A STUDY OF SALTS OF CARTILAGE PROTEOGLYCAN AGGREGATES

S. M. Bychkov, V. N. Bogatov,
and S. A. Kuz'mina

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Much of the protein-chondroitin-keratin-sulfate (PCKS; the aggregated, insoluble fraction) in cartilage is contained in the form of a noncovalent compound composed of many (20-100 or more) macromolecules of this proteoglycan with one macromolecule of hyaluronic acid (HUA). These proteoglycan aggregates (PA) are formed through interaction of the hyaluronate-binding site, located on the end of the protein rod of the PCKS, with HUA, and are stabilized by a special binding protein. The structure of PA has largely been explained, but their biological role has received little study [5]. It has been suggested that one of the functions of PA is regulation of calcification [12, 13]. One of the many problems which must be studied in order to elucidate the biological role of PA is that of the degree to which the individuality of these macromolecular monomers is preserved after their incorporation into PA. The presence of many sulfate and carboxyl groups creates an exceptionally high negative electric charge on the PA. In this connection the results of studies of various salts of PA may be a first approach to the solution of this problem.

In the investigation described below acid and normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA were studied by infrared (IR) spectroscopy [6].

EXPERIMENTAL METHOD

PA were isolated from the cartilaginous rings of the bovine trachea by extraction with a 4 M solution of guanidine hydrochloride [8-12]. This salt was isolated by lyophilization from the solution of the guanidine salt of PA, purified by dialysis. The acid form of PA ($PA\text{-}Hn_n^+$) was obtained by treatment of a solution of this salt with the H^+ form of a cation-exchange resin (Amberlite IR-124, from Serva), followed by lyophilization. The $PA\text{-}Hn_n^+$ is well preserved *in vacuo* above phosphic anhydride.

Acid Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA were prepared by treating a solution of $PA\text{-}H_n^+$ with the corresponding salt form of this cation-exchange resin and lyophilization of the solution of the resulting salt. Normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA were obtained from aqueous solutions of $PA\text{-}H_n^+$ by precipitation at −5°C with five volumes of ethanol, containing CH_3COONa (saturated), CH_3COOK (10%), $CaCl_2$ (10%), and $MgCl_2$ (10%) respectively. The precipitates were separated by centrifugation (1500g), washed with ethanol to remove the salts, and then with ether, and dried *in vacuo* above paraffin and then above P_2O_5. Normal salts of PA can be obtained from solutions of acid salts by precipitating them with ethanol by the method described above.

IR spectra within the 4000-200 cm^{-1} range were obtained from dry preparations. All details of this method were described by the writers previously [2-4], and in the present investigation it was used unchanged.

EXPERIMENTAL RESULTS

The preparations of PA salts obtained are shown in Table 1. The serial number of the preparations in Table 1 corresponds to that of the spectra in Fig. 1.

A strong band of absorption of valences and other overlapping oscillations (symmetrical and asymmetrical) of methylene and hydroxyl groups, of a N–H bond and of other groups and

Research Laboratory of Biological Structures, Ministry of Health of the USSR, Moscow.
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TABLE 1. Relationship between Cations and Anionic Groups in Salts of PA

No.	Acid and acid salts	No.	Normal salts
I	PA-H_n^+	IV	Na_n^+ – PA
II	Na_m^+ – PA-H_{n-m}^+	VII	K_n^+ – PA
III	K_m^+ – PA–H_{n-m}^+	VIII	Ca_m^{2+} – PA
IV	$Ca_{m/2}^{2+}$ – PA–$H_{n-m/2}^+$	IX	$Mg_{n/2}^{2+}$ – PA
V	$Mg_{m/2}^{2+}$ – PA-$H_{n-m/2}^+$		

Legend. Results of analysis (in Oe) of PA-H_n^+: nitrogen 4.30, hexosamine 27.1, hexuronic acids 23.00, sulfate 10.15, hexosamines/hexuronic acids = 1.18.

