DEGRADATION OF

CHONDROITIN SULFATE A

BY

BEE VENOM HYALURONIDASE

BY

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DEGRADATION OF CHONDROITIN SULFATE A BY BEE VENOM HYALURONIDASE

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ABSTRACT

The action of bee venom hyaluronidase on chondroitin sulfate A was investigated. After purification, the mucopolysaccharide was degraded by the enzyme into mainly the hexasaccharide GAl $\xrightarrow{B}$ 3 Gal NAc (SO_3H)1 $\xrightarrow{B}$ 4 GAl $\xrightarrow{B}$ 3 Gal NAc (SO_3H)1 $\xrightarrow{B}$ 4 GAl $\xrightarrow{B}$ 3 Gal NAc (SO_3H) having $[\alpha]_D - 4.2^0$. Two other minor oligosaccharide components could be detected, and on the basis of their relative mobilities were designated as tetra - and octa-saccharides. The hexasaccharide product did not exhibit any unsaturation and had N-acetyl galactosamine content of 29%. The enzyme also hydrolysed chondroitin sulfate C and heparin, and its activity was free of desulfation.

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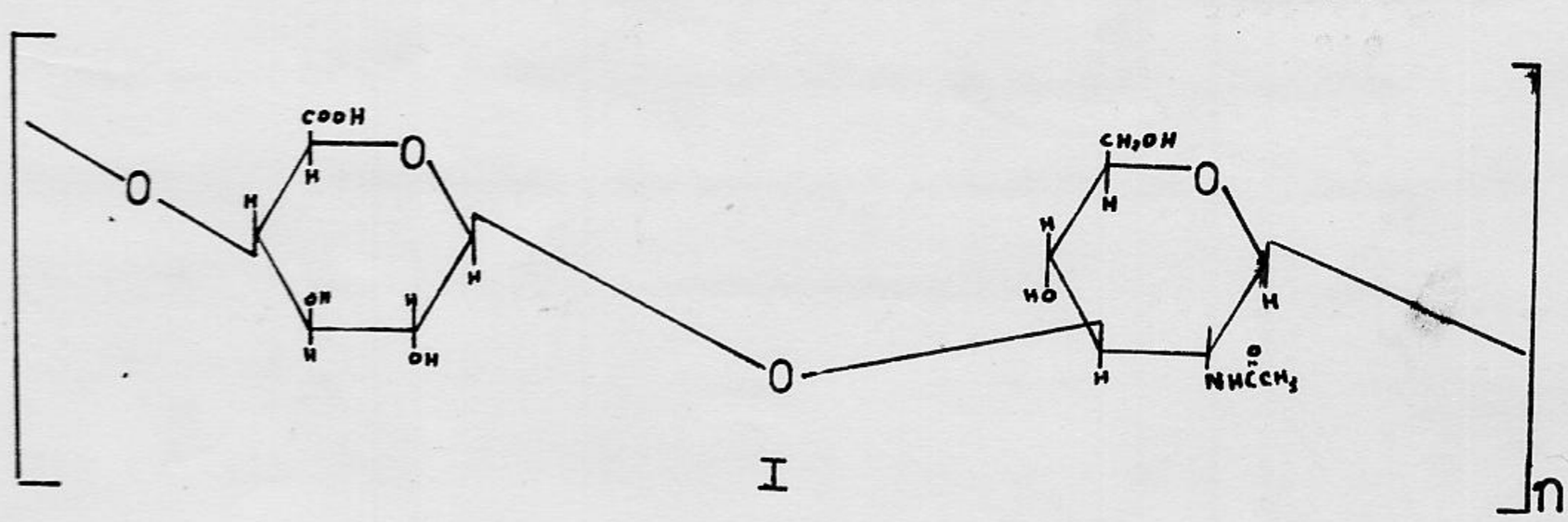
INTRODUCTION

Among the most widely studied mucopolysaccharides are hyaluronic acid, the chondroitin sulfates and heparin. They are of wide distribution in the animal tissues, and because of the important role they seem to play, their chemistry has recently excited much interest. These mucopolysaccharides exhibit structural similarity, and in some cases the difference lies only in the fine structure.

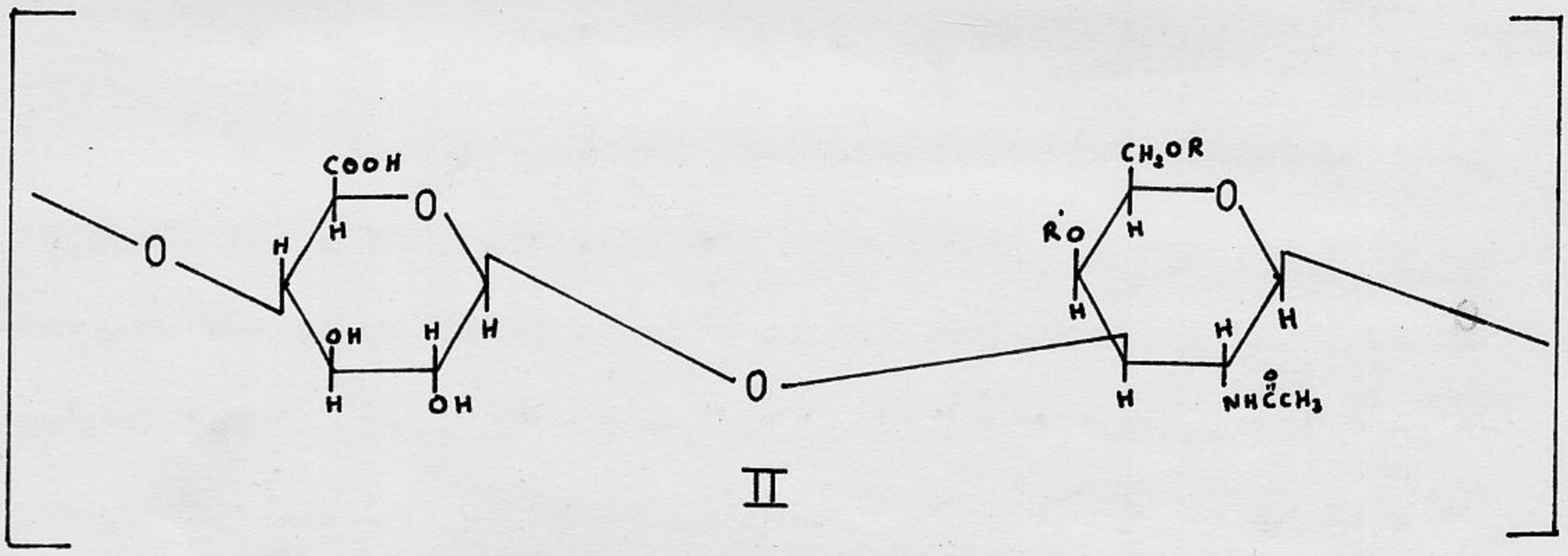
Structure

Hyaluronic acid, $[\alpha]_D$ - 68.8 to - 78.2°, is a non-sulfated polysaccharide consisting of equimolar proportions of N-acetyl-D-glucosamine and D-glucuronic acid residues. In its native state it is often found associated with other polysaccharides and almost invariably in the form of hyaluronate-protein complex. It is an unbranched polymer in which the monosaccharide residues alternate in β-(1 ⟶ 3) glucuronidic[1] and β-(1 ⟶ 4) N-acetylglucosaminidic linkages[2,3]. The repeating structural unit is the disaccharide N-acetyl hyalobiuronic acid (I).



I

Three different chondroitin sulfates have been distinguished and designated A, B and C. Because of the striking similarity, the structures of chondroitin sulfates A, $[\alpha]_D$ -28 to -32°, and C, $[\alpha]_D$ -16 to -22°, will be presented together. Each is an unbranched polymer consisting of D-glucuronic acid, D-galactosamine, acetyl and sulfate residues in equimolar amounts. The repeating structural unit (II) in chondroitin sulfates A and C has the structure:



II

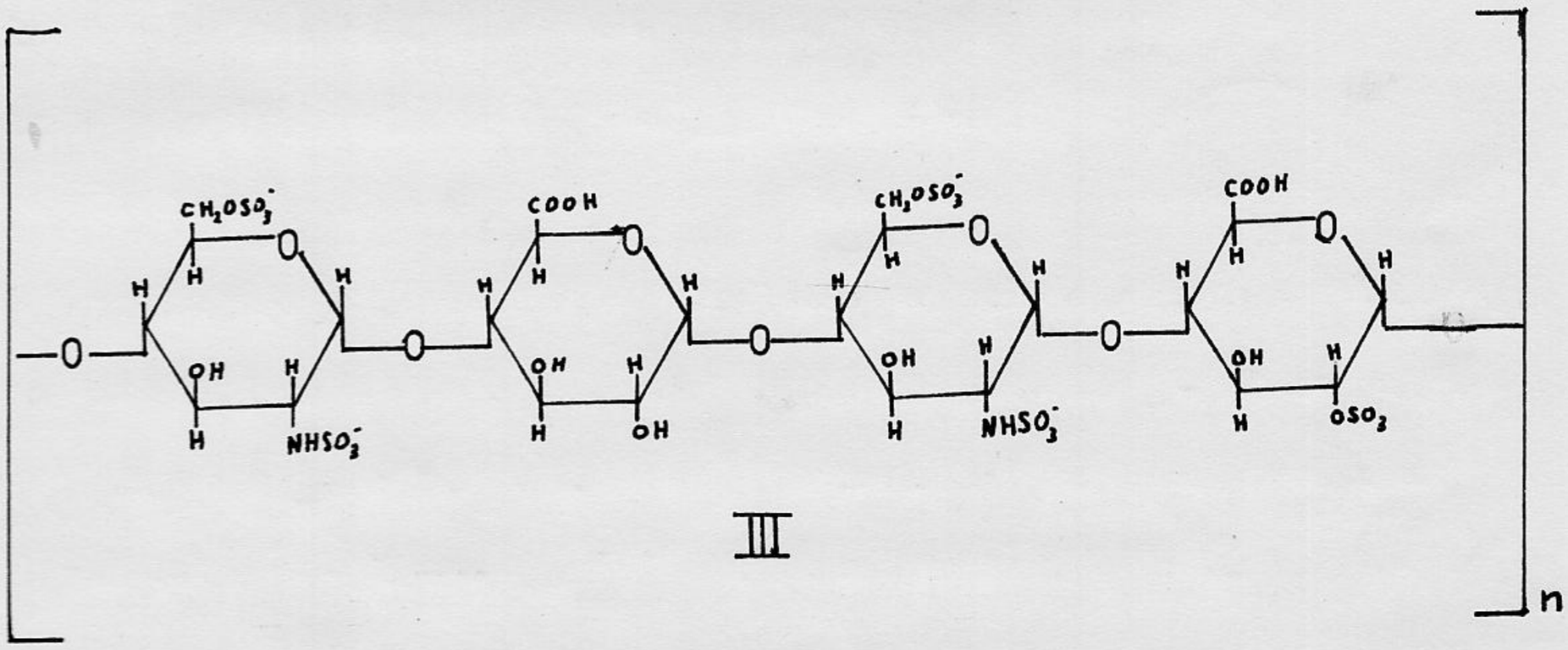
in chondroitin sulfate A R = H and R' = SO_3H

in chondroitin sulfate C R = SO_3H and R' = H

In the polysaccharides the monosaccharide residues alternate, and the infrared studies of Orr[4] indicate that the D-galactosamine moieties are N-acetylated. The glucuronidic linkages have been shown to be β-(1 ⟶ 3)[5,6,7] and the hexosaminidic linkages β-(1 ⟶ 4)[8,9]. Enzymatic studies by Hoffman, Meyer and Linker[10] proved that the sulfate groups were attached to the galactosamine moieties only. The two mucopolysaccharides differed only in the position of the sulfate groups[4,11,12]; in chondroitin sulfate A the sulfate group was allocated to the C - 4 position of the hexosamine moiety and in chondroitin sulfate C to the C - 6 position.

Chondroitin sulfate B has $[\alpha]_D$ - 55 to -63° and its structure differs from that of chondroitin sulfate A only in having L-iduronic acid instead of D-glucuronic.

Heparin, $[\alpha]_D$ +48°, is an unbranched, highly sulfated mucopolysaccharide consisting of D-glucosamine[13,14,15] and D-glucuronic acid[14,16] as monosaccharide residues. It is characterized by having N-sulfated[17,18] but not N-acetylated glucosamine moieties[19,20]. On the basis of available evidence[21-30], the probable structure of the basic unit is represented by (III) in which all glycosidic linkages have the α-(1 → 4) - configuration. However the possibility of some of the glucuronidic linkages in the polysaccharide having a (1 → 6) - structure and/or the β-configuration cannot yet be excluded.



