TISSUE PROTEOGLYCANS STUDIED BY INFRARED SPECTROSCOPY

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The study of infrared (IR) spectra of several individual proteoglycans, in the form of their acids, and acid and normal salts with various bases, has revealed group characteristic absorption frequencies of many representatives of this group of biopolymers [1-4, 6]. The aim of the present investigation was to study proteoglycans present in tissues by direct IR-spectroscopy of the tissues themselves, using data on group characteristic absorbances found in the IR-spectra of standard preparations of individual proteoglycans in order to identify these biopolymers.

EXPERIMENTAL METHOD

Of all the tissues and their individual structures that contain proteoglycans the following were chosen for study: bovine tracheal cartilage, and rabbit cornea, sclera, vitreous body, and lens of the eye. Complexes of lysozyme with various proteoglycans [7] also were investigated in order to determine the effect of electrovalent binding of a protein with proteoglycans on the IR-spectra of the latter. As standard preparations of individual proteoglycans we used the following, isolated from various tissues [1-4, 8, 9] in the form of highly purified normal Na-salts of hyaluronic acid (HUA), a natural complex (soluble) protein-chrondroiting-keratin-sulfate (PCKS), aggregates of proteoglycans (AP) from cartilage, and two fractions of heparin, one of which contained three (HP-3) and the other four (HP-4) sulfuric acid residues per unit of glucosamine residue [3]. Results of analyses of the proteoglycan preparations and complexes of lysozyme are given in Tables 1 and 2. Commercial preparations (from "Sigma") of Na-salts of chondroitin-4- (CS-4) and chondroitin-6-sulfate (CS-6) and of dermatan-sulfate (DS), which were not analyzed, also were used.

IR-spectra were obtained from lyophilized minced tissues and dry preparations of proteoglycans mixed with KBr in the ratio of 1:300, and pressed in the form of tablets 13 mm in diameter with a force of 10 tons. The spectra were recorded on a "Perkin-Elmer" model 577) spectrophotometer at 20°C in the 4000-400-cm^{-1} region. The signal to noise ratio was 100:1. The spanning speed was 50 $cm^{-1} \cdot min^{-1}$. Absorption bands on the spectrograms were identified against standard tables [10, 15].

EXPERIMENTAL RESULTS

A wide band with maximum at 3450 cm^{-1}, and with shoulders at 3100 and 2800 cm^{-1} of symmetrical and asymmetrical valency oscillations of methylene and free carboxyl groups, of the N–H bond, and of certain other structures and bonds was found in the high-frequency range in the IR-spectra of HUA, PCKS, CS-4, CS-6, DS, HP-3,

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TABLE 1. Results of Analyses of Standard Preparations of HUA, PCKS, AP (in %), HP-3, HP-4 (in mmoles × 2^{-1} anion) (M ± m, n = 8)

Preparation	Nitrogen	Hexosamine (A)	Hexuronic acid (B)	Sulfate group (C)	C/A	Protein, by calculation
HUA (from human umbilical cord)	3,00±0,10	42,00±0,12	43,00±0,10	0,00	—	1,20
PCKS (from bovine tracheal cartilage)	4,20±0,70	28,40±0,50	28,00±0,10	14,60±0,50	—	14,60
AP (from the same material)	3,80±0,10	29,40±0,10	26,20±0,15	11,20±0,50	—	12,00
HP-3 (from bovine lung)	1,20±0,12	1,40±0,13	1,40±0,10	4,20	3,00	2,30
HP-4 (from the same material	1,23±0,11	1,30±0,10	1,20±0,13	5,30	4,00	1,00

Legend. Protein content in preparations HP-3 and HP-4 expressed as total content (mmoles) of amino acid residues.