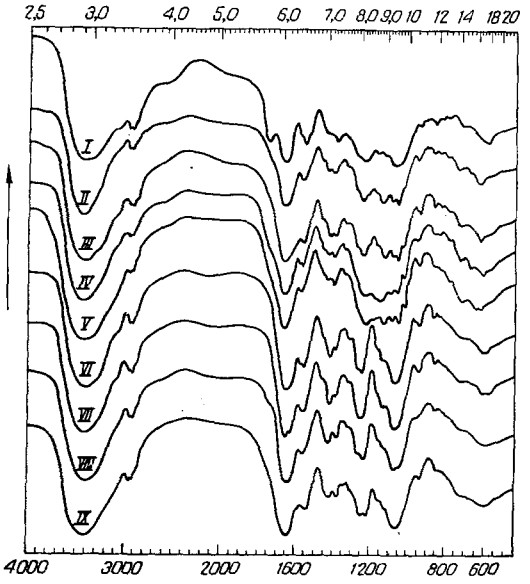

Fig. 1. IR absorption spectra of the acid form (I), and of acid (II-V) and normal (VI-IX) Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA (see also Table 1). Abscissa: above – wavelength (in μ), below – wave numbers (in cm^{-1}); ordinate, transmission (in percent).

bonds is present in the IR spectra of PA-H_n^+ and of all the PA salts studied in the 3800-2700 cm^{-1} band (Fig. 1). The difference between the spectra of this region of frequencies relates to the shoulder at 3100 cm^{-1}, which is poorly defined in the case of the acid Ca^{2+}-salt of PA and absent altogether in spectra of the acid and normal Mg^{2+}-salts. The wide shoulder at 2700-2400 cm^{-1}, which can be attributed to hydrogen bonds [3, 4, 6, 7] arising between carboxyl groups of glucuronic acid residues, is found in the spectra of PA-H_n^+ and all acid salts of PA but is absent in the spectra of all normal salts of PA. Hence it can be concluded that protons of sulfuric acid residues in acid salts of PA are replaced by one of the above-mentioned cations, whereas protons of the carboxyl groups of the glucuronic acid residues remain unsubstituted. In normal salts of PA all protons are replaced by cations of the corresponding metal. However, because of the very complex steric features of the PA macromolecule the possibility cannot be ruled out that during the formation of acid salts a certain proportion of protons of sulfate groups may remain unsubstituted, but in the case of normal salts of PA, for the same reasons some of the carboxyl groups may not be converted into carboxylate ions.

The band of valence oscillations of the carbonyl group of the carboxyl at 1745 cm^{-1} is well defined only in spectra of PA-H_n^+. In the spectra of all acid salts and of normal Ca^{2+}- and Mg^{2+}-salts of PA there is a weak shoulder in this part of the spectrum. These bands and shoulder are absent, however, in spectra of normal Na^+- and K^+-salts of PA. The presence of this shoulder in spectra of normal Ca^{2+}- and Mg^{2+}-salts of PA is probably due to the fact that these cations are distributed among two carboxyls and form intramolecular Ca^{2+}- and Mg^{2+}-bridges, leading to incomplete overlapping of the 1745 and 1645 cm^{-1} bands. It must be pointed out that a difference was observed between the above-mentioned acid salts of PA and the acid Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of the soluble fraction of PCKS and heparin, in whose spectra a distinct band is present at 1745 cm^{-1} [3, 4].

The band at 1645 cm^{-1} ("amide" I), due to oscillations of the carbonyl group of the acetamide residue of N-acetylhexosamines, is present only in the spectra of PA-H_n^+. This band merges in the spectra of all salts of PA with the 1745 cm^{-1} band so that a wide band is formed with a maximum at 1645 cm^{-1}. In the cases of acid and normal K^+-salts of PA this band has a shoulder at 1645 cm^{-1}. Oscillations of C—N, C—C—O, and C—N—R bonds (the band at 1550 cm^{-1}, "amide" II) are very clearly defined in the spectra of PA-H_n^+. The 1550 cm^{-1} band is much weaker in all the other salts of PA. The shoulder at 1450-1400 cm^{-1} and the band at 1370 cm^{-1} for combined valence and deformation oscillations of the carbonyl and hydroxyl groups respectively are present only in spectra of PA-H_n^+. In spectra of all acid salts of PA there is a wide band of average intensity with maxima at 1410 and 1370 cm^{-1}, whereas in the spectra of normal salts of PA there is a somewhat stronger band with clearly defined maxima at 1420 and 1370 cm^{-1}, probably connected with replacement of the proton of the carboxyl groups by the cation of the metal, i.e., by the conversions of these groups into carboxylate ions.