III

Enzymatic Degradation of Mucopolysaccharides

Many enzyme preparations having mucopolysaccharase activity have been reported in literature[31]. The action of many of these enzymes on hyaluronic acid has been widely studied, but information about their mode of action on sulfated mucopolysaccharides is scanty. Table I shows the substrate specificities of those

mucopolysaccharases whose mode of action has been, at least, investigated in part.

1. Testicular hyaluronidase. Hahn[32] showed that purified testicular hyaluronidase hydrolysed hyaluronic acid yielding a complex mixture of oligosaccharides. Later, Weissman et. al.[33] analysed the oligosaccharide mixture present in the 24 hour hydrolysate of the purified enzyme, and found it to consist of a series ranging from di-to tetradeca-saccharide. Each oligosaccharide differing from the succeeding one ~~below~~ by the disaccharide unit-N-acetylhyalobiuronic acid. During the hydrolytic process, only the $C_1 - O$ bond in the glucosaminidic linkages was attacked[34]. As the hydrolysis time was prolonged, the products underwent a progressive shift towards the lower members of the series, until finally the exhaustive hydrolysate obtained with relatively large amounts of the enzyme consisted predominantly (90%) of the tetrasaccharide together with small amounts of N-acetylhyalobiuronic acid[35]. By using isolated oligosaccharides as substrates, it was shown that testicular hyaluronidase mainly catalysed transglycosyl reactions, and during transglycosylation, only one type of hexose-aminidic linkage similar to that present in hyluronic acid was formed. Furthermore, the rate of reaction was found to increase markedly with increasing chain length of the substrate. The production of low molecular weight oligosaccharides however is due to a relatively slow hydrolytic activity. Since testicular hyaluronidase preferentially attacks a glucosaminidic bond which is at least two disaccharide units from either end of the chain,

Table I. Substrate Specificity and Mechanism of Action of Hyaluronidases.

Mucopolysaccharase from	Mode of action	Substrate			
		C.S. A & C.	C.S. B.	Heparin	Hyaluronic acid
Testes	I	+	-	-	+
Snake Venom					+
Proteus Vulgaris	I	+	-	-	+
Pneumococci	II	-	-	-	+
Streptococci	II	-	-	-	+
Staphylococci	II	-	-	-	+
Clostridium Welchii	II	-	-	-	+
Flavobacterium Heparinum					
(i) Unadapted	II	+	-	-	+
(ii) Adapted to heparin	II	+	-	+	+
(iii) Adapted to C.S. B.	II	+	+	-	+
Leech	III				+

I : Attacks Hexosaminidic Linkage .

II : Attacks Hexosaminidic Linkage and Eliminates H_2O .

III : Attacks Hexuronidic Linkage .

it has been classified as an endohexosaminidase.

Testicular hyaluronidase hydrolyses chondroitin and desulfated chondroitin sulfates A and C, giving a mixture of oligosaccharides which have mobilities on paper similar to those obtained from hyaluronic acid[36]. It also hydrolysed chondroitin sulfates A and C, and the major end product afforded by exhaustive treatment consists of sulfated tetrasaccharide[10]. From the production of mixed oligosaccharides by the transglycosyl action on mixtures of hyaluronic acid and chondroitin sulfates A and C, it was shown that the enzyme acts on both polysaccharides by the same mechanism.

Testicular hyaluronidase does not, however, act on chondroitin sulfate B.

2. Snake Venom hyaluronidase. It was found that the hyaluronidase of snake venom hydrolysed hyaluronic acid to a mixture of oligosaccharides identical with those produced by the action of testicular hyaluronidase[2]. It also acted on chondroitin.

3. Hyaluronidase from Proteus vulgaris. A partially purified hyaluronidase preparation from P. vulgaris was shown to hydrolyse hyaluronic acid into a mixture of oligosaccharides that were identical with those obtained by the action of the testicular enzyme[37]. In its action on chondroitin sulfate A, however, the Proteus enzyme differed from testicular hyaluronidase by degrading this substrate about six times as fast as hyaluronic acid, and secondly only one end product, indentified as N-acetylchondrosin sulfate (disaccharide) was formed by exhaustive digestion.[38,39]

Heparin was not acted upon by Proteus enzyme.

A chondrosulphatase was also isolated from extracts of P. valgaris. While this enzyme did not attack the polysaccharide itself, it desulphated N-acetylchondrosin sulfate and other higher sulfated oligosaccharides[40].

4. Bacterial hyaluronidases. On account of the great similarity in the mode of action of the pneumococcal, staphylococcal, streptococcal and clostridium welchii enzymes, they were collectively termed "bacterial hyaluronidases". By exhaustive hydrolysis with the bacterial hyaluronidase, hyaluronic acid was quantitatively converted into a single end product which was identified as 3-O-(β-D-4:5-glucoseenpyranosyl uronicacid)-2-deoxy-2-acetamido-D-glucose. Partial hydrolysis of hyaluronic acid with the bacterial enzyme afforded a series of oligosaccharides which differed from those obtained with testicular hyaluronidase by having a 4:5-unsaturated uronicacid residue at the terminal nonreducing end[2]. The mechanism of the enzyme action seems to involve cleavage of the glucosaminidic bond by an elimination reaction, thus introducing double bond in the liberated molecules.

The bacterial hyaluronidase also degraded chondroitin and desulfated chondroitin sulfates A and C by the same type of mechanism[36]. In contrast with testicular hyaluronidase, the bacterial enzyme does not attack chondroitin sulfates A and C, and it also has no action on chondroitin sulfate B and heparin.

5. Hyaluronidase of Flavobacterium heparinum. The hexose-aminidase obtained from extracts of F. heparinum differed from the bacterial hyaluronidase in that, in addition to hyaluronic

acid, it also hydrolyzed chondroitin sulfates A and C with the formati-on of unsaturated sulfated oligosaccharides as intermediates[41]. The enzyme preparation had no action on heparin or chondroitin sulfate B. F. heparinum could be adapted to act on heparin[42], and later, Hoffman, Linker and Meyer[41], succeeded in adapting the organism to hydrolyse chondroitin sulfate B yielding an unsaturated sulfated oligosaccharide mixture.

6. Leech hyoluronidase. Extracts of leech were shown to contain a hyaluronidase which degraded hyaluronic acid into a mixture of oligosaccharides having glucuronic acid at the reducing end[43]. The end product obtained by exhaustive digestion mainly consisted of a tetrasaccharide[44]. The enzyme exhibited no transglycosylase activity, and was shown to be a β-endoglucuronidase.

The hyaluronidase of bee venom[45] has been shown to be a β-endohexosaminidase which hydrolysed hyaluronic acid into mainly tetrasaccharide, $[\alpha]_D$ - 52.2°, and hexasaccharide, $[\alpha]_D$ - 66.1°. The oligosaccharides were of the saturated type, had N-acetylglucosamine at the reducing end, and were composed of equimolar proportions of N-acetyl-glucosamine and glucuronic acid. The action of the enzyme is free of elimination and transglycosylase activity.

Purpose of the Work

The degradation of hyaluronic acid by bee venom hyaluronidase was studied. In this work, the degradation of the sulfated mucopolysaccharide, chondroitin sulfate A, was investigated. Since the hydrolase action of the enzyme is free of the undesirable side

reactions (elimination and transglycosylation), the enzyme may prove useful in structural determination of mucopolysaccharides.

DISCUSSION

In the structural investigation of mucopolysaccharides, considerable difficulties are encountered in their acid hydrolysis to small oligosaccharide units for the purpose of methylation and periodate oxidation studies. Enzymatic degradation of polysaccharides has been found to be more effective, and in general has the following advantages:

1. Due to the enzyme specificity, attack is directed to one glucosidic linkage, and is not random.

2. The size specificity of the enzyme allows the isolation of the oligosaccharide(s) in high yield, and thus the need for large amounts of the polysaccharide is avoided.

3. The resultant oligosaccharide mixture is less complex and hence could be resolved more easily.

4. Acid hydrolysis often results in the simultaneous hydrolysis of substituent groups (such as acetyl and sulfate), and this raises a new problem of assigning their origional sites.

Bee venom contains a hyaluronidase[52], which has been found to hydrolyse hyaluronic acid into mainly a tetrasaccharide and a hexasaccharide[45]. This enzyme is a β-endohexosaminidase, and on account of the fact that its hydrolase activity is free of objectionable side reactions (transglycosylation, elimination and desulfation), its application in structural studies seems to be promising. In this study, the action of the enzyme on the sulfated mucopolysaccharide chondroitin sulfate A was investigated. Chondroitin sulfate A was chosen as the substrate

because of its structural similarity to hyaluronic acid. It differs from the latter only in having N-acetyl-D-galactosamine carrying $-OSO_3H$ group at C_4 instead of the N-acetyl-D-glucosamine moiety.

Preliminary Examination of the Hydrolysate Obtained with Commercial Chondroitin Sulfate.

To test the hyaluronidase activity of bee venom, commercial chondroitin sulfate isolated from bovine trachea was incubated for 72 hours with bee venom in 0.15 M - NaCl/phosphate-citrate buffer (pH 4.7). The hydrolysate was examined by paper chromatography using the developer 1-butanol/acetic acid/water (44/16/40 v/v, single phase system). This developer was found to give the most satisfactory resolution of acidic oligosaccharides[33]. Developers with less water were found to give lower mobilities while those containing more water resulted in streaking. The pattern of the oligosaccharide products obtained is illustrated in Fig. 8 , and are compared with the hydrolysate of testicular hyaluronidase. The oligosaccharides obtained with bee venom hyaluronidase were designated I, II and III and had relative R glucuronolactone values of 0.33, 0.22 and 0.12 respectively. From the relative intensity of the spots, it was observed that oligosaccharide II was the major component. While oligosaccharide III was present in trace amounts. It was also significant to observe that all three oligosaccharides had similar mobilities to corresponding spots obtained with the testicular hyaluronidase digest.

Isolation and Purification of Chondroitin Sulfate A.

Since the hyaluronidase of bee venom exhibited hydrolase

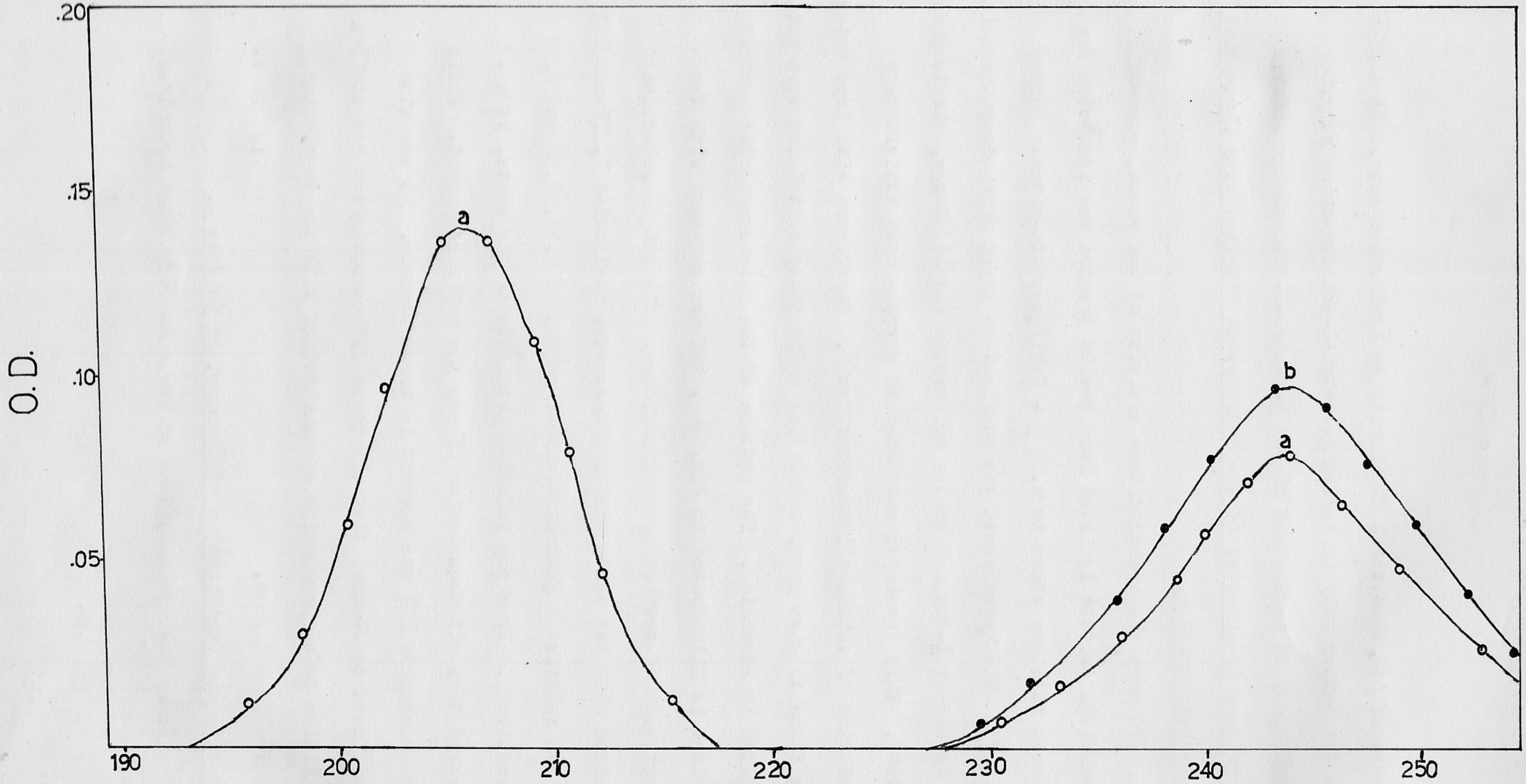


Fig. 1 Characterization of the aminosugar component of commercial chondroitin sulfate (from bovine trachea).
(a) Calibration of column with glucosamine-galactosamine mixture
(b) Elution profile of polysaccharide hydrolyzate.

activity on the commercial chondroitin sulfate preparation, it was decided to investigate its action on a pure chondroitin sulfate A preparation.

