A STUDY OF SOME SALTS OF PROTEOGLYCANS

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The overwhelming majority of glycosaminoglycan components of the proteoglycans contain residues of hexuronic and sulfuric acids. The exceptions are hyaluronic acid (HUA) and chondroitin, which contain glucuronic acid residues only, and keratan sulfate, containing only ester-bound sulfuric acid. The biochemical roles of the carboxyl and sulfate groups are believed not to be identical. However, this problem has received little study, partly because of the lack of methods of obtaining proteoglycans in the form of free acids and of their various salts [4, 9].

The object of this investigation was to develop methods of obtaining HUA and protein-chondroitin-keratan sulfate (PCKS), chosen because of the difference in their acid components, in the form of free acids, and also to obtain Na^+, K^+, Ca^{++}, and Mg^{++} salts of these proteoglycans and to study these biopolymers by infrared (IR) spectoscopy.

EXPERIMENTAL METHOD

The original highly purified preparations of K^+ salts of HUA and PCKS were isolated from human umbilical cords and bovine tracheas respectively [5, 6]. The K^+ salts of HUA and PCKS were converted into acids by treating aqueous solutions of these salts with the H^+ form of the cation-exchange resin Amberlite IR-124 (from Serva), followed by lyophilization of the resulting solutions of these acids. To obtain salts of HUA and PCKS they were redissolved in water. HUA was precipitated from solution at −10°C with 5 volumes of ethanol containing CH_3COONa (saturated), CH_3COOK (10%), $CaCl_2$ (10%), and $MgCl_2$ (10%). After the mixtures had been allowed to stand (4°C, 18 h) the precipitates were separated by centrifugation at 1500g and washed with ethanol and ether, after which they were dried *in vacuo* above P_2O_5. Acid salts of PCKS were obtained by treatment of its solution with the Na^+, K^+, Ca^{++}, and Mg^{++} forms of the above-mentioned cation-exchange resin, followed by lyophilization of the salt solutions. Normal salts of PCKS were obtained from aqueous solutions of this acid or acid salts of the given proteoglycan by precipitation with ethanol containing the corresponding cation, as for obtaining salts of HUA.

In their external appearance the precipates of HUA salts differed sharply from each other: The Na^+ salt forms a precipitate consisting of small, short threads, the K^+ salt has the appearance of interconnected traits, the Ca^{++} salt has the appearance of a thin network woven from fine threads, and the precipitate of the Mg^{++} salt consists of long, twisted bands. The precipitates of all acid and normal salts of PCKS had no particular features.

IR spectra were obtained from the dry preparations of proteoglycans mixed with KBr in the ratio of 1:300. Tablets 13 mm in diameter were pressed with a force of 10 tons. The spectra were recorded on a Perkin-Elmer model 577 spectophotometer at 20°C within the 4000-200 cm^{-1} band. The scanning speed was 50 $cm^{-1} \cdot min^{-1}$ and the signal-to-noise ratio was 100:1 [1-3].

Special experiments showed that lyophilization had no effect on the IR spectra of the proteoglycans and their salts.

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TABLE 1. Symbols Designating the HUA, PCKS, and Their Salts (t- and p-equivalents)

No.	HUA and its salts	PCKS			
		No.	acid and acid salts	No.	normal salts
I	$R—(COOH)_n$	VI	$(HSO_3)_m—R'—(COOH)_n$	XI	$(NaSO_3)_m—R'—(COONa)_n$
II	$R—(COONa)_n$	VII	$(NaSO_3)_m—R'—(COOH)_n$	XII	$(KSO_3)_m—R'—(COOK)_n$
III	$R—(COOK)_n$	VIII	$(KSO_3)_m—R'—(COOH)_n$	XIII	$[Ca(SO_3)_e]_{m/2}—R'—[(COO)_2\times\times Ca]_{n/2}$
IV	$R—[(COO)_2Ca]_{n/2}$	IX	$[Ca(SO_3)_2]_{m/2}—R'—(COOH)_n$	XIV	$[Mg(SO_3)_2]_{/m2}—R'—[(COO_2)\times\times Mg]_{n/2}$
V	$R—[(COO)_2Mg]_{n/2}$	X	$[Mg(SO_3)_2]_{m/2}—R'—(COOH)_n$		

EXPERIMENTAL RESULTS

All preparations of HUA, PCKS, and their salts which were obtained are detailed in Table 1, to which reference must be made when the figures are examined.

