INFRA-RED SPECTROSCOPIC STUDIES OF SOME POLYSACCHARIDES

by

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INTRODUCTION

The mucopolysaccharides have been classified[1] according as to whether they contain both hexosamine and hexuronic acid residues, only one or other of these sugar derivatives, or neither. The polysaccharides of the first class include hyaluronic acid and certain pneumococcal polysaccharides containing only acetic acid in addition to the hexosamine and hexuronic acid, a group of closely related sulphated polysaccharides with approximately one molecule of sulphuric acid per disaccharide unit, and the more highly sulphated anti-coagulant, heparin.

This investigation has been concerned with hyaluronic acid and certain of the monosulphate esters. Hyaluronic acid has previously been obtained from many sources with constant properties as regards chemical analysis, optical rotation and specificity towards enzymes; it has therefore been concluded that hyaluronic acid is a single substance and it is believed to be a long-chain polymer with N-acetylglucosamine and glucuronic acid residues alternating and joined to each other by 1:3 β links[2].

The sulphated polysaccharides are believed to be polymers of N-acetylhexosamine and glucuronic acid with one sulphate group per disaccharide unit. Those in which the hexosamine has been shown to be galactosamine are designated as chondroitin sulphuric acid and those with glucosamine as mucoitin sulphuric acid, but optical rotation data and enzyme experiments, as detailed below, show differences between samples even within this classification. Chondroitin sulphuric acid has been obtained from a variety of cartilaginous sources with very similar properties although it has been pointed out[3] that the analytical figures differ somewhat, and the optical rotations reported for neutral solutions vary from $-19°$ to $-32°$[4,5]. Chemical investigation of the structure of one of these samples, assumed to a single component, has shown the glucuronic acid residues to have a glycosidic link at position 4 and the sulphate to be on the N-acetylgalactosamine residue, although its position and that of the other glycosidic link could not be determined[6]. A material chemically identical with these samples has been obtained from skin[7]; this had, however, a rotation of $-55°$ and, unlike the materials from cartilage, was not hydrolysed by testicular hyaluronidase[8]. Similarly, two samples of mucoitin sulphuric acid have been reported, one from pig gastric mucosa[9] and one from cornea[10]; these had optical rotations in neutral solutions of $-36°$ and $-51°$ respectively, and could be further differentiated since only the latter was hydrolysed by pneumococcal hyaluronidase.

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The first problem was to characterise these polysaccharides in terms of structurally homogeneous components. The use of infrared spectroscopy in the characterisation of large organic molecules and various polymeric materials is well known and seemed particularly suitable for these polysaccharides. Firstly, small differences of stereochemistry give rise to appreciable spectral differences, as has already been shown for simple sugars[11]; secondly, it is possible to identify known components in a mixture and also to determine the number of components in a mixture, provided their relative amounts vary in different samples; thirdly, the position and intensity of certain infrared absorption bands is characteristic of specific molecular groupings and can be used to determine structure, both qualitatively and quantitatively.

Some preliminary results obtained on hyaluronic acid from umbilical cord and chondroitin sulphuric acid from cartilage have already been reported[12], in which the spectra were correlated with the presence of particular molecular groupings and shown to be consistent with their generally accepted structures. Similar studies have since been carried out by LEVINE, STEVENSON AND KABLER[13] on pneumococcal polysaccharides, in which a large number of serologically distinct types were shown to have different spectra with the exception of only one pair. The present results are an extension of those reported earlier on hyaluronic and chondroitin sulphuric acids, so that the identity or otherwise of various preparations from other sources can be established; a more detailed correlation between spectra and structure is also presented.