Usually chondroitin sulfate A occurs in tissues in admixture with other hexosamine and uronic acid-containing polysaccharides. Hence it was decided to examine the purity of the commercial preparation by characterization of the hexosamine present. A column of Dowex 50 WX8 (H^+ form) was first calibrated with authentic D-glucosamine and D-galactosamine hydrochlorides, and then the acid hydrolysate of the commercial chondroitin sulfate was chromatographed on the column. Gardell[47] showed that D-glucosamine and D-galactosamine could be separated by cation exchange chromatography on a resin column. Later[53], it was shown that the volume of eluant required to elute a particular aminosugar was constant under certain conditions. The results obtained (Fig. 1) by eluting the Dowex 50 W (H^+ form) column with 0.3 N-HCl indicate that D-galactosamine was the only hexosamine present. The polysaccharide was therefore considered free of any glucosamine-containing mucopolysaccharide.

Commercial chondroitin sulfate (isolated from bovine trachea) consists mainly of chondroitin sulfate A in admixture with chondroitin sulfate C. So it is essential to isolate chondroitin sulfate A from the polysaccharide mixture. Previous methods of separating the individual chondroitin sulfates were based on their differential solubilities in different solvent systems. These methods did not provide clear cut separation, and often the preparations obtained were not pure. In 1965, Barker and coworkers[48] described anion exchange

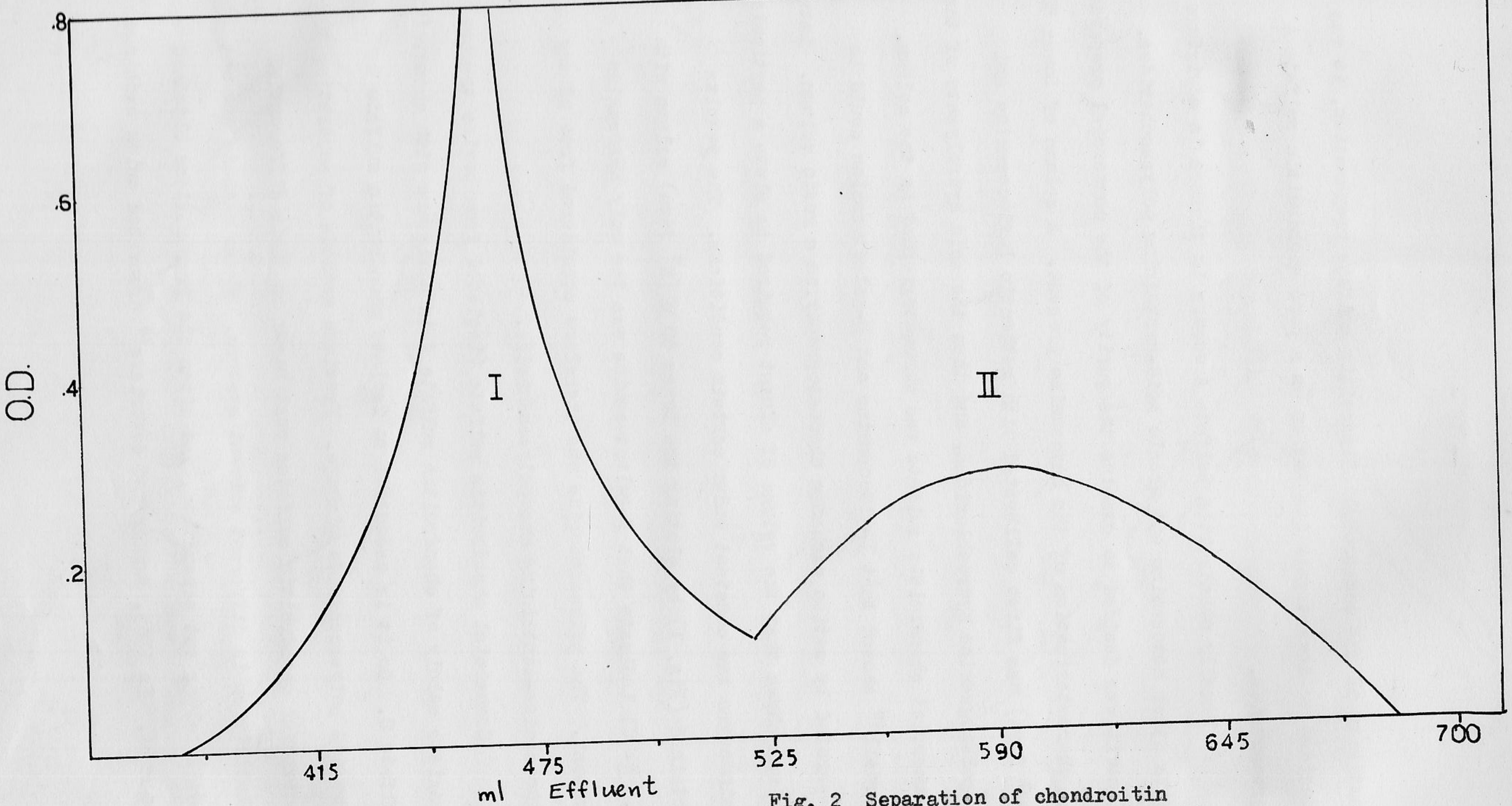


Fig. 2 Separation of chondroitin Sulfate A from C on Deacidite FF (Cl^- form).
I = Chondroitin Sulfate A
II = Chondroitin Sulfate C

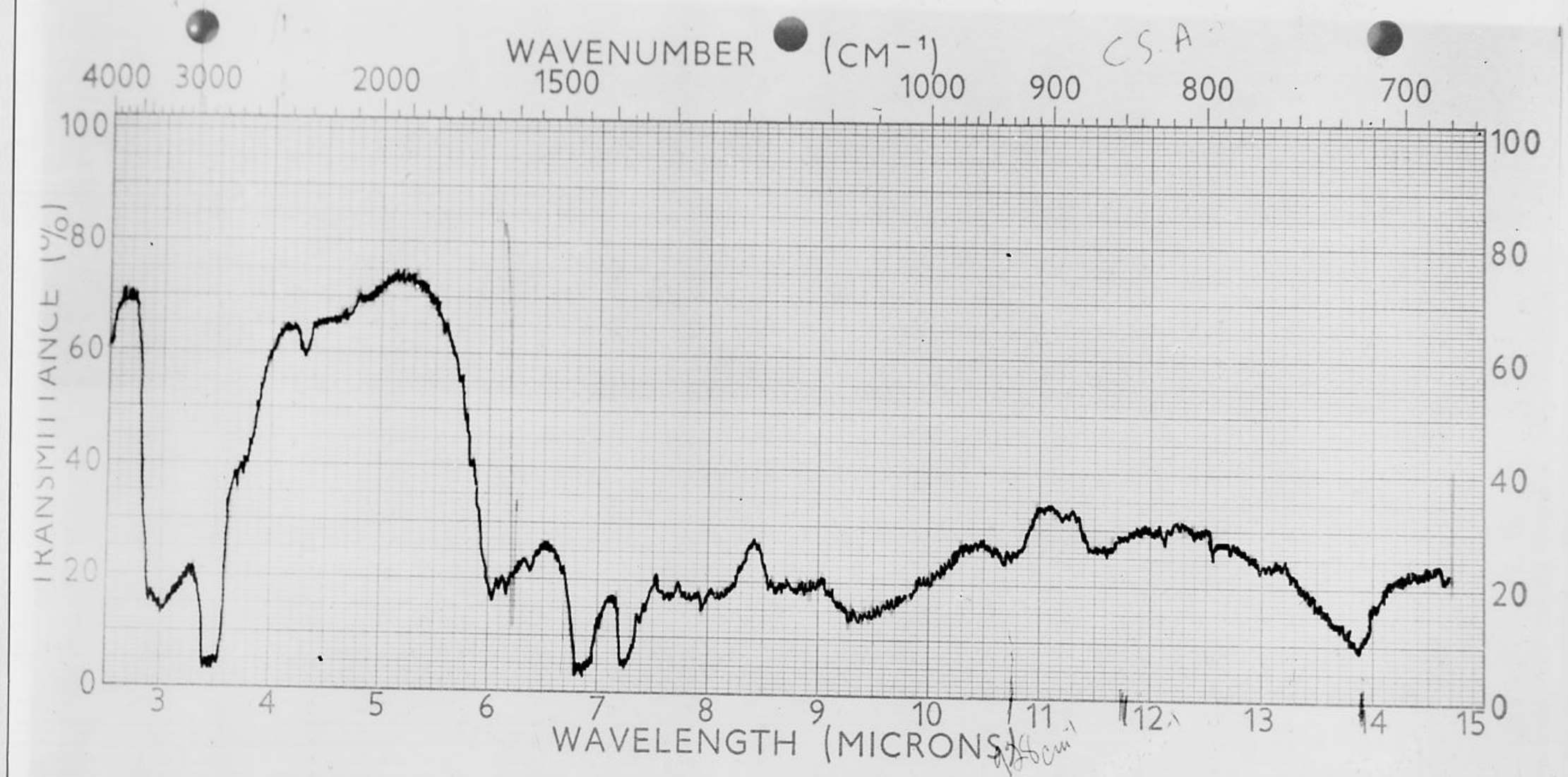


Fig. 3 Infrared spectrum of chondroitin sulfate A after isolation.

chromatography on Deacidite FF (Cl^- form) as an effective method of resolving acidic mucopolysaccharides. It afforded sharp separation of tissue mucopolysaccharides and was adopted in separating chondroitin sulfate A from the commercial preparation. In this work a column of Deacidite FF(Cl^- form ; 150 - 200 mesh; 7 - 9% cross linking) was employed, and the polysaccharides were eluted with NaCl solutions of gradually increasing concentrations. The fractions obtained (Fig. 2) were scanned by the carbazole colorimetric method. Sharp separation of chondroitin sulfate A (peak I) from chondroitin sulfate C (peak II) was achieved. Chondroitin sulfates A and C are isomers that differ only in the position of the $-OSO_3H$ group. In the former it occupies the axial position on C_4, while in the latter, it occupies the equatorial position on C_6. To ascertain further the identity of the faster polysaccharide fraction as chondroitin sulphate A, its infrared absorption spectrum was recorded (Fig. 3). The spectrum was distinguished by exhibiting (1) absorption at 1560 cm^{-1} and 1650 cm^{-1} attributable to the N-H deformation and C=O streching of the N-acetyl groups (2) the S=O streching vibrations at 1230 - 1260 cm^{-1} (3) in the finger-print region 700 - 1000 cm^{-1} the unique bands at 928 cm^{-1}, 852 cm^{-1} and 725 cm^{-1} which are characteristic of chondroitin sulfate A. This confirmed the identity of fraction I as chondroitin sulfate A.

Resolution of the Oligosaccharides Produced by Hydrolysis of Chondroitin Sulfate A by Bee Venom.