HP-4, and AP. A band at 1640-1550 cm^{-1} (Amide I) was present in the same spectra, with overlapping absorbances of the carboxylate ion and C–O group (1725 cm^{-1}) of primary and secondary amides. Contiguous with it was a band (Amide II) of absorbances of valency oscillations N–H, C–N, and C–O, at 1545 cm^{-1}. All these spectra contained a band at 1450 cm^{-1} of deformation oscillations of –OH and valency oscillations of the C–O groups, as well as a shoulder at 1370 cm^{-1} of overlapping deformation oscillations of methylene and methyl groups. In spectra of sulfate-containing proteoglycans, valency oscillations of R-SO3 were represented by a clear band at 1245 cm^{-1} and the same oscillations of S–O, and C–O–S by a band at 850 cm^{-1}. Summation of the 1245-cm^{-1} band with absorbance of carboxyl groups of dicarboxylic amino acids, present in covalently bound protein components of proteoglycans was observed. (In our previous communication [9] on IR-spectra of CS-4, CS-6, DS, and other proteoglycans the 1245-cm^{-1} band was not indicated in the text due to an oversight). All absorbances due to sulfate groups were absent from the IR spectra of HUA. Structures of R–C–O–C–R type were clearly found as absorbances of 1150 and 1000 cm^{-1} only in spectra of HUA. In spectra of sulfate-containing proteoglycans, however, these absorbances were represented by a weak shoulder, but they contained a band at 1125 cm^{-1} not present in the spectrum of HUA, so that it can be ascribed to a not yet explained absorbance connected with the sulfate group. Two-dimensional deformation oscillations of primary and secondary alcoholic hydroxyl groups in the HUA spectrum were represented by a band with a maximum at 1070 and 1040 cm^{-1}, whereas in spectra of sulfate-containing proteoglycans these bands were stronger, due to summation with absorbances of the S–O group, which could be distinguished particularly clearly in the cases of HP-3 and HP-4. In the latter this band has a maximum at 1040 and 1000 cm^{-1}. Extraplanar deformation oscillations of any hydroxyl group in all the spectra were represented by a band at 950-920 cm^{-1}. Absorbances of oscillations of the hydrogen bond between carboxyl and acetamide (in the case of HP-3 and HP-4, sulfamide) groups (Amide V) in the spectra of PCKS, CS-4, HP-3, and AP were represented by a band at 750-700 cm^{-1}. In the spectra of HUA, CS-6, DS, and HP-4 this hydrogen bond is represented by a shoulder in the same part of the spectrum.

It must be emphasized that in the 1400-400 cm^{-1} interval ("fingerprints") of the IR-spectra of the proteoglycans studied, just as in the IR-spectra of many other substances, a large number of overlapping absorbances of C–C, C–O, C–N, and other bands, determining the particular structural features of a given substance and difficult to differentiate, are concentrated.

The main differences between the IR-spectra of the above-mentioned proteoglycans, qualitatively speaking, can be reduced to the fact that no bands at 1245 and 1125 cm^{-1} are present in the spectrum of HUA, and if the band at 1150 cm^{-1} is present, the spectra of sulfate-containing proteoglycans will contain bands at 1245 and 1125 cm^{-1}, and instead of the band at 1150 cm^{-1} there will be a more or less distinct shoulder.

TABLE 2. Results of Analyzes of Complexes of Lysozyme with Proteoglycans (in %) (M ± m, n = 7)

Complex	Nitrogen content		Content of proteoglycan in complex
	of proteoglycan	of complex	
Lysozyme-CS (-4; -6)	2,20±0,01	10,30±0,05	4,40
Lysozyme-PCKS	4,30±0,03	5,80±0,07	9,20
Lysozyme-AP	4,10±0,15	5,55±0,07	9,40
Lysozyme-HP-3	1,20±0,02	6,24±0,05	8,50
Lysozyme-HP-4	1,20±0,03	7,00±0,03	7,70

Legend. Nitrogen content in lysozyme 14.60 ± 0.06%.

In the IR spectra of complexes of lysozyme with CS (a mixture of CS-4 and CS-6), PCKS, HP-3, and HP-4, containing 4.40-9.40% of proteoglycans (Table 2), absorbances of lysozyme and of the proteoglycans within the range 3700-2800 cm^{-1} coincide although there is a small decrease in amplitude of individual bands compared with the spectra of lysozyme and the proteoglycans separately. A strong band at 1650 cm^{-1} could be distinguished in the spectra of the complexes as a result of fusion of absorbance of the carboxylate ion and the Amide I band; the Amide II band was present and the amplitude of the band at 1245 cm^{-1} was significantly reduced. Bands at 1150 and 1125 cm^{-1} were present only in spectra of the complex of lysozyme with CS and AP. Amide V absorbance was present in the spectra of all complexes of lysozyme.

