$ and follows a similar course whether the salt concentration is decreased by straight dilution or by dialysis (Fig. 2).

The finding that lanthanum salts of protein–polysaccharides are soluble in the presence of high concentrations of $LaCl_3$ but are precipitated in the presence of low concentrations of the salt is noteworthy. Sokoloff (1963) investigated the deformability and elastic recovery of the cartilage covering the head of the canine tibia when immersed in 0.085M solutions of various salts. In the presence of univalent salts only a slight deformity could be induced on compression of the cartilage, and recovery was complete when the load was removed. Compression in bivalent salt solutions produced a much greater deformity, but again recovery was complete. In the presence of $LaCl_3$, however, although the deformity produced by compression of the cartilage was of the same order as for experiments with bivalent cations, it was irreversible when the load was removed. The results of our experiments suggest that the protein–polysaccharide of the cartilage would precipitate in the tissue in the presence of 0.085M-$LaCl_3$. It seems likely that the loss of elastic recovery in the cartilage after exposure to these conditions may be associated with the formation of the insoluble lanthanum salt of protein–polysaccharide in the tissue. This evidence therefore supports the idea that a particular conformation of protein–polysaccharide in the extracellular matrix of weight-bearing cartilages is required for the tissue to recover from physiologically imposed stress.

Other investigators have used lanthanum salts in dilute solution as an electron-dense stain for demonstrating protein–polysaccharides in tissue sections (Khan & Overton, 1970; Shea, 1971). Khan & Overton (1970) used 0.075M-$La(NO_3)_3$ solutions to study the electron-microscopic appearance of developing chick cartilage. The observations on the

precipitation of $LaCl_3$-extracted protein–polysaccharide reported above support their suggestion that electron-dense granules seen in cartilage after this treatment are in fact attributable to protein–polysaccharide. It should be noted, however, that macromolecules other than protein–polysaccharide are extracted by 0.5M-$LaCl_3$, although a proteoglycan fraction can be isolated from the extract similar to that isolated from other high-ionic-strength extracts of the tissue (Mayes *et al.*, 1973).

Mechanism of extraction of cartilage protein–polysaccharide by inorganic salt solutions

The results presented in Table 1 and Fig. 1 confirm and enlarge the findings reported by Sajdera & Hascall (1969). However, a satisfactory hypothesis has not yet been advanced to explain why particular metal-ion salts exhibit characteristic profiles for the efficiency of extraction of protein–polysaccharide from bovine nasal cartilage with various concentrations of the salt. In particular, the reasons for a decreasing solubilization of protein–polysaccharide from the tissue when the extracting salt solution is increased beyond a certain optimum value have not so far been explored.

Since a critical ionic strength is required before a particular salt solution effects extraction of protein–polysaccharide from the tissue in excess of that in control experiments, Sajdera & Hascall (1969) have proposed that a dissociative mechanism may account for the extraction, with polyionic interactions between protein–polysaccharides themselves, or other proteins of the cartilage matrix being displaced.

The isolation of a glycoprotein that promotes the aggregation of proteoglycan subunit fraction to form large macromolecular complexes has been reported by Hascall & Sajdera (1969), although the exact nature of the factor promoting aggregation has since been disputed (Mashburn & Hoffman, 1971; Tsiganos *et al.*, 1972). Nevertheless the dissociation of some form of macromolecular aggregate of the type suggested by Hascall & Sajdera (1969) during the extraction of protein–polysaccharide from the tissue would appear to be one form of ionic interaction that may be displaced by the ions of the extracting salt. However, Hascall & Sajdera (1969) also demonstrated that much lower salt concentrations were required to dissociate the isolated complex *in vitro* than were required to solubilize large amounts of protein–polysaccharide from the native cartilage. Rosenberg *et al.* (1970) have also drawn attention to the fact that the salt concentrations required to solubilize large amounts of protein–polysaccharide from the tissue are unusually high compared with those usually required to dissociate ionic bonds. Thus a hypothesis based on a simple dissociation of intermolecular ionic interactions would appear to be an oversimplification of the mechanism by which protein–polysaccharide is extracted from cartilage with high-ionic-strength solutions. Moreover it does not account for the decrease in solubilization of protein–polysaccharide when optimum salt concentrations are exceeded.

In attempting to resolve some of the discrepancies of the simple dissociation hypothesis discussed above we have examined various properties of the salt solutions used for extraction of protein–polysaccharide. These have included conductivity measurements and assessment of activity coefficients, calculation of hydrated-ion radii and charge-to-surface ratios and certain thermodynamic parameters of the ions present in the extracting salt solutions. We consider that, of these factors, a thermodynamic approach may be of use in extending the scope of the dissociation hypothesis.

The enthalpy of hydration, ΔH_f^0(aq.), of an ion is an indication of the affinity of that ion for water molecules. Values for this parameter for the ions of salts used to extract protein–polysaccharide from cartilage are given by Rossini *et al.* (1952). The relevant data are shown in Table 2.