A 72 hours hydrolysate was prepared by large scale treatment of pure chondroitin sulfate A with bee venom hyaluronidase. Upon

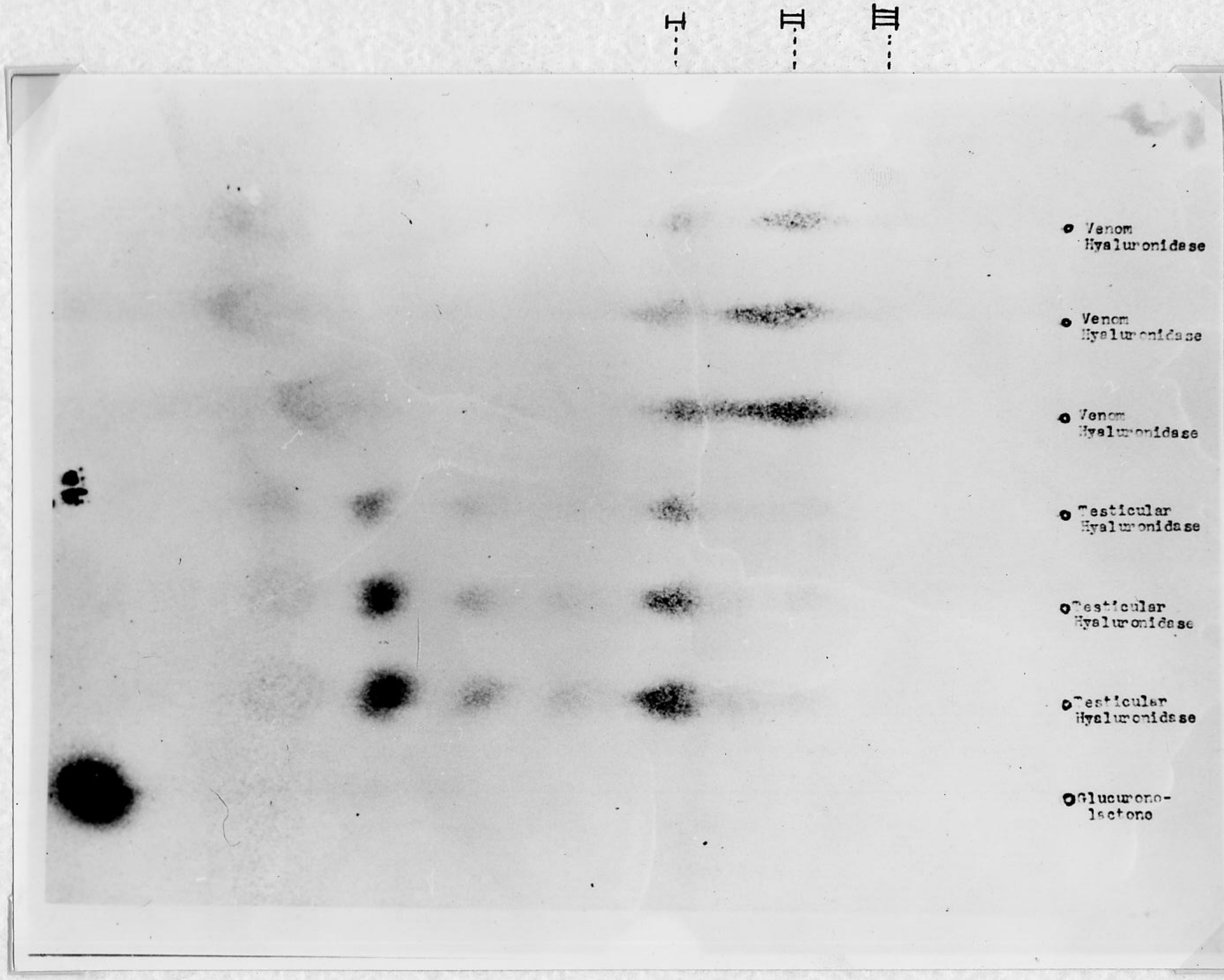


Fig. 4 Oligosaccharide components obtained by degrading chondroitin sulfate A with bee venom hyaluronidase.

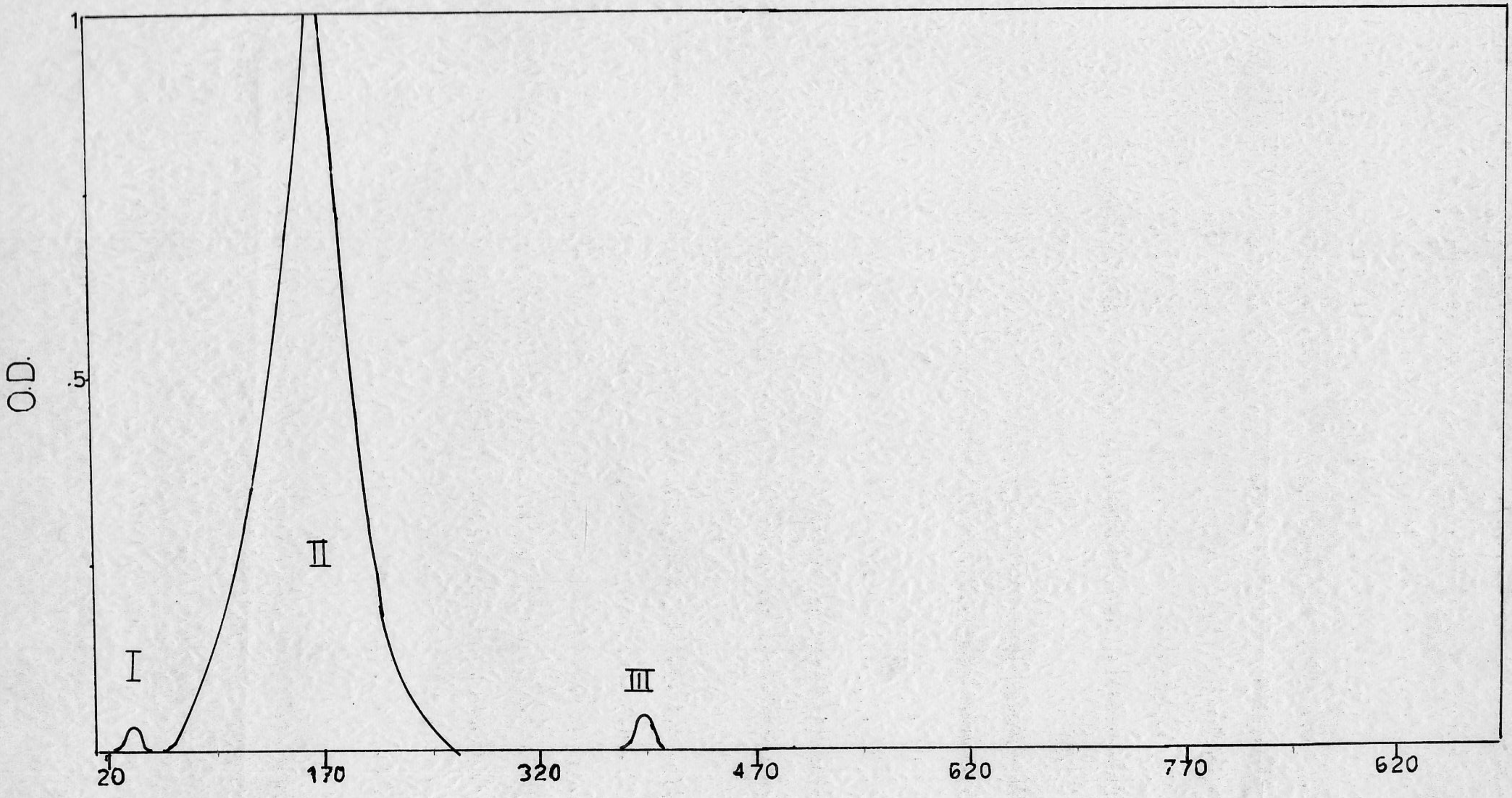


Fig. 5 Oligosaccharide components obtained by degrading chondroitin sulfate A with bee venom hyaluronidase.

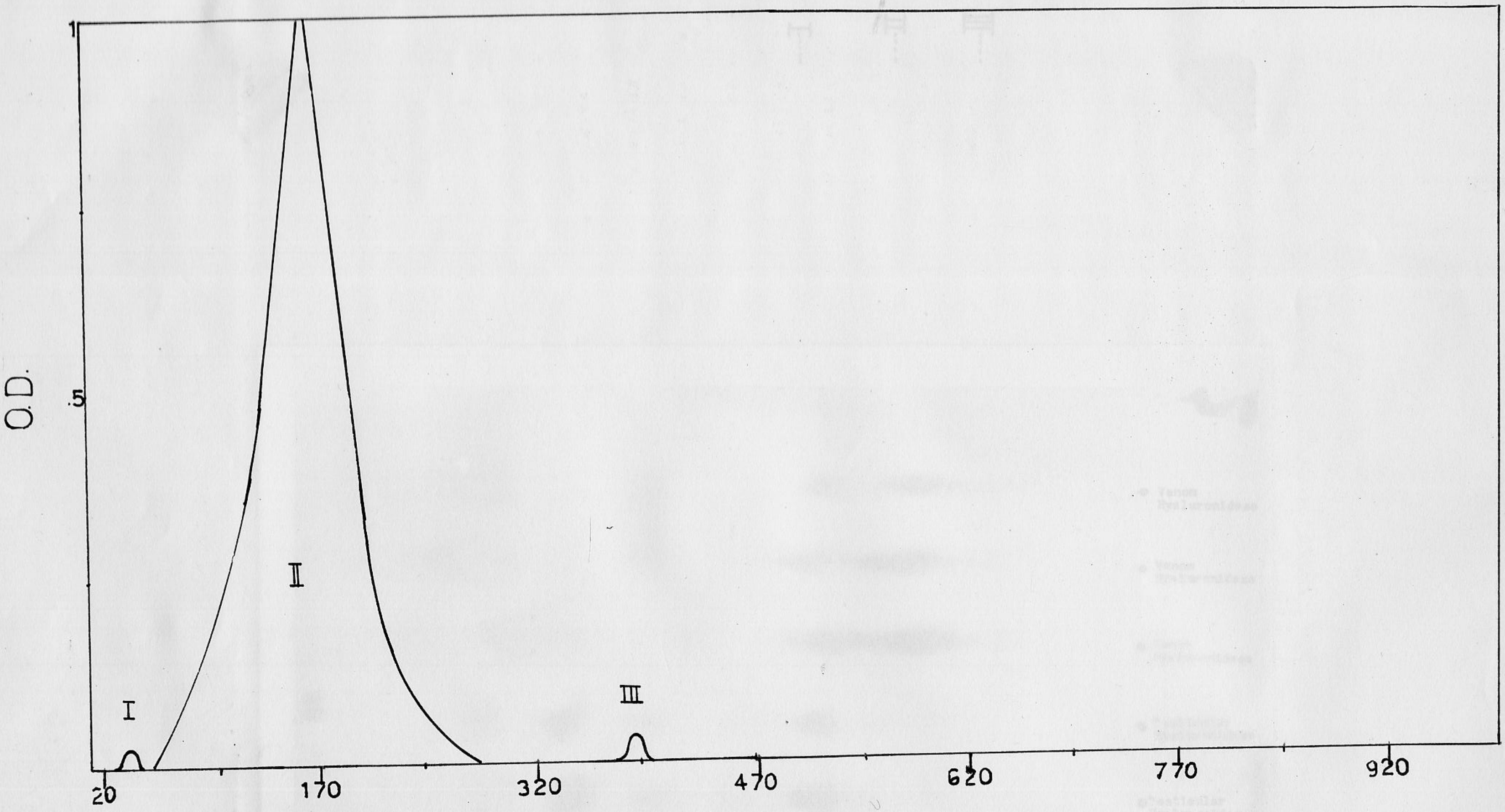


Fig.5 Oligosaccharide components obtained by degrading chondroitin sulfate A with bee venom hyaluronidase.

chromatographic examination (Fig. 4), the hydrolysate gave the same series of oligosaccharides I, II and III which had the same R glucuronolactone values that were previously indicated (0.33, 0.22 and 0.12 respectively), and the same relative intensities.

Furthermore, the three spots also showed similar mobilities to corresponding spots obtained from testicular hyaluronidase hydrolysate.