Bands at 1220 and 850 cm^{-1}, due to valence oscillations of S=O and C—O—S groups, are present in spectra of all the PA preparations studied. Considerable differences, depending on the nature of the cation and its position, are observed in the structure of this part of the spectra. In spectra of PA-H_n^+ and of the acid and normal K^+- salts of PA these bands are weaker than in spectra of the other salts of PA. Spectra of acid Ca^{2+}- and Mg^{2+}-salts of PA have a wide band in the 1300-950 cm^{-1} region with maxima at 1220, 1070, and 1040 cm^{-1} and, in addition, they have several shoulders, whereas in spectra of normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts the band at 1220 cm^{-1} is clearly defined; in spectra of the Ca^{2+}- and Mg^{2+}-salts, moreover, this band has two maxima, and it is weaker than in the spectra of normal Na^+- and K^+-salts, probably due to binding of one bivalent cation with two sulfate groups.

The structural features of the 1220 cm^{-1} band in the spectra of PA salts noted above probably also occur in spectra of Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts (acid and normal) of the nonaggregated, soluble fraction of PCKS and heparin, studied previously, for their acid salts also form hydrogen bonds on account of carboxyl groups [3, 4].

Within the 1400-900 cm^{-1} interval of the IR spectra, besides the absorption bands noted above, absorption bands of valence oscillations of C—C, C—O, and C—N bonds and many deformation oscillations also are concentrated by them, so that there is considerable overlapping of the bands of the spectra. Nevertheless, the whole structure of the spectra within this interval of frequencies reflects the individual features ("fingerprints") of the various substances, including proteoglycans and their monomers [1-4].

The band of oscillations of primary and secondary alcohol hydroxyls at 1070-1075 cm^{-1} in the spectra of PA-H_n^+ and of acid Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA is present only in the form of maxima in the wide 1400-900 cm^{-1} band, whereas it is very clearly defined in spectra of normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA.

A narrow interval of the spectrum between 1150 and 1125 cm^{-1}, difficult to identify, in which PA-H_n^+ has a band at 1150 cm^{-1} and a shoulder at 1125 cm^{-1}, whereas normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA have only shoulders at 1150 and 1125 cm^{-1}, is interesting. In spectra of acid Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA, however, there is one interesting wide band at 1125 cm^{-1}. According to the results of previous investigations, in the IR-spectra of HUA and of its Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts there is only a band at 1150 cm^{-1}, whereas in spectra of the nonaggregating fraction of PCKS, and of its acid and normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts a band at 1150 cm^{-1} and a shoulder at 1125 cm^{-1} or, conversely, a shoulder at 1150 cm^{-1} and a band at 1125 cm^{-1} are present depending on the nature of the cation [3]. Hence it follows that the ratio of the intensities of absorption at these two frequencies is determined by the nature and position of the cations in the macromolecular monomers forming the PA.

The band at 920 cm^{-1} of extraplanar deformation oscillations of hydroxyl groups in the spectra of PA-H_n^+ and of all acid and normal Na^+-, and K^+-salts of PA is sufficiently clearly defined, but in the cases of normal Ca^{2+}- and Mg^{2+}-salts it is represented by a shoulder. In the two latter salts, some of the hydroxyls of the N-acetylhexosamines and of glucuronic acid may perhaps form intramolecular hydrogen bonds, thus reducing the intensity of the 920 cm^{-1} band. A similar phenomenon also is found in salts of nonaggregating (soluble) PCKS [3].

The study of IR absorption spectra of PA-H_n^+ and of acid and normal Na^+-, K^+-, Ca^{2+}-, and Mg^{2+}-salts of PA showed that HUA and PCKS, which are macromolecular monomers of PA, preserve to a certain degree their individual properties as regards the formation of salts with inorganic cations, intramolecular hydrogen bonds, and so on. Nevertheless, the differences mentioned above were found between the IR spectra of aggregating and nonaggregating (soluble) PCKS. It is still not quite clear why replacement of the proton in carboxyl groups of acid salts of PA by the above-mentioned cations should be accompanied by sharp changes in structure of the IR spectra within the 1400-900 cm^{-1} range formed by normal salts of PA. Because of the exceptional complexity and macromolecular nature of PA, salt formation by this biopolymer is probably accompanied by cooperative effects and, in addition, steric and other factors may also play an essential role [1]. Despite difficulties in the identification of certain absorption bands in IR spectra of proteoglycans and PA, these spectra can be used to investigate these biopolymers.

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