EXPERIMENTAL

Preparation of materials (by HARRIS, MALMGREN AND SYLVÉN, except where otherwise indicated). The preparation and chemical analyses of the hyaluronic acid from umbilical cord[14] and the Rous sarcoma[15] have already been described; the sample from the human myxoma was isolated in a similar way to that from the Rous sarcoma. The sample of chondroitin sulphuric acid from articular cartilage was prepared by the method of EINBINDER AND SCHUBERT[3] and further purified by ion exchange and electrodialysis. The three samples from nuclei pulposi were similarly isolated, except that alkali was used in the extraction of two of the samples[16]. The sample from trachea was originally prepared by Professor J. E. JORPES; part of this material was fractionated by an electrophoretic countercurrent technique (by courtesy of Dr. O. SNELLMAN) and the faster-moving component isolated.

The polysulphated hyaluronic acid was from Leo Pharmaceutical Laboratories, Inc., Hälsingborg, Sweden, and had a sulphur content equivalent to between 2 and 3 sulphate groups per disaccharide unit. The alginic acid was a highly purified specimen from A/S Protan, Drammen, Norway. The polysulphated alginic acid was from Wyeth, Inc., by courtesy of Dr. SCIFTER, Philadelphia, U.S.A.; its sulphur content was equivalent to 3 sulphate groups per disaccharide unit.

Amino group analysis. The free amino contents of the hyaluronic and chondroitin sulphuric acids were determined by the ninhydrin reaction, using the method of MOORE AND STEIN[17]. The spectrophotometric analysis was carried out on a Beckman DU spectrophotometer at 570 mμ in 1 cm cells. 10 mg samples were used, so that 0.05% free amino group per disaccharide unit could be detected.

Infrared data. Spectra were obtained on a Perkin-Elmer Model 12C spectrometer with NaCl prism. Samples were prepared for spectral examination by stripping off the thin film formed on a glass plate by casting from aqueous solution; neutral solutions were used to prepare the polysaccharides in their salt forms (carboxyl groups ionized) and dilute HCl solutions for the acid forms (free carboxylic acids). These films did not scatter the radiation and were measured against a rocksalt plate blank; the spectra were finally plotted as optical density against wavenumber, so that their shapes are independent of the film thickness.

In the quantitative measurements only relative values of the ratio of the intensities of the bands at 1736 and 1560 cm^{-1} are required, so measurement of quantities proportional to the true intensities is sufficient; graphical separation of the bands is then not necessary, provided the overlap from neighbouring bands can be allowed for. The following method of measurement was therefore used. On the optical density-frequency plot, the points at 1815 and 1490 cm^{-1} were joined by a line and the areas between this and the curve measured between 1815 and 1736 cm^{-1} and between 1560

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and 1490 cm^{-1}. As the bands are well separated, these two quantities should be proportional to the true intensities of the bands centred at 1736 and 1560 cm^{-1} respectively.

Optical rotation data. These were obtained on a Schmidt and Haensch three-prism polarimeter reading to 0.005°. A 10-cm cell was used and the solutions were about 2 g/100 ml; the standard error of each quoted value is about ± 1°.