IR-spectra of cartilage within the frequency band 3700-2800 cm^{-1} contained all absorbances characteristic of PCKS and AP. These spectra also contained Amide I and Amide II bands, a band at 1245 cm^{-1}, and shoulders at 1150, 1125, and 850 cm^{-1}, and Amide V. The total PCKS and AP preparation extracted from this same specimen of tissue by 3 M $MgCl_2$ solution had a spectrum completely identical with that of standard preparations of PCKS and AP. The RI-spectrum of the cartilage residue obtained after removal of PCKS and AP from it was characterized by a much lower amplitude of the absorbances belonging to spectra of these proteoglycans. The band at 1245 cm^{-1} was particularly weakened.

Direct IR-spectroscopy of the vitreous body, cornea, lens, and sclera of the rabbit eye revealed a band at 3700-2800 cm^{-1} in their spectra, as in all the spectra examined above, with all the shoulders present on it, and Amide I and Amide II bands, of which the latter was more marked in the case of the sclera. Amide II was represented least strongly in the spectrum of the vitreous body. Absorbance of valency oscillations of $R-COO^-$ (1400-1370 cm^{-1}) was represented most clearly in the spectrum of the lens. The band at 1245 cm^{-1} and all other absorbances due to the sulfate group were found in spectra of the cornea, lens, and sclera, whereas in the spectrum of the vitreous body only a weak shoulder at 1245 cm^{-1} was observed. Of the two characteristic absorbances at 1150 and 1125 cm^{-1} in the spectrum of the cornea the former was represented by a band, whereas the latter overlapped with absorbance at 1080 cm^{-1}. Both bands were present in the spectrum of the sclera, with two corresponding shoulders in the spectrum of the lens. Amide V band was present in varied amplitude in spectra of the lens, vitreous body, and sclera, whereas in the case of the cornea, this band overlaps with other stronger bands.

Comparison of the IR-spectra of the tissues and also of lysozyme-proteoglycan complexes with IR-spectra of standard preparations of proteoglycans revealed, besides common absorbancies for all these biopolymers, others characteristic of individual proteoglycans or characteristic of their separate groups. In the investigations of hyaline cartilage by direct IR-spectroscopy, the adequacy of the group absorbances revealed was proved by IR-spectroscopic analysis of proteoglycans isolated preparatively from this tissue. Group characteristics of absorption of proteoglycans that are components of lysozyme-proteoglycan complexes, revealed by IR-spectroscopy, were confirmed by IR-spectra obtained beforehand for each separate proteoglycan.

It follows from the fact that the IR-spectrum of the vitreous body contains a weak shoulder at 1245 cm^{-1}, and that absorbance at 1150 cm^{-1} is present as a shoulder on the 1100-cm^{-1} band, that the vitreous contains HUA and a certain number of sulfated proteoglycans [11, 12, 14]. Absorbance at 1245 cm^{-1} and other bands due to oscillations of the sulfate group, and also at 1125 cm^{-1}, overlapped by a band at 1100 cm^{-1}, in the spectrum of the cornea proves that tissues of the cornea contain sulfated proteoglycans. Well-defined absorbances at 1245, 1150, and 1125 cm^{-1} (shoulder) in the IR-spectrum of the sclera are proof that the sclera contains proteoglycans of the AP type [13]. The presence of the same absorbances in the IR-spectrum of the lens is in good agreement with the discovery that it contains heparin-sulfate and chondroitin-4-6-sulfates [16].

In other investigations [5] of human articular cartilage and synovial membrane by direct IR-spectroscopy, comparative analysis of the spectrograms revealed AP in the former and HUA and heparin in the synovial membrane.

It should be pointed out that far fewer separate absorption bands due to proteoglycans could be distinguished in the IR-spectra of all tissues studied in the present investigation, and also in articular cartilage, synovial membrane, tissue structures, and lysozyme-proteolytic complexes in the 1400-900-cm^{-1} range than in the spectra of standard preparations of these biopolymers. There is every reason to accept that the factor causing these differences in the spectra is the protein bound to the proteoglycans electrovalently. In the IR-spectra of lysozyme–proteoglycan complexes, in which this protein was bound electrovalently by its own basic groups with the acidic groups of the proteoglycan the number of individual absorbances in the 1400-900 cm^{-1} range, just as in the tissue spectra, was significantly less than in spectra of standard proteoglycans. In tissues and tissue structures, the proteoglycans contained in them are joined by their acidic groups by an ionic bond with various bases, including proteins, and this seems to be the main cause of the decrease in the number of separate absorbances in the tissue IR-spectra within the 1400-900-cm^{-1} range. This conclusion is supported by the fact that the structure of this region of IR-spectra of acid and normal Na-, K-, Ca-, and Mg-salts of PCKS and AP depends to a large extent on each of these cations [2-4]. Probably one macromolecule of a proteoglycan (especially a sulfated one) in the tissues can bind with different bases through ionic interactions. This is confirmed by the fact that after removal of AP from cartilage with a 4 M solution of guanidine hydrochloride, the isolated macromolecules have free hydroxyl groups, i.e., they are acid salts [7].