A band of valency symmetrical and asymmetrical oscillations of free hydroxyl, methylene, and other groups could be sharply distinguished in the IR spectra of HUA and PCKS within the 3800-2700 cm^{-1} band (maximum 3480 cm^{-1}). In the region of 2700-2200 cm^{-1} in these spectra there was a distinct shoulder, caused by hydrogen bonds formed between the carboxyl groups of the glucuronic acid residues as the result of intermolecular interaction between proteoglycans [7, 8]. Both spectra contained a distinct band at 1740 cm^{-1} of valency oscillations of the carbonyl group of the carboxyl, a shoulder at 1420 cm^{-1} and a band at 1370 cm^{-1} of the combined valency oscillations of the carbonyl and deformation oscillations of the hydroxyl group of the carboxyl. Well marked bands at 1650 and 1550 cm^{-1} ("amide I" and "amide II") indicate oscillations of the carbonyl group of the acetyl residue of N-acetyl-hexosamines and C-N, C-C-O, and C-N-R bonds respectively [1-3, 7]. A band with complex structure with maxima at 1070 and 1030 cm^{-1}, a composite band in the 1190-950 cm^{-1} region, oscillations of the primary and secondary alcoholic groups, and the adjacent band of valency oscillations of C-O-C bonds at 1150-1060 cm^{-1} could be distinguished in the spectra of HUA and PCKS. The band at 955-890 cm^{-1} of the noncoplanar deformation oscillations of all hydroxyl groups in the spectra of HUA and PCKS was very ill defined. The difference between the IR spectra of HUA and PCKS is that the spectrum of the former contained a clearly defined band at 1150 cm^{-1}, whereas the spectrum of the latter also had a shoulder at 1125 cm^{-1}. The spectrum of PCKS of course includes a band at 1220 cm^{-1}, shoulders at 1260-1230 cm^{-1}, and a band at 850 cm^{-1} of valency oscillations of S=O and C-O-S groups respectively (Table 1; Fig. 1, spectra I and IV).

In the IR spectra of the Na^+, K^+, Ca^{++}, and Mg^{++} salts of HUA the shoulder at 2700-2200 cm^{-1} (hydrogen bonds) and the band at 1740 cm^{-1} disappeared, but bands at 1660-1600 and 1410-1370 cm^{-1} of symmetrical and asymmetrical valency oscillations of the carboxylic ion appeared. Of the 1740 cm^{-1} band only a shoulder remained in the spectrum of the Mg^{++} salt of HUA, which was absent in spectra of the other above-mentioned salts of HUA; this can be explained by the relatively smaller reduced mass compared with that of the remaining salts of HUA. In the spectrum of the K^+ salt of HUA the band at 1660-1600 cm^{-1} is slightly duplicated, and the "amide II" band is represented by a shoulder. The 1150-950 cm^{-1} band in spectra of all salts of HUA has maxima at 1070 and 1030 cm^{-1}, which in the case of the Na^+ salt are more clearly defined. In these spectra there is also a distinct band at 920 cm^{-1} (in the case of the Mg^{++} salt it is rather weaker), which is virtually absent in the spectrum of HUA itself (Table 1; Fig. 1, spectra I-V). The Mg^{++} salt of HUA evidently has some distinguishing features from the other salts of HUA mentioned above.