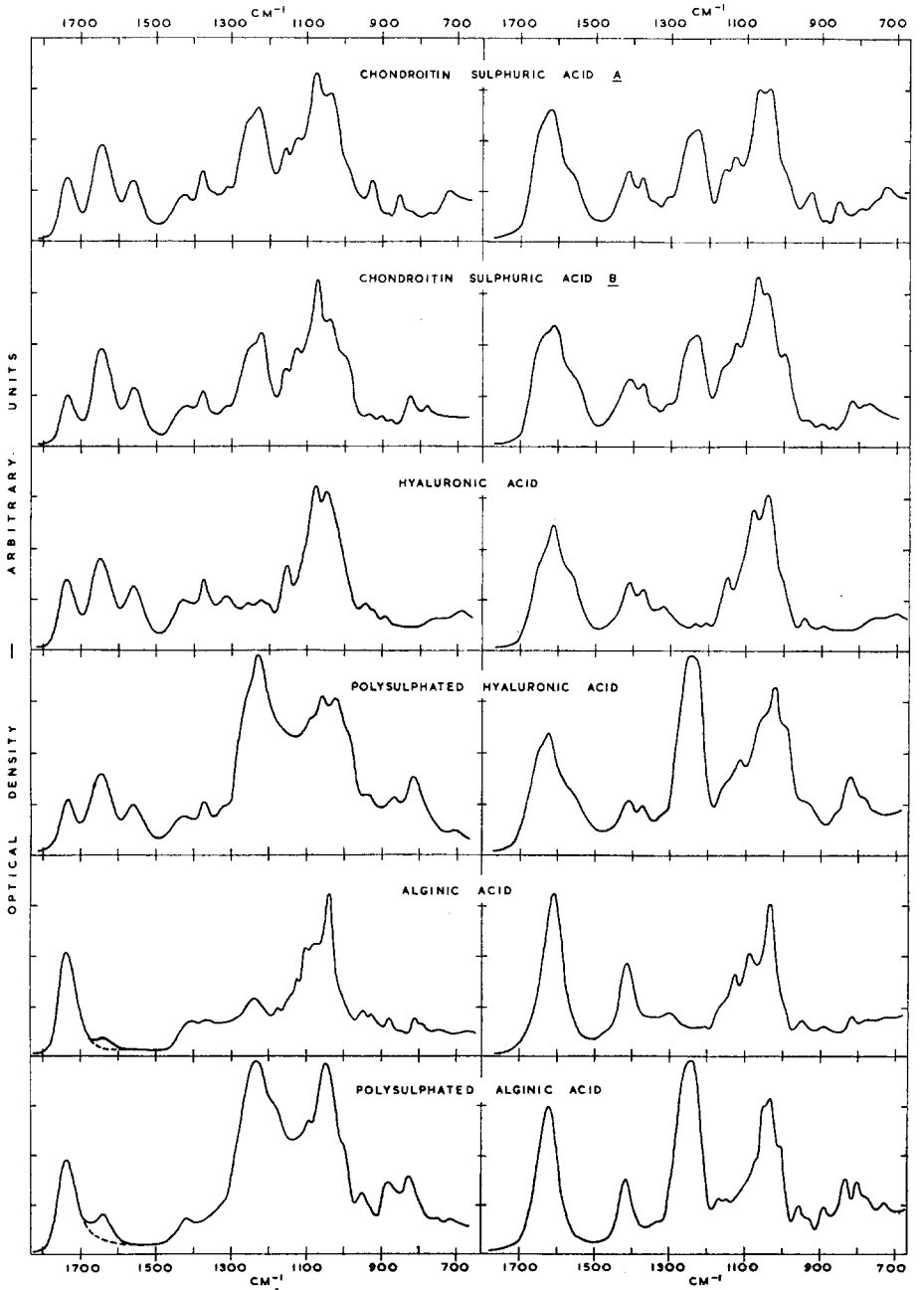

Fig. 1. The infrared spectra of the polysaccharides, on the left as free carboxylic acids, and on the right as salts.

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RESULTS

Qualitative studies. The spectra were first compared qualitatively for the presence or absence of bands not common to all samples, especially at frequencies below 1000 cm^{-1} where differences due to the molecular skeleton should become apparent.

Four samples of hyaluronic acid were measured: two of these were from umbilical cord, one from a Rous chicken sarcoma and one from a human myxoma. Except for some slight intensity variations, these four samples gave identical spectra, those in the figure deriving from one of the samples from umbilical cord.