Covalently bound protein components of the proteoglycan macromolecule evidently have no significant role in the reduction of the number of individual absorbances within the 1400-900-cm^{-1} region of the IR-spectra. Standard proteoglycans used in this study, while differing considerably in their content of covalently bound protein component in their macromolecules, have a considerable number of individual absorbances in their IR-spectra within the 1400-900-cm^{-1} range, but this number is reduced after electrovalent binding of these molecules with proteins.

Comparison of the group characteristics of absorbances in tissue IR-spectra with those in IR-spectra of standard proteoglycans can thus reveal the presence of particular members of these biopolymers in the tissues, can establish the position of hydrogen bonds in their macromolecules, and can shed light on the character of interactions of these macromolecules with tissue protein components.

REFERENCES

1. S. M. Bychkov, V. N. Bogatov, and S. A Kuz'mina, Byull. Éksp. Biol. Med., No. 11, 561 (1980).
2. S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 9, 301 (1981).
3. S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 12, 680 (1981).
4. S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No 11, 52 (1982).
5. S. M. Bychkov, M. M. Zakharova, S. A. Kuz'mina, et al., Byull. Éksp. Biol. Med., No. 11, 554 (1987).
6. S. M. Bychkov and S. A. Kuz'mina, Vopr. Med. Khim., No. 1, 19 (1986).
7. S. M. Bychkov and S. A Kuz'mina, Byull. Éksp. Biol. Med., No 6, 687 (1986).
8. S. M. Bychkov and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 5, 475 (1991).

9. S. M. Bychkov and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 12, 480 (1991).
10. A. Smith, Applied IR-Spectroscopy [Russian translation], Moscow (1982).
11. W. S. Allen, E. C. Otterbein, and A. H. Nordi, Biochim. Biophys. Acta, **198**, 167 (1977).
12. U. B. G. Laurent, Studies on Indigenous Sodium Hyaluronate in the Eye, Upsala (1982).
13. U. B. G. Laurent, Exp. Eye Res., **36**, 493 (1983).
14. U. B. G. Laurent, Acta Ophthalm. (Copenhagen), **63**, 302 (1983).
15. F. S. Parker, Infrared Spectroscopy in Biochemistry, Biology, and Medicine, New York (1971).
16. P. Speziate, A. Bardoni, M. E. Tira, et al., Ital. J. Biochem., **28**, 317 (1979).

ACTION OF METRAZOL ON REGULATION OF THE $GABA_A$ RECEPTOR-CHANNEL COMPLEX

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Most of the epileptogenic activity of metrazol (pentylenetetrazol, PTZ) is due to its ability to inhibit selectively the Cl^- channel of the $GABA_A$ receptor complex, blockade of which is known to be one of the principal mechanisms of neuronal hyperactivity [1]. However, this conclusion is mainly drawn from indirect data. In the only study in which a direct method of determination of activity of the GABA channel-receptor complex was used, namely determination of the inflow of $^{36}Cl^-$ into synaptoneurosomes, the action of PTZ was not investigated in detail and its kinetic parameters were not determined [2].

In the investigation described below the kinetic parameters of inhibition of the muscimol-dependent inflow of $^{36}Cl^-$ into synaptosomes by PTZ were determined, slowing of desensitization of the $GABA_A$ receptor complex under the influence of PTZ was demonstrated, and the effect of PTZ on dependence of the effect of muscimol on concentration was analyzed.

EXPERIMENTAL METHOD

Synaptoneurosomes were obtained by the technique of Hollingsworth [5] with certain modifications: in particular, instead of expressing the brain tissue homogenate through a teflon filter with pore diameter of 10 μ with a syringe in order to separate cells that were not disintegrated, the more sparing procedure of successive filtration of the preparation through a series of kapron gauze strainers with decreasing mesh size was used.

Noninbred male albino rats weighing 180-200 g were decapitated, the cerebral cortex was isolated, and it was homogenized manually (five frictions) at 0-4°C in a glass homogenizer with teflon pestle in Krebs–Ringer medium

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