The glycosaminoglycan moieties of the protein–polysaccharide in the tissue carry many anionic sites ($-OSO_3^-$ and $-CO_2^-$) and these are probably associated with Na^+ counterions. It is noteworthy that the chloride salts of Na^+, K^+ and Cs^+ do not show any tendency towards optimum extraction peaks in solubilizing protein–polysaccharide with increasing concentration of the salt. The enthalpies of hydration of Na^+, K^+ and Cs^+ are similar to that of Cl^-. However, the enthalpy values of Li^+, Mg^{2+}, Ca^{2+} and La^{3+} are in excess of those for Na^+ and Cl^-, and the chloride salts of these species show characteristic extraction peaks for solubilizing protein–polysaccharide from cartilage. This suggested that there may be a relationship between the hydration energy of a metal ion and the molar concentration of the chloride salt of the ion at which optimum solubilization of protein–polysaccharide occurs. The limited results so far available, from our extraction experiments and those of Sajdera & Hascall (1969) carried out with inorganic salts, indicate that the molar concentration of the salt at which maximum solubilization of protein–polysaccharide occurs is directly related to log ΔH_f^0(aq.) for each metal ion (see Fig. 3).

The correlation between the hydration energy of ions and the optimum concentration at which their respective salts extract protein–polysaccharide from the tissue may explain the characteristic extraction profiles of the salts (Fig. 1). One litre of solution will contain 55.5 molecules of water. Thus for a 1.0M solution of LiCl (1mol of Li^+, 1mol of Cl^-), for example, there will be 27.8 molecules of water available to each ion. However, in a 6.0M solution

Table 2. *Enthalpies of hydration of various ions present in salt solutions used to extract protein–polysaccharides from bovine nasal cartilage*

Enthalpy, H, is the standard heat of formation (kJ/mol) of a given substance from its elements at a reference temperature of 298.16°K (25°C). The standard state for a solute in aqueous solution is taken as the hypothetical ideal state of unit molality, in which state the enthalpy of the solute is the same as that in infinitely dilute solution. The enthalpy of hydration ΔH_f^0(aq.) of an ion, $Z^{\pm x}$, therefore describes the energy released in the process:

$$Z^{\pm x}(g) + \infty H_2O(l) = Z^{\pm x}(aq.)$$

where $Z^{\pm x}$(g) represents the ion in the gaseous state, l is liquid and $Z^{\pm x}$(aq.) the solvated ion in aqueous solution. The magnitude of ΔH_f^0(aq.) for a particular ion is a direct measure of the affinity of that ion for water. ΔH_f^0 values are those given by Rossini *et al.* (1952). The molarity at which the chloride salt of an ion extracts a maximal amount of protein–polysaccharide from the cartilage (see Table 1) is given in the last column.

$Z^{\pm x}$	ΔH_f^0(aq.) at 298.16°K/25°C (kJ/mol)	Molarity (M)
Li^+	−278.18	6.0
Na^+	−239.43	—
K^+	−250.97	—
Cs^+	−247.46	—
Mg^{2+}	−461.51	3.0
Ca^{2+}	−542.44	2.0
Ba^{2+}	−537.84	1.8
La^{3+}	−736.52	0.5
Ce^{3+}	−726.48	0.5
Cl^-	−167.28	—

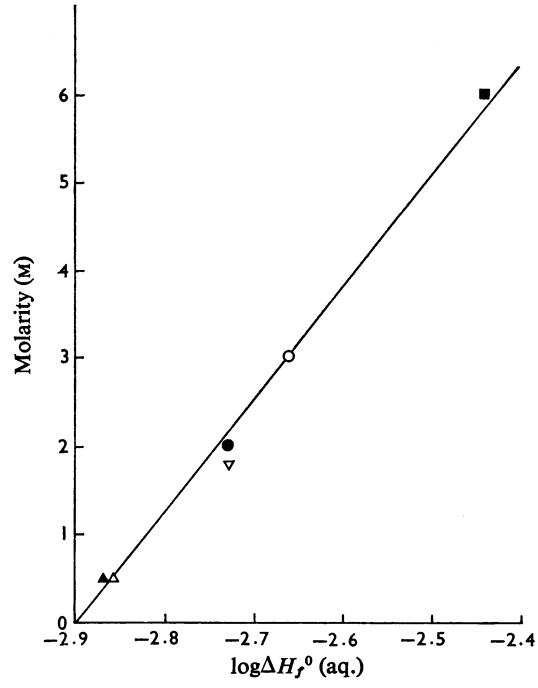

Fig. 3. *Plot of the log values of enthalpy of hydration for various cations against the molar concentration of their chloride salts maximally effective in extracting protein–polysaccharide from bovine nasal cartilage*

ΔH_f^0(aq.), the enthalpy of hydration for a cation (see Table 2). Molar concentration refers to that at which the chloride salt of a particular cation extracts a maximal amount of protein–polysaccharide from the cartilage (see Table 1). ■, LiCl; ○, $MgCl_2$, ●, $CaCl_2$; ▽, $BaCl_2$; ▲, $LaCl_3$; △, $CeCl_3$.

