A STUDY OF VARIOUS HEPARIN SALTS

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Besides glucosamine residues, the heparin macromolecule also includes residues of iduronic and glucuronic acids and large quantities of sulfate groups, which endow it with strong acid properties, and it also possesses a unique spectrum of biological activity. Accordingly, the study of heparin salts is particularly interesting [6, 10, 12]. However, the absence of methods of obtaining the various acid and normal salts of heparin is a substantial handicap.

The aim of the present investigation was to develop methods of obtaining acid and normal Na^+, K^+, Ca^{++}, and Mg^{++} salts of two individual heparin fractions, one of which contains three (HP-3) and the other four (HP-4) sulfate groups per dimer of the macromolecule [7-9], and to study these salts by IR spectroscopy.

EXPERIMENTAL METHOD

The original highly purified preparations HP-3 and HP-4 were obtained in the form of normal K^+-salts by the method described previously [9]. Acid salts HP-3 and HP-4 were obtained by treating solutions of original preparations with the H^+ form of a cation-exchange resin (Amberlite IR-124, from Serva, West Germany). After removal of the Amberlite the acids HP-3 and HP-4 formed in the solutions were converted into Na^+, K^+, Ca^{++}, and Mg^{++} salts by the corresponding salt form of this resin. Solutions of the salts thus obtained were separated from the resin by centrifugation and lyophilization. Normal salts of HP-3 and HP-4 also were prepared from solutions of the acid forms of these fractions by precipitation with 5 volumes of ethanol containing CH_3COONa (saturated), CH_3COOK (10%), $CaCl_2$ (10%), and $MgCl_2$ (10%). After 18-20 h (4°C) residues of the salts were separated on a centrifuge (see above), washed with ethanol (to remove the salts) and ether, and dried above P_2O_5 *in vacuo*. Neither HP-3 nor HP-4 could be isolated as free acids because of their exceptionally hygroscopic properties.

The IR spectra of dry preparations of salts of HP-3 and HP-4, mixed with KBr in the ratio of 1:300, were obtained at 20°C on a Perkin-Elmer model 577 spectrophotometer. Full details of the method were described in [3-6].

EXPERIMENTAL RESULTS

Analysis of original preparations of normal K^+ salts of HP-3 and HP-4 showed that they are highly purified and that the ratio between the monomers agrees with the theoretical values (Table 1). The content of covalently bound amino acids in HP-3 and HP-4 was 2.22 and 0.90% respectively. Dicarboxylic amino acids and hydroxyamino acids greatly predominated.

All the preparations of salts obtained are listed in Table 2, which gives the ratios between cations and anions in acid and normal salts of HP-3 and HP-4. The identification number of the salts (Roman numerals) in Table 2 are identical with those of their spectra in Figs. 1 and 2.

In the IR spectra of all acid and normal salts of HP-3 and HP-4 in the 3700-2800 cm^{-1} region there was a strong band (maximum about 3400 cm^{-1}) with shoulders at 3100 and

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TABLE 1. Results of Analysis (in pmoles/mg g anion of Preparations of Heparin Fractions (M ± m); n = 5

Component	HP-3	HP-4
Nitrogen	1,19±0,01	1,20±0,01
Amino sugar (A)	1,41±0,00	1,31±0,01
Sulfate group (B)	4,46±0,05	5,29±0,03
Hexturonic acids (C)	1,45±0,02	1,37±0,03
B/A	3,16	4,03
C/A	1,02	1,04

TABLE 2. Ratio between Cations and Anions in Dimers of Salts of HP-3 and HP-4 (HP-3-$[(HSO_3)_3$-R-COOH$]_n$; HP-4-$[(HSO_3)_4$-R-COOH$]_n$; Disaccharide Repeating Structural Unit — HP-3 and HP-4 Dimer — Inside Square Brackets