The method employed for resolving these oligosaccharide products was the same as that previously used for resolving the testicular hyaluronidase digests of hyaluronic acid[33]. It is based upon adsorbing the acidic oligosaccharides of the bee venom hydrolysate on the anion exchanger Dowex 1 x 8 ($H - COO^-$ form), followed by stepwise elution of the column with formic acid solutions of gradually increasing concentrations. The fractions were scanned by the carbazole colorimetric method of Dische[49]. Prior to application on the column, the enzymatic hydrolysate was boiled and centrifuged to get rid of the enzyme present by denaturation and precipitation. The anion exchange chromatography effected sharp separation of the three oligosaccharide fractions (Fig. 5) which had the same structure but different molecular sizes. From 375 mg of pure chondroitin sulfate A, oligosaccharide I weighed 1 mg and was the fastest component; oligosaccharide II (102 mg) was the major fraction; oligosaccharide III was present in small amounts also (2 mg) and had the lowest mobility. Hence oligosaccharide II was the major fraction and it constituted about 95% of resolved material. Since oligosaccharides I and III were obtained in low yields, further chemical investigations could not

be carried on these two fractions.

The homogeneity of oligosaccharides I, II and III was examined by spotting 1% solution of each oligosaccharide on paper chromatograms that were developed with 1-butanol/acetic acid/water (44/16/40 v/v). Each oligosaccharide moved in the form of 1 spot only indicating homogeneity and sharp separation by the anion exchange chromatography.

Molecular Weight Determination of Oligosaccharide II.

The alkaline iodine oxidation method as modified by Jeanloz and Forchielli[50] was adopted for the molecular weight determination. A standard experiment using N-acetyl-D-glucosamine was first carried out. The results shown in table 2 and Fig. 9 indicated that after 15 - 25 hours the reaction was complete as evidenced from the fact that one equivalent of the sugar reduced one mole of iodine. Since the iodine uptake by N-acetylglucosamine proceeded stoichiometrically after 15 - 25 hours, the method was applied for the molecular weight determination of oligosaccharide II. Upon treatment of the oligosaccharide in the alkaline hypoiodite reaction under the same conditions employed for the N-acetyl-D-glucosamine standard, the experimentally found molecular weight was in the range of 1407 - 1471. This result was within about 3.6% of the theoretical value expected from a hexasaccharide (1395). Oligosaccharide II was therefore designated as a hexasaccharide. This also indicates that in its action on chondroitin sulfate A, bee venom hyaluronidase has the preferred size specificity corresponding to hexasaccharide, i.e., the enzyme acts on the glycosidic linkage which is six monosaccharide units away from the reducing end.

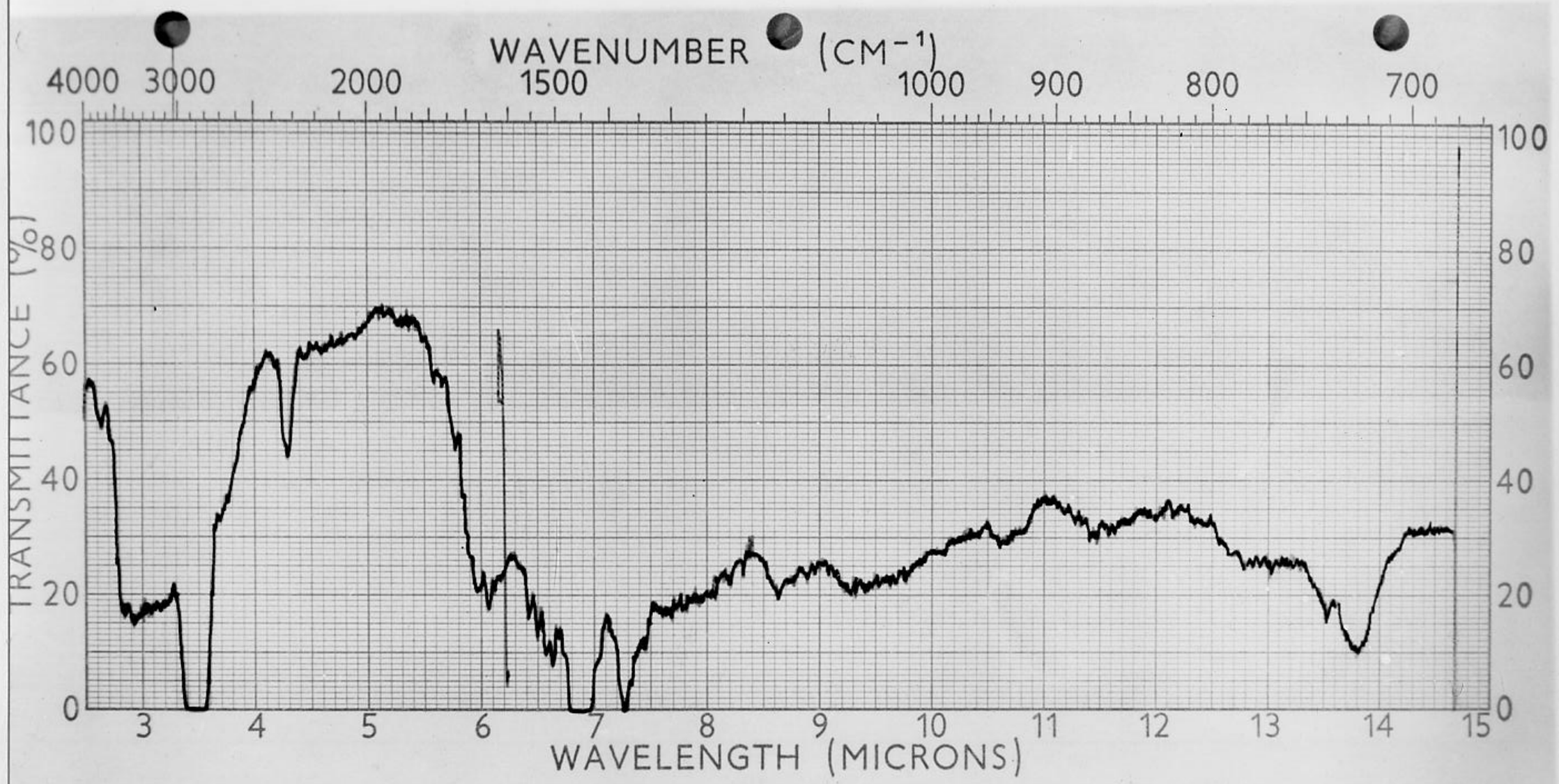


Fig. 6 Infrared spectrum of the hexasaccharide.

Measurement of optical rotation revealed that the hexasaccharide had $[\alpha]_D$ - 4.2°.

Infrared Spectrum of the Hexasaccharide.

The infrared absorption spectrum of the hexasaccharide (free acid form) was similar to that of chondroitin sulfate A particularly in the fingerprint region (Fig. 6). The hexasaccharide exhibited absorption in the 3300 - 3500 cm^{-1} attributable to O - H and N - H stretching, and at 2900 cm^{-1} due to C - H stretching. The characteristic absorption bands appeared at 1560 - 1565 cm^{-1} (N - H deformation) and 1635 - 1665 cm^{-1} (C = O stretching) indicative of N-acetyl groups. The C = O stretching vibration of unionized carboxylic acid or lactone appeared at 1730 cm^{-1} region. The presence of the sulfate ester group was indicated by absorption at 1230 - 1265 cm^{-1} and the characteristic absorption bands in the fingerprint region at 725, 855 and 930 - 935cm^{-1} confirmed the axial position of the sulfate ester group on C_4 of the N-acetyl-galactosamine moiety.

Further evidence for the presence of the sulfate ester group in position 4 of the N-acetyl-galactosamine moiety was obtained from the following two experimental findings:

1. The hexasaccharide was hydrolysed with N-HCl for 2 hours at 100° to effect desulfation. When the hydrolysate was treated with $BaCl_2$ it gave an immediate positive test.

The unhydrolysed oligosaccharide, under similar conditions, gave negative test.

2. As substitution in position 4 of the N-acetyl-galactosamine moiety suppresses the color formation in the Elson-Morgan reaction[54,55], the

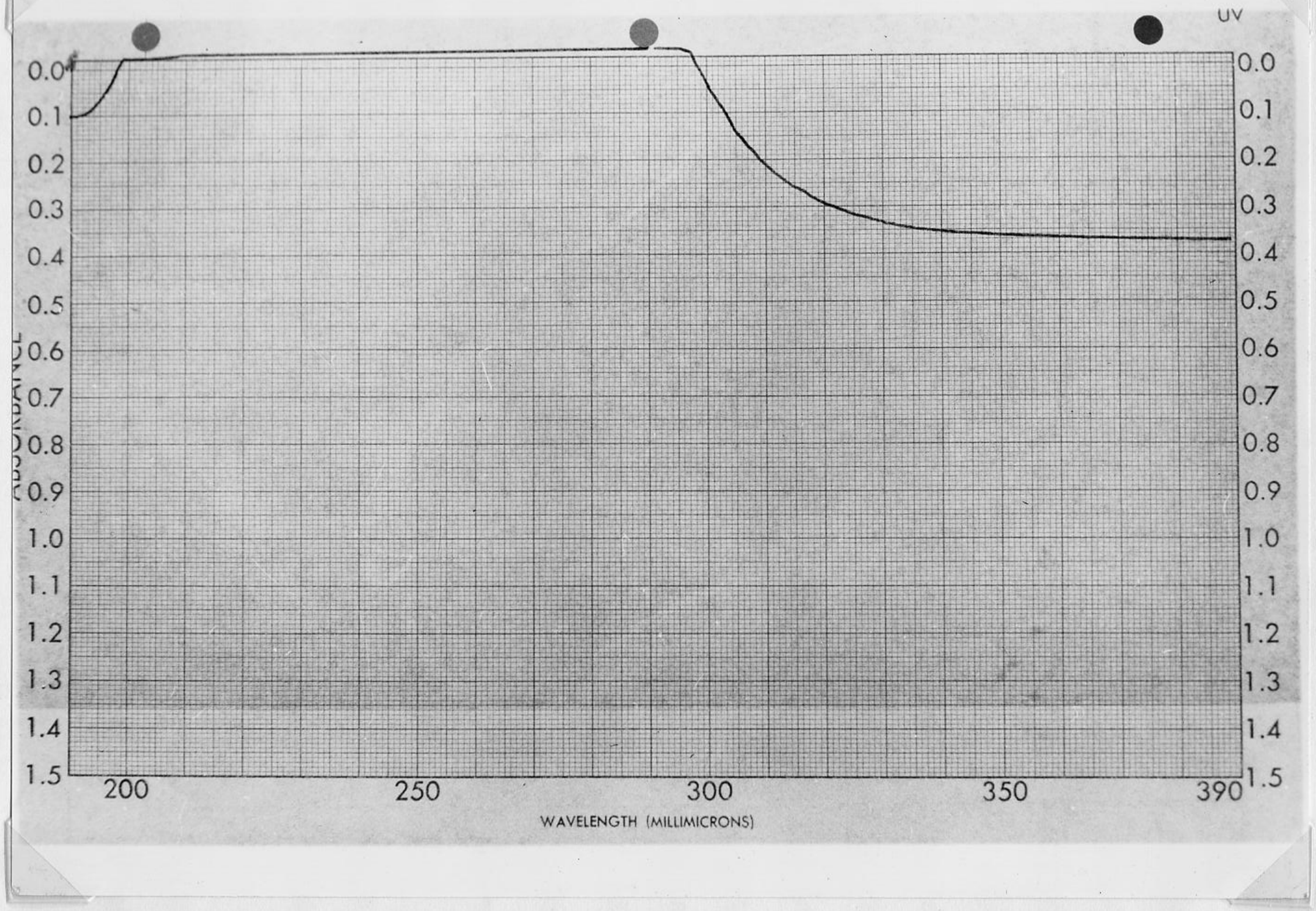


Fig. 7 U.V. spectrum of the hexasaccharide.

untreated hexasaccharide did not react with Ehrlich's reagent. The desulfated and partially de-N-acetylated oligosaccharide resulting from brief acid treatment however, gave a positive reaction in Elson-Morgan test.

Absence of Unsaturation in the Hexasaccharide.

Certain bacterial hyaluronidases cleave the hexosaminidic linkage by an intramolecular elimination reaction leading to unsaturation in the $\Delta^{4,5}$ - position of the uronic acid moiety. The bromine absorption and the ultraviolet spectrum (Fig. 7) of the hexasaccharide were determined. The oligosaccharide was of the saturated type as evidenced from; (1) lack of consumption of any bromine, while under the same conditions the bromine uptake by dihydropyran was very rapid. (2) No ultraviolet spectral absorption was detectable at 232 mμ. This indicated absence of α , β- unsaturation in the glucuronic acid moiety. It is obvious therefore that the hyaluronidase of bee venom hydrolyses the galactosaminidic linkage utlizing a water molecule from the medium, and in this respect, its action is similar to that of testicular hyaluronidase.