(6mol of Li^+, 6mol of Cl^-), there will be only 4.6 molecules of water available for each ion. Considering the enthalpy of hydration, the affinity of Li^+ ion for water is considerably greater than that of the Cl^- ion. Hence the Li^+ ion would be expected to co-ordinate more water molecules than the Cl^- ion when the solvent becomes restricted. The result of this would be that water molecules would be removed from the hydration shells of ions with lower ΔH_f^0(aq.) values, such as Cl^-, to satisfy the greater affinity of Li^+. This would also apply to the Na^+ ions associated with the anionic groups of the protein–polysaccharide and proteins of the tissue suspended in the LiCl solution.

Na^+ and Cl^- with depleted hydration shells would be liable to form $Na^+ \cdot Cl^-$ ion pairs in this situation, with the more hydrated Li^+ replacing Na^+ as the counterion of the anionic groups of the tissue macromolecules. Such replacement may induce a conformational change in one or more of these macromolecules, leading to their release from the tissue into solution. We suggest that optimum solubilization of protein–polysaccharide from the tissue with 6.0M-LiCl represents the salt concentration at which this effect is maximal.

With higher concentrations of LiCl, for example a 7.0M solution in which there would be rather less than 4.0 molecules of water per ion, even Li^+ would have depleted hydration shells and so form $Li^+ \cdot Cl^-$ ion pairs, thus decreasing the number of ions in solution and decreasing the efficiency of extraction of macromolecules from the tissue.

A similar reasoning may be applied to explain the extraction profiles of bivalent and tervalent salts able to solubilize large amounts of protein–polysaccharide from the cartilage. Extraction of the tissue with univalent salts other than LiCl solubilizes only

relatively small amounts of protein–polysaccharide, even in high concentrations of the salt. This is predictable from the enthalpy–optimum-salt-concentration relationship shown in Fig. 3. By considering the enthalpy of hydration of K^+, for example, a KCl solution would be expected to show an optimum extraction peak for protein–polysaccharide at a salt concentration of 6.25M. However, a saturated solution of the salt occurs at 4.6M. LiCl differs from the other univalent salts tested in having a higher ΔH_f^0(aq.) for Li^+ and in its greater solubility.

Sajdera & Hascall (1969) observed that a saturated solution of *S*-methylisothiouronium chloride extracts 80% of the total protein–polysaccharide from the cartilage and suggested therefore that decreased extraction by high concentrations of other salts was unlikely to be due to a decrease in the amount of water available for the solvation of protein–polysaccharide. The hypothesis presented above, however, is concerned primarily with the behaviour of the extracting ions as their hydration shells become depleted in increasingly concentrated solutions.

The mechanism by which organic salts extract protein–polysaccharide from cartilage may be more complex than that for inorganic salts. Guanidinium chloride, for example, shows a much broader extraction profile with increasing concentration than that observed for inorganic salts (Sajdera & Hascall, 1969). Moreover, concentrated solutions of guanidinium chloride and its structural analogue *S*-methylisothiouronium chloride are capable of forming strong hydrogen bonds with macromolecules. They may therefore effect additional conformational changes in the tissue components to those induced by inorganic ions.

The nature of the association between cartilage protein–polysaccharides and other macromolecules in the tissue is little understood at the present time. Protein–polysaccharides may form dimers (Rosenberg *et al.*, 1970), aggregate with glycoproteins (Hascall & Sajdera, 1969) or can be associated with collagen (Brandt & Muir, 1971). A conformational change induced in any of these molecules by an extracting ion, as postulated above, could result in a disruption of stereospecific molecular interactions with consequent liberation of protein–polysaccharide from the tissue.

Possible changes in the conformation of defined protein–polysaccharides associated with different inorganic counterions have not been studied to date. However, $CaCl_2$ has been shown to induce large changes in the optical rotation of collagen (Vankataraman, 1960), and Anderson & Sajdera (1971) obtained ultrastructural evidence of disaggregation of collagen fibrils in bovine nasal cartilage after extraction with 1.9M-$CaCl_2$. Finally it is known that many proteins, particularly of the globular variety, have a net free energy of structure stabilization of only 42–84kJ/mol (10–20kcal/mol) (Timasheff, 1970). Thus small changes in the stabilization free energy in various parts of such a molecule, induced by counterion substitutions, may result in local changes in conformation sufficiently great to disrupt stereospecific interactions with other molecules. The low viscosity of the glycoprotein promoting protein–polysaccharide aggregation (Hascall & Sajdera, 1969) suggests that it may be a globular protein.

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