Samples of chondroitin sulphuric acid from three different sources were originally examined. These were bovine trachea, articular cartilage of young calves, and bovine nuclei pulposi; the polysaccharide was obtained from this latter source in three fractions by a graded extraction procedure[16]. All samples gave very similar spectra from 1000 to 1800 cm^{-1}, but differences were found below 1000 cm^{-1}. In this region, the samples from trachea and articular cartilage were very similar to each other, but differed from the three samples from nuclei pulposi (themselves all very similar) by the presence of additional bands, which suggested the former samples to be mixtures. This was confirmed by the counter-current electrophoresis of the sample from trachea, for a faster-moving component was obtained, the spectrum of which, below 1000 cm^{-1}, only contained the original bands not present in the samples from nuclei pulposi. This spectrum is shown in the figure as chondroitin sulphuric acid A, while the spectrum of chondroitin sulphuric acid B is of the sample from nuclei pulposi obtained by the mildest extraction procedure. As the only amino-sugar indicated in all samples was galactosamine, these two materials must be regarded as isomers. In order to correlate these findings with those for previously reported samples, optical rotation figures were obtained in neutral solution (calculated in terms of free acid). The sample from trachea when fractionated had $[\alpha]_D$ — 26.3°, and that from nuclei pulposi — 15.5°. The unfractionated materials from trachea and articular cartilage had $[\alpha]_D$ — 19.1° and — 20.5° respectively. These results thus support the infrared findings that these unfractionated materials are mixtures of the two isomers.

As an aid to the study of the structure of these isomers, spectra were obtained from samples of polysulphated hyaluronic acid, alginic acid and polysulphated alginic acid. These spectra are also shown in the figure and will be discussed below.

Quantitative studies. Attention was then directed to the quantitative study of the spectra, for the intensities of those bands characteristic of specific molecular groupings should be proportional to the amount of the respective group present. The basis of group analysis has been well established, for example in the carbonyl groups of steroids[18], where band intensities (measured as band areas) have been shown to be constant for similarly placed groups in different molecules. This should apply in the case of the characteristic bands in these polysaccharides for their frequencies of absorption do not alter, so that the groups must be in the same environment in different samples. Unfortunately, these samples could not be measured in solution and the difficulty of measuring accurately the thickness of thin cast films precluded any possibility of absolute measurements, although application of the pressed potassium bromide technique[19,20] may overcome this difficulty.

Measurements of relative intensity were of course still possible and were carried out on the bands at 1736, 1648 and 1560 cm^{-1} appearing in films cast from acid solution.

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The presence of moisture in the films gave rise to absorption at 1640 cm^{-1}, and due to the difficulty of drying them completely, the intensity of the 1648 cm^{-1} band cannot be attributed with certainty to the polysaccharide alone. The effect of moisture may easily be seen in the spectrum of alginic acid and polysulphated alginic acid, where the full curve is the spectrum of the air-dried film; drying over phosphoric oxide did not readily remove all the moisture, and the dotted curve was obtained by casting from heavy water solution so that any residual moisture in the dried film would not absorb in this region. The bands at 1736 and 1560 cm^{-1} in the original polysaccharides are, however, only very slightly overlapped by this water band and their intensities should be proportional to the amounts of the groups absorbing at the two positions.

The band at 1736 cm^{-1} must be due entirely to the carboxylic acid group, for in the salt this band is replaced by one at lower frequencies due to the ionised carboxyl group and there is no residual absorption in any sample, such as would arise from O-acetyl groups if these were present. The monosubstituted amide group gives rise to the bands at 1648 and 1560 cm^{-1}, but any free amino groups present will also absorb near the latter position. Their presence could not be deduced readily and accurately from the spectra, but the weak colours produced in the ninhydrin reaction showed them to occur to only a small extent. The sample of hyaluronic acid from human myxoma did, however, give a colour equivalent to about 3% free amino groups per disaccharide unit, but all other samples had values, on the same basis, of about 0.5% or less. The band at 1560 cm^{-1} can therefore be attributed to N-acetyl groups alone, and the ratio of band intensities at 1736 and 1560 cm^{-1} proportional to the hexuronic acid to hexosamine ratio. The values of this intensity ratio, relative to that of hyaluronic acid from umbilical cord, are given in Table I and are discussed below. Each value in the Table represents the mean of at least three individual measurements; from the variation of these values, it is concluded that the average has a standard error of about $\pm$ 0.05.