Salts of HP-3				Salts of HP-4			
No.	acid	No.	normal	No.	acid	No.	normal
I	$Na_3(SO_3)_3$—R—COOH	V	$Na_3(SO_3)_3$—R—COONa	IX	$Na_4(SO_3)_4$—R—COOH	XIII	$Na_4(SO_3)_4$—R—COONa
II	$K_3(SO_3)_3$—R—COOH	VI	$K_3(SO_3)_3$—R—COOK	X	$K_4(SO_3)_4$—R—COOH	XIV	$K_4(SO_3)_4$—R—COOK
III	$Ca_3[(SO_3)_3$—R—COOH$]_2$	VII	$Ca_3[(SO_3)_3$—R—COO$]_2$Ca	XI	$Ca_4[(SO_3)_4$—R—COOH$]_2$	XV	$Ca_4[(SO_3)_4$—R—COO$]_2$Ca
IV	$Mg_3[(SO_3)_3$—R—COOH$]_2$	VIII	$Mg_3[(SO_3)_3$—R—COO$]_2$Mg	XII	$Mg_4[(SO_3)_4$—R—COOH$]_2$	XVI	$Mg_4[(SO_3)_4$—R—COO$]_2$Mg

2500 cm^{-1}, due to overlapping valency oscillations (symmetrical and antisymmetrical) of methylene and free hydroxyl groups, the N—H bond, and certain other groups and bonds. In the spectra of the acid Ca^{++} salt of HP-4 this band was slightly split and had maxima at 3525 and 3400 cm^{-1}. In the spectra of the acid Na^+, K^+, and Ca^{++} salts of HP-3 and also of the acid Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-4 in the 2800-2500 cm^{-1} interval a shoulder was present, which varied in size and could be ascribed to hydrogen bonds [11, 13]. Neither the normal salts of HP-3 and HP-4 nor the acid Mg^{++} salt of HP-3 had any shoulder in their spectra in this region (Fig. 1, I-IV and Fig. 2, IX-XII).

All the acid salts of HP-3 had bands of valency oscillations of the carbonyl group of the carboxyl residue in their spectra at 1745 cm^{-1}, oscillations due to C—N, C—C—O, and C—N—R bonds (amide II) at 1550 cm^{-1}, and oscillations of the R—SO_3^- group at 1245 and 600 cm^{-1} [1-5, 11]. Bands at 1125 cm^{-1} and within the range 1100-950 cm^{-1} (maxima at 1050 and 1020 cm^{-1}) of overlapping oscillations of primary and secondary alcohol hydroxyl groups, and also non-planar deformation oscillations of the hydroxyl group (925 cm^{-1}) also were clearly visible in the spectra of these salts. Bands at 1645 and 1380 cm^{-1} also were present (see below). In the spectra of acid salts of bivalent metals with HP-3 the R-SO_3^- group band had an ill-defined maximum at 1220 cm^{-1} with adjacent shoulder (Fig. 1, I-IV).

Normal Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-3 had no band at 1745 cm^{-1} in their spectra but they did have a strong band at 1645 cm^{-1} due to absorption by the carboxylate ion, and also bands at 1420, 1380, and 1125 cm^{-1}. The intensity of the 1550 cm^{-1} was sharply reduced in the spectra of normal salts of HP-3, especially the Ca^{++} and Mg^{++} salts. The 600 cm^{-1} band in the spectra of the Ca^{++} and Mg^{++} salts was much wider than in the spectra of the acid salts of these same metals. The 940 cm^{-1} band was weaker in the normal salts of HP-3 than in its acid salts (Fig. 1, V-VIII).

Spectra of acid Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-4 had a band at 1745 cm^{-1}, which was weaker in the case of salts of the bivalent metals, and also bands at 1650 and 1550 cm^{-1}. There was a clear band at 1380 cm^{-1} in the spectra of the acid Na^+, K^+, and Mg^{++} salts of HP-4, whereas in the spectrum of the Ca^{++} salt of HP-4 only a shoulder remained of this band. In the 1300-1000 cm^{-1} range a band with complex structure including only a shoulder instead of the band at 1240 cm^{-1}, was present in the spectra of acid Na^+ and Ca^{++} salts of HP-4, and in the narrow interval 1200-1100 cm^{-1} there was a band at 1140 cm^{-1} and a shoulder. In the case of the Mg^{++} salt of HP-4 there was a wide band of simple structure without a shoulder at 1240 cm^{-1} in this same region. A band at 1240 cm^{-1} and one at 1100 cm^{-1} with a shoulder at 1150 cm^{-1} were present in the 1300-1000 cm^{-1} range in the spectra of the acid K^+ salt of HP-4. Acid Na^+, K^+, and Ca^{++} salts of HP-4 had a pointed band at 600 cm^{-1} in their spectra, which was represented by a doublet in the case of the Ca^{++} salt, whereas in the spectrum of the Mg^{++} salt in this region there was a wide band. A shoulder was present