Quantitative Determination of the Hexosamine.

The galactosamine content of the hexasaccharide was quantitatively determined by the Elson-Morgan reaction as modified by Rondle and Morgan[51]. This involved treatment of the free aminosugar with alkaline 2,4-pentanedione to form the chromogens which were then reacted with p-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent). The red color produced had absorption maxima at 530 mμ, and the extinction coefficient of

the color produced by D-galactosamine is about 90% of that produced by D-glucosamine.

Little is known about the nature of the chromogens formed upon heating the hexosamine with 2,4-pentanedione in an alkaline medium. Of the chromogens suggested 2-methylpyrole which is volatile with steam is the most important. It seems that the reaction conditions such as pH during condensation with acetylacetone, temperature, time, concentration of reagents and the presence of interfering agents affect the reaction. Since it was found that non-nitrogenous sugars, when present alone do not interfere with the Elson-Morgan reaction, the glucuronic acid residues of the hexasaccharide should not affect the intensity of the color produced.

The hexasaccharide was hydrolysed with 0.04 N-HCl for 6 hours at 100 - 110°. These conditions were found to be optimal for the maximum liberation of the aminosugar with the least destructive effect. The galactosamine content of the hexasaccharide as determined under these conditions was 29%, which is close to the theoretical value (30%).

Evidence of β(1 ⟶ 3)-Glucuronidic Linkage.

It was shown that when the C_4 position on the hexosamine moiety is substituted, the Elson-Morgan reaction will not take place[54,55]. Substitution at the C_3 position, on the other hand, results in a pinkish-red color that has the absorption maxima at 510 mμ instead of 530 mμ. The hexasaccharide was consequently treated with N- H_2SO_4 for 110 minutes at 90° to effect partial de-N-acetylation and desulfation. After reacting the hydrolysate with the Elson and Morgan

reagents, maximum absorbance appeared at 512 mμ. This result indicated that the glucuronidic linkage in the hexasaccharide, like that in chondroitin sulfate A, was 1 ⟶ 3. On this basis, the hexasaccharide might prove useful as a model compound for future structural studies, and the elucidation of the mechanism of action of other mucopolysaccharases.

Action on Other Mucopolysaccharides.

Chondroitin sulfate C was found to be hydrolysed by bee venom hyaluronidase giving similar spots to those obtained from chondroitin sulfate A. Unlike testicular hyaluronidase and other hydrolases, bee venom hyaluronidase degraded heparin giving two oligosaccharide spots on paper chromatographic examination of the hydrolysate. Further structural work is necessary to elucidate the identity of the oligosaccharide products obtained from heparin and chondroitin sulfate C. The fact that bee venom hyaluronidase degraded heparin is of appreciable significance, since it may prove valuable in studying the structure of this mucopolysaccharide.

EXPERIMENTAL

I. General Methods. Experiments 1 - 3

Experiment 1. Paper Chromatography.

Paper chromatographic analyses were effected on Whatman No. 1 paper. The chromatograms were developed for 10 - 72 hours with 1-butanol/glacial acetic acid/water (44/16/40 v/v)- single phase system.

After downward irrigation, the chromatograms were dried, and the saccharide spots detected with one of the following spray reagents:

a) Elson-Morgan and Morgan-Elson reagents[45] for the detection of certain aminosugars and their N-acetyl derivatives respectively.

b) Silver nitrate[46]. The dry chromatograms were first passed rapidly through a solution of silver nitrate in acetone, and allowed to dry in air. Spraying with 0.5 N ethanolic sodium hydroxide brought up reducing and non-reducing sugars as black spots. When required as permanent records, the chromatograms were fixed by soaking in 40% sodium thiosulfate solution followed by thorough rinsing with water.

Experiment 2. Infrared Analysis.

All samples were dried (4 hours in vacuum at 60°C over P_2O_5) and the spectra were determined on a Perkin-Elmer Infracord, Model 137, using nujol-mulls.

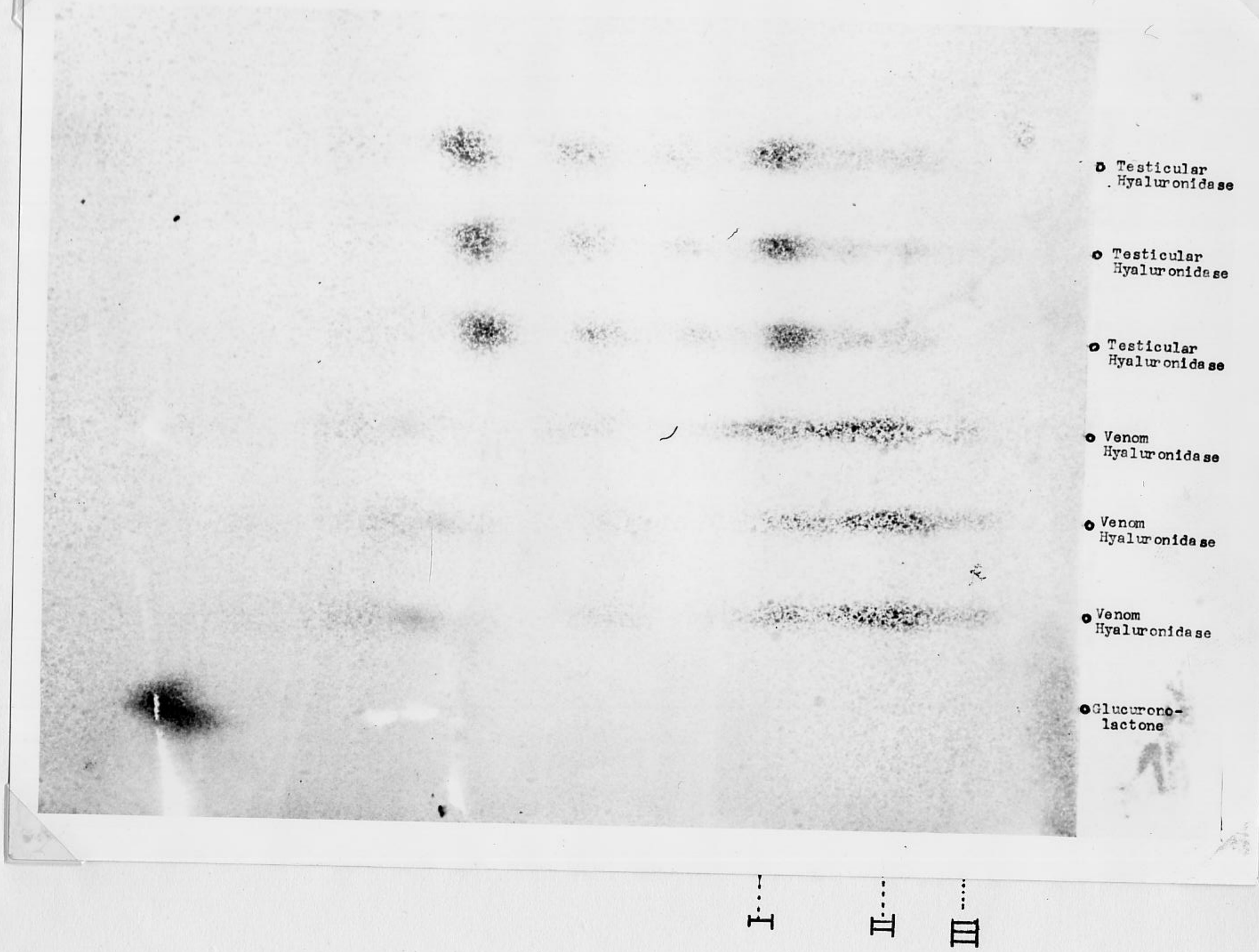


Fig. 8 Paper chromatographic examination of the hydrolysate obtained with commercial chondroitin sulfate.

Experiment 3. Specific Rotation Measurements.

All samples were dried in vacuo over P_2O_5 for 4 hours at 60°C, and adequate amounts were dissolved in M - NaCl. The specific rotations were calculated after measuring the optical rotation of the solutions using Perkin-Elmer Model 141 polarimeter and 1 cm. cells. The observed rotations were recorded automatically.

II. Degradation of Chondroitin Sulfate A by Bee Venom Hyaluronidase.

Experiment 4. Preliminary Examination of the Action of Bee Venom Hyaluronidase on Commercial Chondroitin Sulfate[45].

To 10 mg of commercial chondroitin sulfate (from bovine trachea) in 0.3 ml of 0.05 M-phosphate-citrate buffer (pH = 4.7), 0.5 mg of bee venom dissolved in 0.2 ml of 0.15 M-NaCl was added. The resultant solution was incubated for 72 hours at 37° with occasional shaking. The hydrolysate was then centrifuged, and after concentration to half its volume under reduced pressure, the clear solution was examined by paper chromatographic analysis. The chromatograms were developed with the organic phase of 1-butanol/ acetic acid/water (44/16/40 v/v) for periods ranging between 18 to 72 hours. The reference saccharides applied were D-glucuronolactone and the hydrolysate obtained from the same commercial chondroitin sulfate with testicular hyaluronidase. The hydrolysate of bee venom hyaluronidase gave a series of 3 spots: Oligosaccharide I was the fastest (R glucuronolactone 0.33); oligosaccharide II had (R glucuronolactone 0.22) and oligosaccharide III had the lowest mobility (R glucuronolactone 0.12) see Fig. 8 . From their

relative intensities, oligosaccharides I and II were the major components, with II predominating. Oligosaccharide III was present in very small amounts. Oligosaccharides I and II had similar mobilities to corresponding oligosaccharides obtained by the action of testicular hyaluronidase on the same polysaccharide.

Experiment 5. Examination of the Purity of Commercial Chondroitin Sulfate by Characterization of the Aminosugar Moiety[47].

A. Preparation of Column:

40 g of Dowex 50 w x 8 (200 - 400 mesh) was washed with a total of 85 ml portions of 4 N - HCl to rid the resin of fine particles. The resin was then suspended in 4 N - HCl and poured into a burette half-filled with water and packed with a piece of pyrex glass wool at the bottom. The resin was left to settle overnight and the column was then washed with 0.3 N - HCl for 26 hours, after which time the normalities of inflowing and outflowing solutions were identical.

B. Calibration of the Column:

1 ml of a 0.3 N - HCl solution containing 340 µg of glucosamine hydrochloride and 250 µg of galactosamine hydrochloride was applied to the column. The column was eluted with 0.3 N - HCl at a flow rate of 2 - 3 ml/hour. The glucosamine and galactosamine peaks were spotted by the Elson-Morgan reagents at tubes 78 - 86 and 93 - 102 respectively Fig. 1 .

C. Application of the Material:

3 ml of a 0.3 N - HCl solution containing the hydrolysate

(3 N - HCl for 6 hours at 100°C) obtained from the commercial chondroitin sulfate (bovine trachea) was applied to the column. The solution was allowed to run slowly through, and the walls were washed twice with 1 ml portions of 0.3 N - HCl. The washings were also allowed to pass through slowly. The space above the column was connected to a 0.3 N - HCl reservoir. The level of the latter was adjusted to give a flow rate of 3 - 4 ml effluent/hour.

2.5 ml fractions were collected on an automatic fractions collector (Gallenkamp) starting from the moment the hydrolysate was applied to the column. For the maintenance of the same flow rate the level of the reservoir was occasionally adjusted. Only one aminosugar component corresponding to the authentic galactosamine peak could be detected at tubes 92 - 100 Fig. 1 . This proved that the product was free of any glucosamine-containing impurities (such as hyaluronic acid and heparin).

Experiment 6. Isolation and Purification of Chondroitin Sulfate A by Ion Exchange Chromatography[48].

Deacidite FF (100 - 200 mesh; 7 - 9% cross linking) was heated with 6 N - HCl to rid it of fines, followed by 6 N - NaOH, and then washed three times with 6 N - HCl. The resin was finally washed several times with deionized distilled water. After suspension in deionized distilled water, it was poured as a slurry into a column (75 x 3 cm) one third full of water and packed with a piece of glass wool at the bottom. The column was washed with deionized distilled water until the effluent was neutral.