TABLE I

Material	*Source*	*Ratio of intensities of the band at 1736 to that at 1560 cm^{-1}*
Hyaluronic acid	Umbilical cord	1.00 (standard)
	Rous chicken sarcoma	1.00
	Human myxoma	0.86
Chondroitin sulphuric acid	Fraction from trachea	1.00
	Whole bovine trachea	0.89
	Articular cartilage	0.93
	Nuclei pulposi I	0.71
	II	0.79
	III	0.73
Polysulphated hyaluronic acid	Synthetic	1.05

DISCUSSION

Hyaluronic acid. That the spectra of all hyaluronic acid samples are qualitatively the same suggests only one type of hyaluronic acid to exist. The spectra of the samples from umbilical cord and the Rous sarcoma are also quantitatively identical, but Table I

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shows the sample from the human myxoma to have a lower carboxyl absorption relative to the band at 1560 cm^{-1}. This sample is, however, the one found to contain appreciable amounts of free amino groups; these may have been produced during the extraction by hydrolysis of N-acetyl groups, or they may have arisen from unseparated protein. In either case, the intensity ratio would be affected, so that this quantitative difference may not be significant. Further work on the isolation and purification of this material is being carried out.

The spectrum of hyaluronic acid is entirely consistent with the formulation of this material as a polymer of glucuronic acid-N-acetylglucosamine disaccharide units. In addition to the main band at 1736 cm^{-1}, the carboxyl absorbs weakly around 1230 cm^{-1}, while in the ionised form, these bands are shifted to 1610 and 1410 cm^{-1}. The bands due to the monosubstituted amide at 1648 and 1560 cm^{-1} have already been mentioned, and the band at 1315 cm^{-1} may also be due to this group[21], while the sharp band at 1375 cm^{-1} must be due to the associated methyl group. The strong absorption at 1000–1200 cm^{-1}, characteristic of all sugar derivatives[11], must be due to the C-O stretching modes and C–O–H bending modes. Below 1000 cm^{-1} the absorption is relatively weak but should be characteristic of the particular way in which the molecule is linked together, as the C–O–C bending modes should be appearing. Certain correlations have been noted here for unsubstituted and methyl substituted glucose saccharides[22], but no assignments are possible in the present case.

Chondroitin sulphuric acid. The characterisation of two isomers of this acid with $[\alpha]_D$ values of — 26.3° and — 15.5° suggests that many samples previously reported[5,6] may have been mixtures of these two components. There is still the possibility of yet another isomer existing to account for the high $[\alpha]_D$ value of — 55° reported for a sample from skin[7].

Although the main difference between the spectra of the two isomers occurs in the region characteristic of the molecular skeleton, a difference in the relative intensities of the bands at 1736 and 1560 cm^{-1} has been noted (Table I). The variations between the three fractions from nuclei pulposi are not significant and probably reflect the accuracy of the measurements, while the samples which are mixtures of the A and B isomers have values between those of the separated isomers. These considerations suggest that the differences have not been produced by degradation during isolation but are due to real differences in molecular composition of the two isomers. Furthermore, the constancy of the ratio for hyaluronic acid even on polysulphation supports the conclusion that the ratio is unaffected by differences in the rest of the molecule. As the free amino group content of each isomer has been found to be less than 0.5%, these groups can be neglected and the results therefore indicate about 25% less hexuronic acid, relative to hexosamine, in the B compared to the A isomer.

The assignments of the bands of the two isomers are similar to those of hyaluronic acid. Above 1000 cm^{-1} the only qualitative difference is the presence in the former of an intense band at about 1240 cm^{-1}; this is undoubtedly due to the sulphate group, being correspondingly more intense in the two polysulphated polysaccharides and at the same position as the band assigned to sulphate in simple sodium alkyl sulphates[23]. This band must be due to S = O stretching vibrations analogous to the 1735 cm^{-1} C = O stretching vibration in acetates. Now the acetates have a band at 1240 cm^{-1}, attributed to the C–O–C system[24], and these sulphates would be expected to show absorption due to vibrational modes involving stretching within the C–O–S system at

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correspondingly lower frequencies. Comparison of the spectra of polysulphated hyaluronic acid itself leads to the unequivocal assignment of the COS vibration to the band appearing at about 820 cm^{-1} in both the free acid and the salt of the former. The polysulphated alginic acid also absorbs much more strongly than alginic acid in this region, but the extra absorption seems to be present in at least two bands, at 802 and 834 cm^{-1} in the salt and at 824 and 880 cm^{-1} in the free acid.