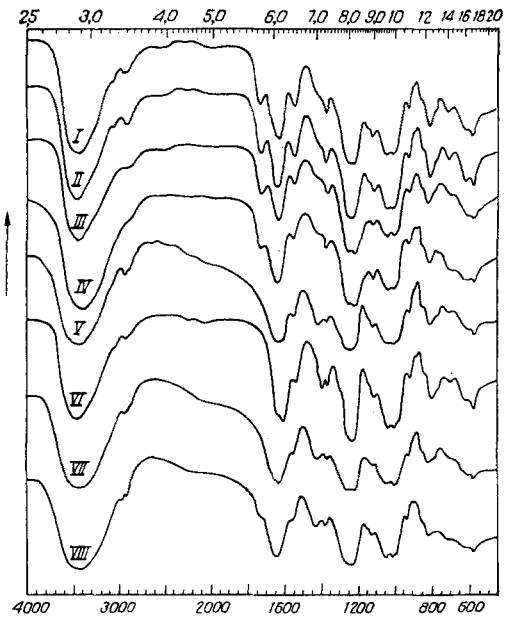

Fig. 1

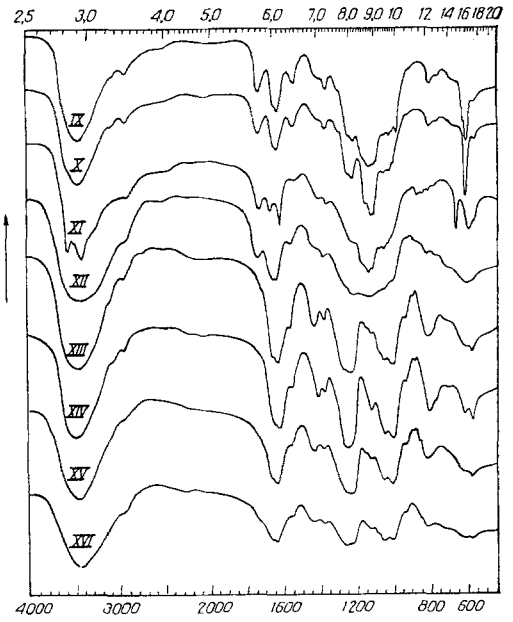

Fig. 2

Fig. 1. IR absorption spectra of acid (I-IV) and normal (V-VIII) Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-3. Roman numerals correspond to salts listed in Table 2. Abscissa: above — wavelength (in μ), below — wave numbers (in cm^{-1}); ordinate, transmission (in %).

Fig. 2. IR adsorption spectra of acid (IX-XII) and normal (XIII-XVI) Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-4. Legend as to Fig. 1.

at 940 cm^{-1} in the spectra of all acid salts of HP-4. Marked differences were found in the structure of the spectra of acid salts of HP-4 due to the nature of the cation, and affecting mainly the region of oscillations of the $R—SO_3^-$ group (Fig. 2, IX-XII).

The band at 1745 cm^{-1} was absent in spectra of normal Na^+, K^+, Ca^{++}, and Mg^{++} salts of HP-4 but an intensive carboxylate ion band was present at 1640 cm^{-1}, which was much weaker in the case of the Mg^{++} salt than in the spectra of the other three normal salts of HP-4. Instead of the 1550 cm^{-1} band only a shoulder remained. Bands at 1420, 1380, and 1240 cm^{-1} (particularly strong for Na^+ and K^+ salts) and at 940 cm^{-1} were present in the spectra of all normal salts of HP-4. The band at 600 cm^{-1} in the spectra of the Na^+ and K^+ salts consisted of a doublet, whereas in the spectra of the Ca^{++} and Mg^{++} salts it was indistinct in shape (Fig. 2, XIII-XVI).

The strong band at 1645 cm^{-1} in the spectra of all salts of HP-3 and HP-4, which overlapped with the carboxylate ion band in the spectra of the normal salts of these biopolymers, cannot be attributed to oscillations of the acetamide group, for even if this group was present in HP-3 and HP-4, it was only in extremely small quantities. More probably this band was due to peptide bonds of the polypeptide components of HP-3 and HP-4 [9, 14]. The band at 1380 cm^{-1}, present in the spectra of all the salts studied, is also difficult to explain. On the basis of the facts described above, and also since a band at 1380 cm^{-1} is present in the IR-spectra of glucuronic acid and potassium glucuronate, which contain no acetyl group [4], it does not seem possible to attribute this band in the spectra of HP-3 and HP-4 to oscillations of this group. The question of the presence of an acetyl group in HP-3 and HP-4 must remain open.