150 mg of commercial chondroitin sulfate (from bovine trachea) was dissolved in about 5 ml of water and the solution applied onto the column. The solution was allowed to run slowly through, and the walls of the column were washed twice with 1 ml portions of distilled water. The washings were also allowed to pass through slowly. The space above the resin was carefully filled with water, and the column was connected to a distilled water reservoir. After adjusting the rate of flow to 80 ml/hour; 10 ml fractions were collected on a Gallenkamp automatic fraction collector. The column was successively eluted with 50 ml of water, 150 ml of 0.5 M - NaCl, 150 ml of 1.25 M - NaCl, 150 ml of 1.5 M- NaCl, 150 ml of 2 M - NaCl, and 200 ml of 4 - M - NaCl. The series of tubes were scanned, and the polysaccharide fractions spotted by using the carbazole reaction[49] as indicated below.

To 1 ml aliquot of each fraction, 6 ml of 87% sulfuric acid (diluted from 98% H_2SO_4, B.D.H. Microanalytical Reagent) was added with cooling in an ice bath. The reaction mixture was then heated for 20 minutes in a boiling water bath and subsequently cooled in tap water. 0.2 ml of a 0.1% ethanolic solution of carbazol (commercial grade recrystallized twice from benzene) was then added, and the solutions allowed to stand for 1 hour at room temperature. The optical density values of the colors developed were read at 535 mμ in a Unicam S.P. 800 spectrophotometer against a blank. The plot of optical densities against test tube No. is shown in Fig. 2 . The tubes in each peak were pooled, dialized against running water for 24 hours to remove NaCl, and lyophilized. The

polysaccharide products consisted of two main fractions. Fraction I was the major fraction and weighed 48 mg while fraction II weighed 18 mg.

A total of 1.05 g of commercial chondroitin sulfate was resolved by two similar fractionations. A total of 335.5 mg of fraction I was obtained.

Experiment 7. Characterization of Chondroitin Sulfate I and II.

Fractions I and II were characterized as chondroitin sulfates A and C respectively on the basis of the following evidence:

1- Barker and coworkers[48] found that chondroitin sulfate A always exhibited faster mobility on Deacidite FF than chondroitin sulfate C. On this basis, fraction I, which was eluted first, should be chondroitin sulfate A.

2- The infrared spectra of fractions I and II were determined. Fraction I exhibited in the finger-print region (700 - 1000 cm^{-1}) absorption bands at 928, 852, and 725 cm^{-1} Fig. 3 , characteristic of chondroitin sulfate A. Fraction II was identified as chondroitin sulfate C since it exhibited the characteristic absorption at 1000, 820, and 775 cm^{-1} ~~Fig.~~ regions.

Experiment 8. Large Scale Degradation of Pure Chondroitin Sulfate A by Bee Venom Hyaluronidase.

375 mg of pure chondroitin sulfate A in 0.05 M -phosphate-citrate buffer (12 ml, pH = 4.7) were exhaustively degraded with 50 mμg of bee venom in 7.5 ml of 0.15 M - NaCl by incubation for

72 hours at 37°. The hydrolysate was then boiled for 3 minutes to precipitate the enzyme by denaturation. After centrifugation, the clear hydrolysate was freeze-dried, and examined by paper chromatographic analysis in butanol/acetic acid/water (44/16/40 v/v). D-glucuronolactone and a testicular hyaluronidase hydrolysate of chondroitin sulfate A were used as references. The oligosaccharides resolved had identical mobilities and relative intensities as those of I, II, and III previously described in the preliminary hydrolytic reaction. Furthermore these three spots had mobilities similar to the tetra-, hexa-, and octa-saccharide spots obtained by the action of testicular hyaluronidase on the polysaccharide Fig. 4 .

Experiment 9. Resolution of the Oligosaccharides by Anion Exchange Chromatography[33].

Dowex 1 x 8, 200 - 400 mesh, was obtained from the manufacturer (Fluka) in the Cl^- form. A batch of about 150 g was first washed with 2 N - HCl to rid it of fine particles. After the resin was allowed to settle, the supernatant liquid was decanted. Following two more such washings, the resin was filtered and washed with water until neutral. The resin was then suspended in water and packed as a slurry into a column (95×1- cm) that was half-full of water and fitted with glass wool at the bottom. After packing, the resin was converted to the $HCOO^-$ form by eluting with 1 M - sodium formate until the effluent gave a negative test for Cl^- with acidic $AgNO_3$ solution. The column was then washed overnight with distilled water.

About 200 mg of the freeze-dried degradation product

obtained from the previous experiment was dissolved in water (about 5 ml) and applied onto the column. The solution was allowed to pass through and the walls were washed with two successive portions of water that were also allowed to pass through slowly. The oligosaccharide components were resolved with 270 ml portions of 0.015 M -, 0.05 M -, 0.15 M-, 0.3 M -, 0.50 M -, 0.80 M - and 1.5 M - formic acid solutions. Eluate fractions of 10 ml were collected on a Gallenkamp automatic fraction collecter at the rate of 10 - 12 drops/min. The tubes were scanned with the carbazol colorimetric method of Dische[49] using 1 ml aliquot portions. The color which developed was measured in a Unicam S.P. 800 spectrophotometer. The plot of optical densities *vs.* test tube number is shown in Fig. 5 . The tubes in each peak were pooled, and after lyophilization, the oligosaccharide products were weighed. Oligosaccharide I (< 1 mg) was collected in tubes 3 - 7; Oligosaccharide II (102 mg) in tubes 9 - 27, and oligosaccharide III (2 mg) in tubes 37 - 42.

Experiment 10. Examination of the Homogeneity of the Oligosaccharide Fractions.

1% aqueous solutions of oligosaccharides I, II and III were prepared. Each solution was analyzed by paper chromatography with D-glucuronolactone as reference in butanol/acetic acid/water (44/16/40 v/v). The paper chromatograms were sprayed with silver nitrate and Elson-Morgan reagents. The silver nitrate spray revealed that each fraction consisted of only one component

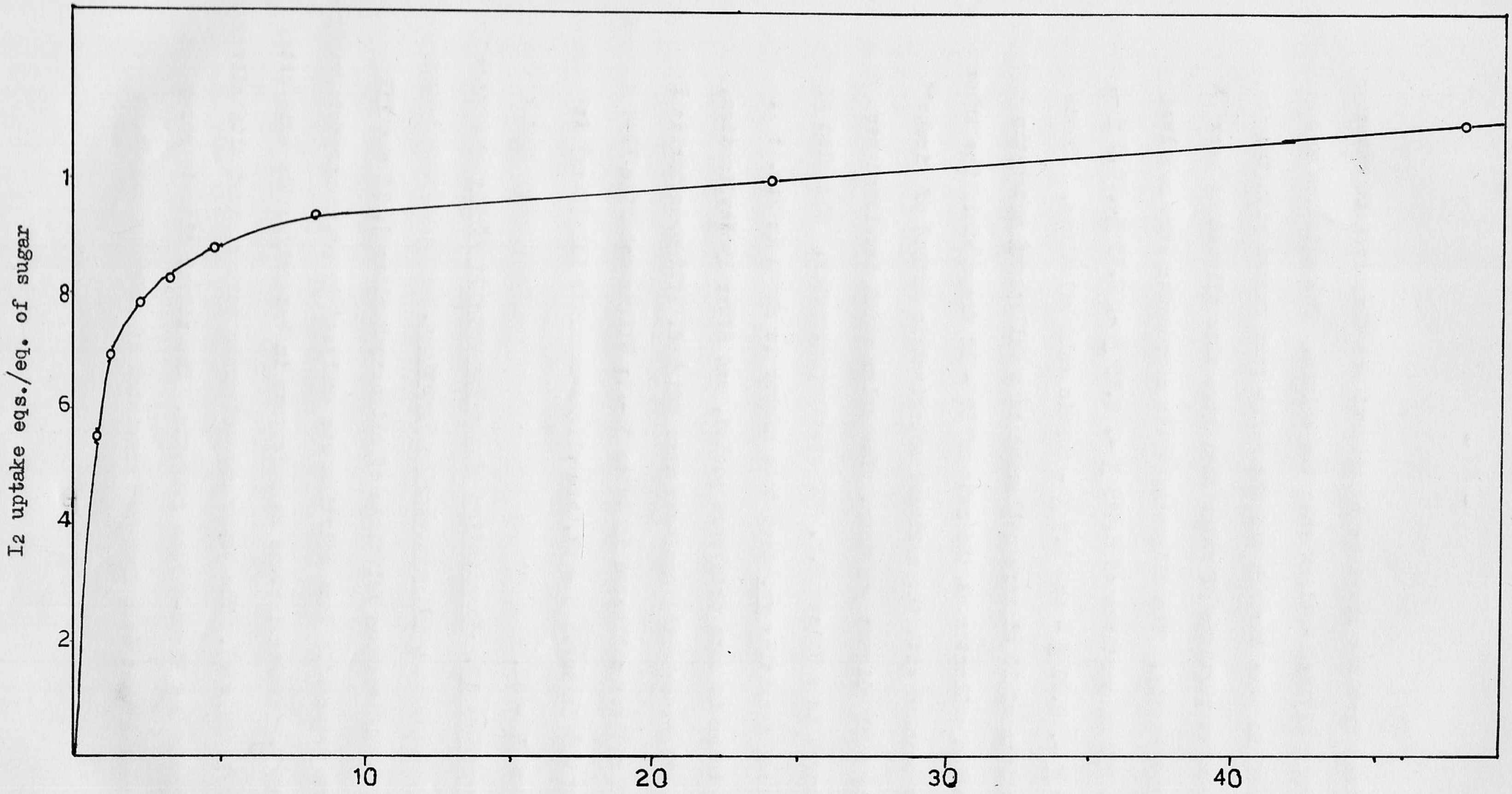


Fig. 9 Hypoiodite oxidation
of N-acetyl-D-glucosamine.

(one spot could only be detected), and that oligosaccharides I, II and III had the relative mobilities 3:2:1.

With the Elson-Morgan Spray no color was developed, and this is attributable to the fact that in chondroitin sulfate A oligosaccharides substitution (-O-SO_3H) at position 4 in the galactosamine moiety suppresses the color formation in this reaction.

Experiment 11. Molecular Weight Determination of Oligosaccharide II.

The hypoiodite oxidation method as modified by Jeanloz and Forchielli[50] was adopted. Under these conditions, the oxidation of N-acetyl-D-glucosamine proceeded stoichiometrically, one equivalent of the sugar consumed one equivalent of iodine.

To a solution of 50.2 mg of N-acetyl-D-glucosamine in 5 ml of water, 5 ml of 0.2 M-Na_2CO_3 and 5 ml of 0.2 M-$NaHCO_3$ were added. To the resultant solution 5 ml of 0.037 N-iodine solution was added, and after mixing, the solution was made up to 50 ml with distilled water. All solutions and distilled water were equilibrated overnight at 0 - 2° before addition. The reaction mixture was kept at 0 - 2° in the dark, and at suitable intervals, 5 ml aliquots were pipetted, acidified with 5 ml of 5 N-H_2SO_4, diluted with water, and titrated with 0.0257 N-sodium thiosulfate using "Thyodene" indicator. The iodine uptake equivalents/equivalent N-acetyl-D-glucosamine was plotted against time Fig. 9 .

Table II

Hypoiodite Oxidation of N-Acetyl-D-Glucosamine

Time (hours)	I_2 uptake eqs./eq. of sugar
0.50	0.560
1.00	0.699
2.00	0.799
3.00	0.833
5.00	0.865
8.00	0.960
24.00	1.001
49.00	1.100

Four samples of the disaccharide II (0.86, 0.97, 1.03 and 1.4 mg) were dissolved each in 5 ml of water, and treated with a mixture (1 ml) consisting of equal parts of 0.2 M-Na_2CO_3 and 0.2 M-$NaHCO_3$, followed by 0.5 ml of 0.037 N-iodine solution. All solutions were cooled to 0 - 2° before addition. After standing at 0 - 2° in the dark for 18 hours, the reaction mixtures were acidified with 5 ml of 5 N-H_2SO_4 each, diluted with water to about 25 ml, and titrated with 0.0257 N-sodium thiosulfate using "Thyodene" indicator. From the iodine consumption, the molecular weights found were in the range of 1407 to 1471.