The analogous acetate band at 1240 cm^{-1} has been studied in 3-acetoxysteroids[25] and differences found which have been correlated with stereochemical configuration. In cases where the acetate is bonded equatorially to the cyclohexane ring, a single band is observed between 1236 and 1242 cm^{-1}, and where it is in a polar position, three bands are observed between 1210 and 1260 cm^{-1} with the same total intensity. Similarly, in 3-hydroxysteroids the band around 1030 cm^{-1}, corresponding to the C–O stretching mode, although always single, is at a higher frequency when the hydroxyl group is equatorial than when it is polar[26]. The pyranose sugar ring is stereochemically very similar to the cyclohexane ring; attached groups will, therefore, have either a polar or equatorial position, and differences in the COS band in the spectra of hexose sulphates can be expected.

If the structure of hyaluronic acid is examined on the accepted basis of its being a 1:3 β linked polymer[2], it will be seen that sulphation at any position will give rise to an equatorial sulphate group (although at the C_6 position it is the C_5–C_6 and not the C–O bond which is equatorial). If the links are 1:4 β, the only other arrangement suggested, an extended and a contracted form are possible, but in each case all the sulphate groups will be in the same position, either equatorial or polar respectively. Sulphation of alginic acid, which is a 1:4 β linked polymannuronic acid, can give rise to either type of sulphate, for of the four free hydroxyl groups in each disaccharide unit, two are in the polar and two in the equatorial position, although the actual configuration will differ for the sodium salt, which is in the extended form, and for the free acid, which is in the contracted form[27]. The difference in the assignment of the COS vibration in the two polysulphates is thus explicable. In polysulphated hyaluronic acid only a single band, that at 820 cm^{-1}, appears as all the sulphate groups are in the same position, almost certainly the equatorial one, while in polysulphated alginic acid the assignment is less certain because both types of sulphate linking can occur.

In the chondroitin sulphuric acids, the absorption due to the COS vibration should still be the most intense in the 700–1000 cm^{-1} region, and as the spectral differences between the isomers is in this region, their structural differences must involve the sulphate group. Now the B isomer has a band at 825 cm^{-1}, similar to that in polysulphated hyaluronic acid, while the only other band of comparable intensity in this region is at 775 cm^{-1}. The A isomer has neither of these bands, but has three bands of comparable intensity at 928, 855 and 725 cm^{-1}. It is, therefore, concluded that the sulphate group in the B isomer has the same configuration with respect to the sugar ring as those in polysulphated hyaluronic acid, that is an equatorial one, while that in the A isomer must be in the other, polar configuration. This difference could arise from differences in glycosidic linkage of identical units, from a different position of the sulphate group alone, or from a combination of these causes. Insufficient structural evidence is available from other sources to decide between these possiblilities, although the fact that the optical rotations of the two isomers are so similar may indicate that the isomerism involves the position of the sulphate group alone.

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Although the structural differences cannot be exactly determined, it has been shown by their infrared spectra that two isomeric chondroitin sulphuric acids exist, that they occur together in trachea and cartilage, and that because of their similar optical rotation this previously used method of characterisation is not sufficiently sensitive to demonstrate the homogeneity of any sample.

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SUMMARY

Samples of hyaluronic acid from umbilical cord, Rous chicken sarcoma and a human myxoma have given infrared absorption spectra which are almost identical, so that it is concluded that only one type of hyaluronic acid exists in these materials. A small quantitative difference has been found, but evidence is presented which suggests that this is due to impurity in the sample from the human myxoma.