Unlike the spectra of all the HUA salts which were studied, the spectra of acid Na^+, K^+, Ca^{++}, and Mg^{++} salts of PCKS have a well marked shoulder at 2700-2200 cm^{-1}, which can be interpreted by the presence of hydrogen bonds, and also 1740 cm^{-1}, "amide I," and "amide II" bands. Hence it follows that the carboxyl groups in acid salts of PCKS are free, on account of which intermolecular hydrogen bonds arise, and the carboxylate ion is absent. In this case these salts were formed by substitution of the protons in the sulfuric acid residues by a cation. The band of valency oscillations of the S=O group in spectra of the acid Na^+ and K^+ salts of PCKS is wider (1300-1180 cm^{-1}) than in the spectra of PCKS, and in the spectra of the same Ca^{++} and Mg^{++} salts it is identical with that in the spectrum of this acid (1320-1180 cm^{-1}). These changes were due to replacement of the protons in the sulfuric residues by a cation, which was less well marked in the spectra of the Ca^{++} and Mg^+ salts than in those

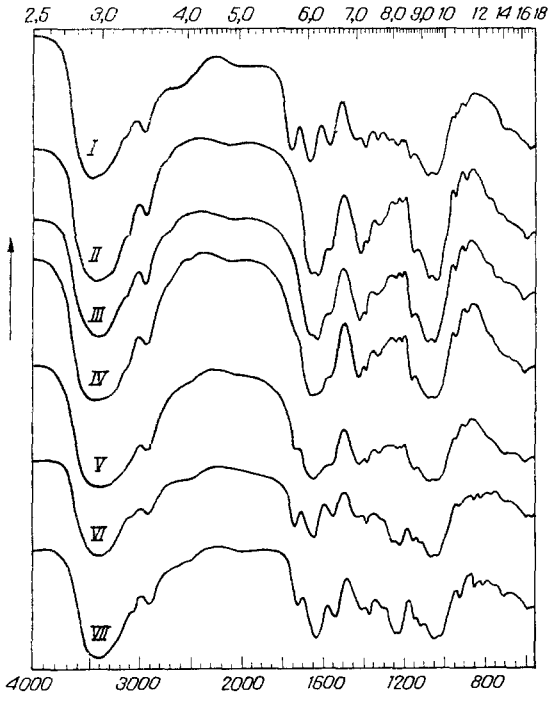

Fig. 1

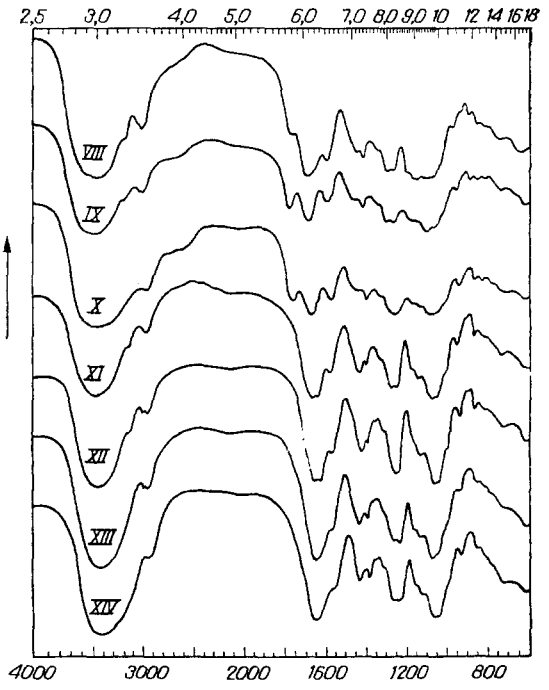

Fig. 2

Fig. 1. IR spectra of HUA, of Na^+, K^+, Ca^{++}, and Mg^{++} salts of HUA, of PCKS, and of the Na^+, K^+, Ca^{++}, and Mg^{++} salts of PCKS. For formulas, see Table 1. Here and in Fig. 2 — abscissa: above — wavelength (in μ), below — wave numbers (in cm^{-1}); ordinate, transmittance (in %).