Experiment 12. Determination of Specific Rotation:

The specific rotation of the hexasaccharide, $[\alpha]_D$ - 4.2°,

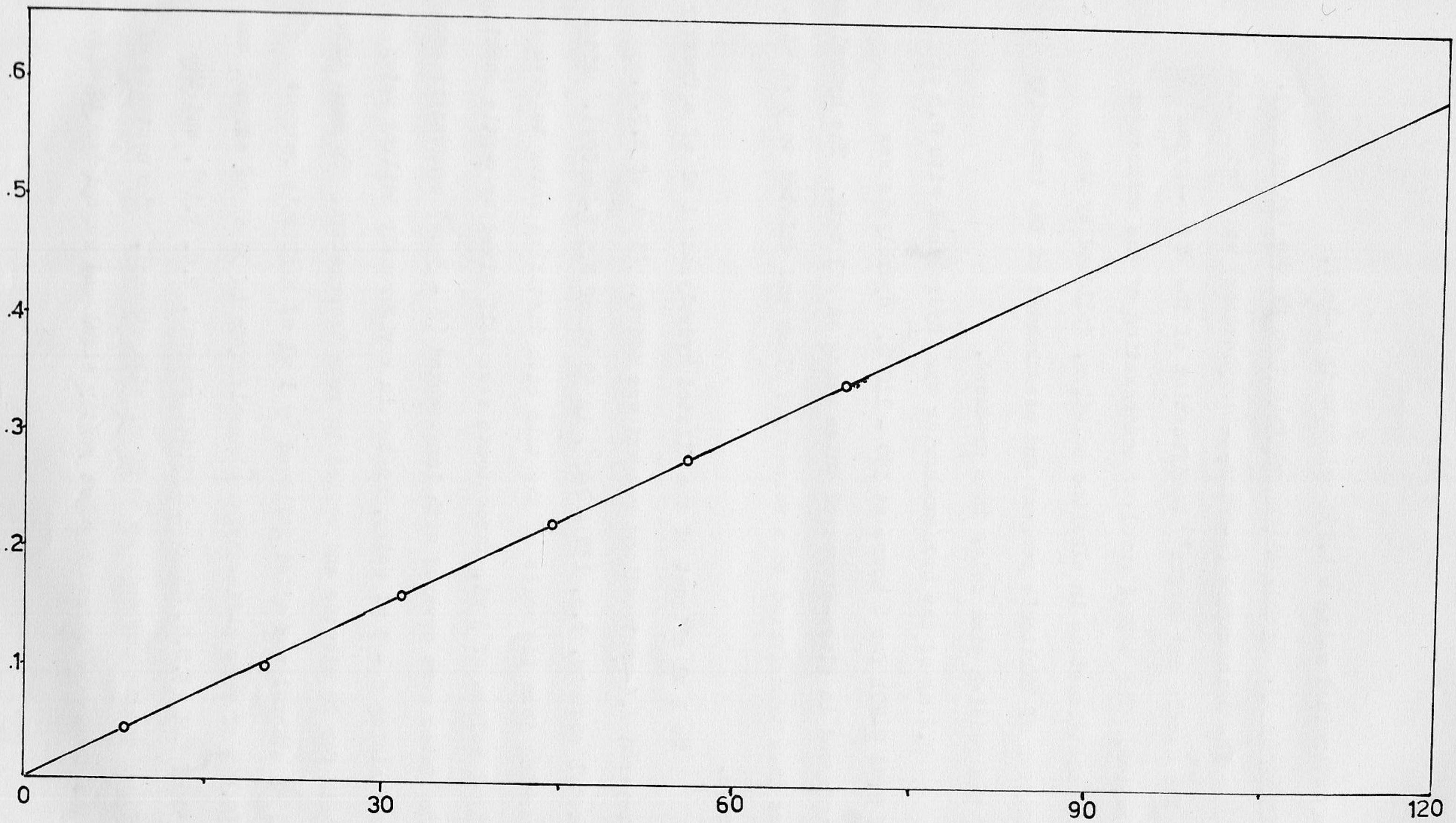
was determined using a 1.15% solution in N-NaCl.

Experiment 13. Quantitative Determination of the Aminosugars.

The aminosugars (2-amino-2-deoxyhexoses) were determined by the Rondle and Morgan[51] modification of the Elson-Morgan reaction. The method involved condensation of the aminosugar with acetylacetone in alkaline solution, followed by the addition of Ehrlich's reagent and measurement of the intensity of the reddish pink color thus produced.

0.4 mg of the hexasaccharide was hydrolysed with 0.5 ml of 0.04 N-HCl for 6 hours at 100 - 110°C. Hydrolysis was performed in glass-stoppered tubes using an oil bath. The hydrolysate was then transferred to a volumetric flask and diluted to 5 ml with water.

To 1 ml aliquot of the diluted hydrolysate 1 ml of a freshly prepared 2,4-pentanedione reagent was added. (1 ml of B.D.H. acetyl acetone, b.p. 138 - 140°, in 50 ml of 0.5 N-Na_2CO_3). After washing down the walls of the tube with distilled water to give a final volume of 3 ml, the contents were well mixed by gentle shaking. The tubes were covered with long-necked (2 - 3 cm) condensers each containing 2 - 3 ml water, heated in a vigorously boiling water bath for 20 minutes and cooled to room temperature. To each tube 5 ml ethanol was added followed by 1 ml of Ehrlich's reagent (0.8 g of pure p-dimethylaminobenzaldehyde in 30 ml of ethanol and 30 ml HCl (B.D.H. Microanalytical reagent, S.G. 1.18), and the final volume was made up to 10 ml with ethanol. The contents of the tubes were thoroughly but gently mixed and warmed for 10 minutes



ug glucosamine

Fig.10 Elson-Morgan Reaction

Calibration Curve for Hexosamines

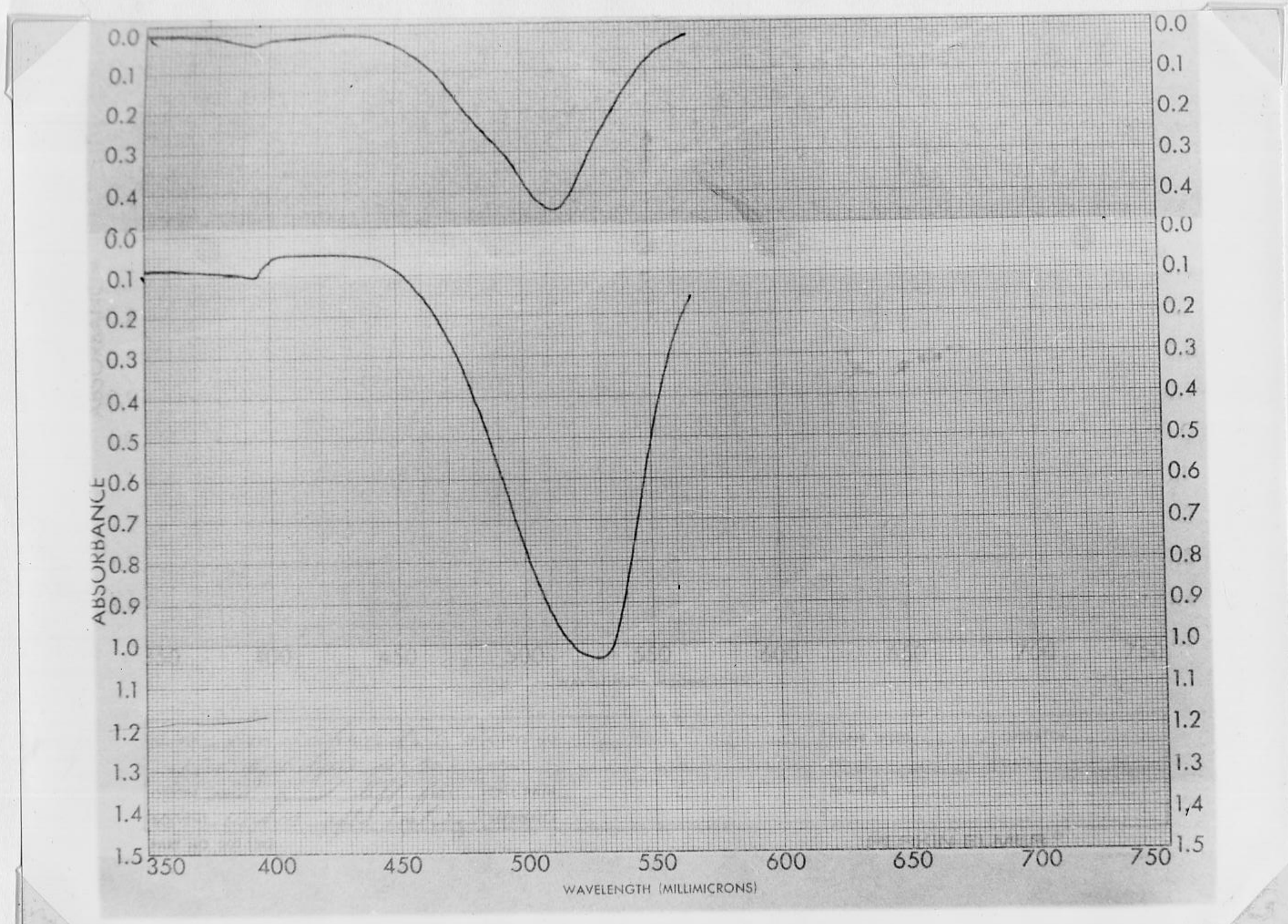


Fig. 11 Absorption maximum of the desulfated and de-N-acetylated hexasaccharide in the Elson-Morgan reaction

in a water bath at 65 - 70° to accelerate liberation of CO_2. After cooling to room temperature, the contents of the tubes were again mixed, and the color intensity at 535 mμ measured in a Unicam spectrophotometer S.P. 800 using 1 cm silica cells.

Standard tubes containing 8 μg, 24 μg, 40 μg, 48 μg, 64 μg and 80 μg of authentic glucose-amine hydrochloride were treated in the manner described above. The calibration curve is shown in Fig. 10 .

Experiment 14. Infrared Analysis of the Hexasaccharide.

The infrared absorption spectrum of the free acid form of the hexasaccharide was recorded.

Experiment 15. Evidence of the 1 ⟶ 3 Glucuronidic Linkage.

2 mg of the hexasaccharide was treated with 0.5 ml of N-H_2SO_4 for 110 minutes at 90° to effect partial de-N-acetylation and desulfation. The resultant hydrolysate was neutralized with Na_2CO_3 and diluted to 5 ml with water. An aliquot of 0.5 ml was then reacted with the Elson-Morgan reagents as described by Rondle and Morgan[51]. Simultaneously, tubes of D-galactosamine hydrochloride and a blank were treated in the same way. The absorption maxima of the chromogen formed by the hexasaccharide (512 mμ) and D-galactosamine hydrochloride (530 mμ) were determined by recording the spectra between 350 - 600 mμ using 1 cm silica cells Fig. 11 .

Experiment 16. Determination of the Ultra-Violet Absorption Spectrum of the Hexasaccharide.

The ultra-violet absorption spectrum of the hexasaccharide

was determined on a solution of 1 mg in 5 ml of water in the range of 200 - 390 mμ. No absorbance was observed in the 235 mμ region.

Experiment 17. Absence of Bromine Absorption.

1 mg of the hexasaccharide was dissolved in 2 ml of absolute and aldehyde-free ethanol, and three drops of a dilute solution of Br_2 in $CHCl_3$ (0.0125 ml/100 ml) was added. After standing in the dark at room temperature, the bromine color was not discharged. Simultaneously, 2 ml of ethyl alcohol containing a trace of dihydropyran discharged the color immediately even after 25 drops of the bromine solution were added.

Experiment 18. Qualitative Test for the Presence of Sulfate Groups.

1 mg of the hexasaccharide was hydrolyzed with 0.5 ml of N-HCl for 120 minutes at 100°. To one drop of the hydrolysate, a drop of saturated $BaCl_2$ solution was added. A precipitate of $BaSO_4$ was formed immediately. When 1 drop of the unhydrolysed acidic solution of the hexasaccharide was similarly treated, no precipitate could be observed.

Experiment 19. Action of Bee Venom Hyaluronidase on other Mucopolysaccharides.

1. Chondroitin Sulfate C.

Hydrolysates of bee venom hyaluronidase and testicular hyaluronidase were prepared by incubating 10 mg of the mucopolysaccharide with each enzyme preparation at 37° for 72 hours in the phosphate-citrate buffer/0.15 M saline mixture. The hydrolysates were examined by paper chromatography. After spraying with $AgNO_3$

spray reagents, the bee venom gave a series of three spots with mobilities similar to those obtained from chondroitin sulfate A and similar to the mobilities of corresponding spots obtained with testicular hyaluronidase. It could also be observed that the spot with intermediate mobility (most likely a hexasaccharide) was the major fraction since it gave the most intense spot.

2. Heparin.

When testicular hyaluronidase and bee venom hyaluronidase hydrolysates obtained by degrading 10 mg of heparin with each were examined by paper chromatography, no hydrolysis was detected with testicular hyaluronidase. Bee venom hyaluronidase, however, degraded the mucopolysaccharide but at a slow rate.

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