Two isomers of chondroitin sulphuric acid, designated A and B, have been characterised. The B isomer, $[\alpha]_D$ —15.5°, has been found alone in samples from bovine nuclei pulposi, but both isomers have been found together in samples from bovine articular cartilage and trachea. From the latter, the A isomer, $[\alpha]_D$ —26.3°, has been obtained by counter-current electrophoresis. The main differences in the spectra are below 1000 cm^{-1} and it is suggested that these arise from a difference in molecular structure which places the sulphate group in an equatorial position with respect to the sugar ring in one isomer and in a polar position in the other. A significant difference in the relative intensities of the bands at 1736 and 1560 cm^{-1} has also been noted, the A isomer having the same ratio as hyaluronic acid, while the 1736 cm^{-1} band is 25% relatively less intense in the B isomer. These two bands are due to carboxylic acid and monosubstituted amide groups respectively, and the relation between their relative intensity and the hexuronic acid to hexosamine ratio is discussed.

RÉSUMÉ

Des échantillons d'acide hyaluronique du cordon ombilical, du sarcome de Rous du poulet et du myxome humain possèdent des spectres d'absorption infra-rouge pratiquement identiques. Ces trois sources fourniraient donc un seul type d'acide hyaluronique. Il existe une faible différence quantitative, mais il semble qu'elle soit due à une impureté présente dans l'échantillon provenant de myxome humain.

L'acide chondroïtine sulfurique se présente sous deux formes isomères, A et B. L'isomère B, $[\alpha]_D$ = 15.5°, a été trouvé seul dans des échantillons de nuclei pulposi bovin, mais les deux isomères existent côte à côte dans des échantillons de cartilage articulaire et de trachée de boeuf. L'isomère A, $[\alpha]_D$ = —26.3°, a été isolé de la trachée par électrophorèse à contre-courant. Les principales différences spectrales se manifestent au-dessous de 1000 cm^{-1} et elles seraient dues à une différence de structure moléculaire, le groupe sulfate se trouvant en position équatoriale par rapport au cycle glucidique dans l'un des isomère et en position polaire dans l'autre. Une différence significative dans les intensités relatives des bandes situées à 1736 et 1560 cm^{-1} est également à notor, l'isomère A ayant le même rapport que l'acide hyaluronique tandis que la bande 1736 cm^{-1} est de 25% moins intense dans l'isomère B. Ces deux bandes sont respectivement dues à des groupes carboxyliques et amides monosubstitués, et la relation entre leur intensité relative et le rapport acide hexuronique/hexosamine est discutée.

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ZUSAMMENFASSUNG

Proben von Hyaluronsäure aus dem Nabelstrang, Rous-Hühner-Sarcom und einem menschlichen Myxom geben beinahe identische Infrarotabsorptionsspektren, sodass gefolgert wurde, dass nur ein Typ Hyaluronsäure in diesen Materialien vorkommt. Ein kleiner quantitativer Unterschied wurde aufgefunden, es wurde aber augenscheinlich gemacht, dass er vermutlich einer Verunreinigung der Probe aus menschlichem Myxom zuzuschreiben war.

Zwei Isomere von Chondroitinschwefelsäure, die mit A und B bezeichnet wurden, wurden charakterisiert. Das B-Isomer $[\alpha]_D$ $-15.5°$ wurde allein in den Proben von Rindernucleipulposi angetroffen, beide Isomeren wurden aber zusammen gefunden in Proben von Rindergelenkknorpeln und -luftröhren. Aus den letzteren wurde das A-Isomer $[\alpha]_D$ $-26.3°$ durch Gegenstromelektrophorese erhalten. Die Hauptunterschiede in den Spektren sind unterhalb 1000 cm^{-1} und es wird vermutet, dass diese von einem Unterschied in der Molekülstruktur herrühren, bei der die Schwefelsäuregruppen sich in einer äquatorialen Stellung bezüglich des Zuckerrings in dem einen Isomer und in einer polaren Stellung im anderen Isomer befinden. Ein bedeutungsvoller Unterschied in der relativen Intensität der Bande bei 1736 und 1560 cm^{-1} wurde ebenfalls festgestellt; das A-Isomer hat das gleiche Verhältnis wie Hyaluronsäure, während beim B-Isomer die 1736 cm^{-1} Bande um relativ 25% weniger intensiv ist. Diese beiden Bande sind Carboxylsäure- bezw. monosubstituierten Amidosäuregruppen zuzuschreiben und das Verhältnis ihrer relativen Intensität zu dem Hexuronsäure-Hexosaminverhältnis wird besprochen.