Fig. 2. IR spectra of Na^+, K^+, Ca^{++}, and Mg^{++} salts of PCKS.

of the Na^+ and K^+ salts, probably as a result of the smaller reduced mass in the case of the bivalent cations (Table 1; Fig. 1, spectra VI and VII; Fig. 2, spectra VIII-X).

In the spectra of all the normal salts of PCKS the shoulder at 2700-2200 cm^{-1} and the band at 1740 cm^{-1} were absent but there was a wide band of oscillations of the carboxylate ion, overlapping the "amide I" band, and since there are no free carboxyl groups in these salts of PCKS, no intermolecular hydrogen bonds were formed. The "amide II" band in spectra of normal Na^+ and K^+ salts of PCKS was represented by a small band, but in the spectra of the same Ca^{++} and Mg^{++} salts only a shoulder was present at the corresponding frequency. The 1240 cm^{-1} band in the spectrum of the Na^+ salt was wider than in the spectrum of the K^+ salt and it had two ill defined maxima. Duplication of the 1240 cm^{-1} band also took place in the spectra of the normal Ca^{++} and Mg^{++} salts, i.e., what happened was the same as in the spectra of the acid salts of PCKS, and for the same reason. The band at 1150 cm^{-1} was represented by a shoulder but that at 1125 cm^{-1} still remained in the spectra of all salts of PCKS. These same spectra also had a wide band in the 1190-950 cm^{-1} interval with two maxima, expressed to different degrees, and a band at 920 cm^{-1}, which was ill defined in the spectrum of PCKS (Table 1; Fig. 2, spectra XI-XIV).

The appearance of a band at 920 cm^{-1} in the spectra of all salts of HUA and PCKS studied was probably due to liberation of the alcoholic hydroxyl groups of the residues of N-acetylhexosamines and hexuronic acids which took part in the formation of hydrogen bonds in HUA and in PCKS, in addition to the same bonds arising between carboxyl groups. Hydrogen bond formation between alcoholic hydroxyl groups is ruled out in macromolecules of salts of HUA and PCKS (both acid and normal). The appearance of a band at 920 cm^{-1} in the spectra of the acid salts of PCKS, in which intermolecular hydrogen bonds, formed between carboxyl groups, were found confirms the hypothesis that these bonds in HUA and PCKS may also arise between the alcoholic hydroxyl groups already mentioned. The presence of a metal cation, even one bound only with sulfuric acid residues, as is observed in PCKS, and in the presence of free carboxyl groups (acid salts of PCKS), in HUA and PCKS prevents the formation of hydrogen bonds through the participation of alcoholic hydroxyl groups of glycosaminoglycan monomers.

IR spectroscopy showed that HUA and PCKS can form macrocomplexes through intermolecular hydrogen bonds, arising between carboxyl groups of glucuronic acid residues and, probably, between alcoholic hydroxyl groups of monomers of these biopolymers. Acid salts of PCKS which contain free carboxyl groups, forming hydrogen bonds, can create macrocomplexes not only on account of such bonds, but also through electrovalency interactions between bivalent cations and sulfate groups belonging to different molecules of the same glycosaminoglycans. Normal Na^+, K^+, Ca^{++}, and Mg^{++} salts of HUA and PCKS cannot form macrocomplexes through the intermediary of hydrogen bonds, but the normal Ca^{++} and Mg^{++} salts of these proteoglycans are macrocomplexes arising as the result of linking through the bivalent metal, each atom of which binds two carboxyl or two sulfate groups of separate molecules of the given proteoglycan. In all salts of HUA and PCKS studied in the present investigation the possibility of participation of coordination bonds in intermolecular interaction cannot be ruled out, as several investigations have shown [4].

The relative diversity of the nature of the bonds concerned in intermolecular interactions in HUA and, in particular, in PCKS, and also their salts, can lead to the formation of macrocomplexes and of more complex supramolecular structures, in which cooperative effects are possible. Probably many biochemical functions of the proteoglycans are determined by the features mentioned above, and this applies above all to the participation of proteoglycans in cation binding by tissues and cells, in the role of active ion-exchange biopolymer.

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