The presence of a band at 1745 cm^{-1} in the spectra of acid salts of HP-3 and HP-4 evidently proves that protons of carboxyl groups of hexuronic acids in these salts are not replaced by Na^+, K^+, Ca^{++}, or Mg^{++}. This is in agreement with the fact that hydrogen bonds were detected only in salts with free carboxyl groups, by means of which intermolecular interaction can take place, with the formation of hydrogen bonds. The absence of the band at 1745 cm^{-1} and shoulder of hydrogen bonds in spectra of the normal salts of HP-3 and HP-4 and the presence of a band of carboxylate ion (1645 cm^{-1}) in them is evidence that in these

cases the proton of the carboxyl group is replaced by a metal, as a result of which intermolecular hydrogen bond formation on account of carboxyl groups is ruled out. The absence of intermolecular hydrogen bonds in the acid Mg^{++} salt of HP-3, correlating with the presence of a band at 1745 cm^{-1} in the spectrum, is probably due to the presence of stearic and other factors in this case, preventing intermolecular interaction.

The band at 820-800 cm^{-1} present in the spectra of all salts of HP-3 and HP-4 is difficult to interpret. It can be tentatively suggested that absorption in this region is connected with the $R\text{-}SO_3^-$ group, for the amplitude and structure of this wave showed parallel changes with the 600 cm^{-1} band [1, 2].

Changes in the 600 cm^{-1} band in the spectra of acid salts of HP-3 and HP-4 dependent on the nature of the cation show that the proton of the sulfate group in these salts is replaced by the corresponding cation [1, 2].

Intermolecular interaction in acid and normal Ca^{++} and Mg^{++} salts of HP-3 and HP-4 can also take place with the aid of these bivalent cations, when one cation reacts with two acid groups belonging to different macromolecules of the given proteoglycan, resulting in the formation of supramolecular complexes. Participation of coordination bonds in this process likewise cannot be ruled out [15].

Within a given heparin fraction, i.e., HP-3 or HP-4 differences in the IR spectra of the salts are thus determined by the cation and by the presence or absence of a free carboxyl group. Differences between the IR spectra of salts of HP-3 and HP-4 with the same cation, however, are determined by the anion. It follows from analysis of the IR spectra of the salts of HP-3 and HP-4 we have studied that differences between these heparin fractions are not confined to their chemical composition, but also extend to their macromolecular structure.

Individual macromolecules of the same heparin fraction in the living organism may perhaps exist in the form of Na^+, K^+, Ca^{++}, and Mg^{++} salts, with the conformational properties peculiar to each of them and with their own particular biological activity, in harmony with the diversity of physiological functions of heparin.

LITERATURE CITED

1. L. Bellamy, Infrared Spectra of Complex Molecules [Russian translation], Moscow (1963).
2. L. Bellamy, New Data on the IR Spectra of Complex Molecules [Russian translation], Moscow (1971).
3. S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 6, 680 (1980).
4. S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 4, 442 (1981).
5. S. M. Bychkov, V. N. Bogatov, and V. N. Kharlamova, Byull. Éksp. Biol. Med., No. 6, 680 (1980).
6. S. M. Bychkov, and M. M. Zakharova, Vop. Med. Khim., No. 3, 227 (1979).
7. S. M. Bychkov and V. N. Kharlamova, Byull. Éksp. Biol. Med., No. 12, 28 (1974).
8. S. M. Bychkov and V. N. Kharlamova, Byull, Éksp. Biol. Med., No. 3, 61 (1975).
9. S. M. Bychkov and V. N. Kharlamova, Byull. Éksp. Biol. Med., No. 3, 289 (1976).
10. R. G. Zhbankov, N. M. Rukina, and T. L. Grinkevich, Khim.-Farm. Zh., No. 5, 45 (1971).
11. A. Cross, Introduction to Practical Infrared Spectroscopy [Russian translation], Moscow (1961).
12. A. M. Ovsepyan, V. V. Kobyakov, and V. P. Panov, Zh. Prikl. Spektrosk., 24, 3022 (1977).
13. G. C. Pimentel and A. L. McClellan, The Hydrogen Bond, Freeman (1960).
14. Yu. N. Chirgadze, Biofizika, 7, 523 (1962).
15. Y. Wistler, Pure Appl. Chem., 49, 1229 (1977).