REFERENCES

1 M. STACEY, *Advances in Carbohydrate Chem.*, 2 (1946) 161.
2 B. WEISSMANN AND K. MEYER, *J. Am. Chem. Soc.*, 74 (1952) 4729.
3 J. EINBINDER AND M. SCHUBERT, *J. Biol. Chem.*, 185 (1950) 725.
4 H. G. BRAY, M. STACEY AND J. E. GREGORY, *Biochem. J.*, 38 (1944) 142.
5 K. MEYER AND E. M. SMYTH, *J. Biol. Chem.*, 119 (1937) 507.
6 M. L. WOLFROM, R. K. MADISON AND M. J. CRON, *J. Am. Chem. Soc.*, 74 (1952) 1491.
7 K. MEYER AND E. CHAFFEE, *J. Biol. Chem.*, 138 (1941) 491.
8 K. MEYER AND M. M. RAPPORT, *Arch. Biochem.*, 27 (1950) 287.
9 K. MEYER, E. M. SMYTH AND J. W. PALMER, *J. Biol. Chem.*, 119 (1937) 73.
10 K. MEYER AND E. CHAFFEE, *Am. J. Opthalmol.*, 23 (1940) 1320.
11 L. P. KUHN, *Anal. Chem.*, 22 (1950) 276.
12 S. F. D. ORR, R. J. C. HARRIS AND B. SYLVÉN, *Nature*, 169 (1952) 544.
13 S. LEVINE, H. J. R. STEVENSON AND P. W. KABLER, *Arch. Biochem. Biophys.*, 45 (1953) 65.
14 B. SYLVÉN AND H. MALMGREN, *Lab. Investigation*, 1 (1952) 413.
15 R. J. C. HARRIS, H. MALMGREN AND B. SYLVÉN, *Brit. J. Cancer*, (in press).
16 H. MALMGREN AND B. SYLVÉN, *Biochim. Biophys. Acta*, 9 (1952) 706.
17 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
18 R. N. JONES, D. A. RAMSAY, D. S. KEIR AND K. DOBRINER, *J. Am. Chem. Soc.*, 74 (1952) 80.
19 M. M. STIMSON, *J. Am. Chem. Soc.*, 74 (1952) 1805.
20 U. SCHIEDT, *Z. Naturforsch.*, 7b (1952) 270.
21 R. D. B. FRAZER AND W. C. PRICE, *Nature*, 170 (1952) 490.
22 S. A. BARKER, E. J. BOURNE, M. STACEY AND D. H. WHIFFEN, *Chemistry and Industry*, (1953) 196.
23 I. M. KLOTZ AND D. M. GREEN, *J. Phys. and Colloid Chem.*, 52 (1948) 961.
24 H. W. THOMPSON AND P. TORKINGTON, *J. Chem. Soc.*, (1945) 640.
25 R. N. JONES, P. HUMPHRIES, F. HERLING AND K. DOBRINER, *J. Am. Chem. Soc.*, 73 (1951) 3215.
26 A. R. H. COLE, R. N. JONES AND K. DOBRINER, *J. Am. Chem. Soc.*, 74 (1952) 5571.
27 K. J. PALMER AND M. B. HARTZOG, *J. Am. Chem. Soc.*, 67 (1945) 1865.

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