STUDIES ON THE STRUCTURE OF HEPARAN SULPHATE

by

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TO MY DEAR BROTHERS

STALWART, SHEPHERD AND SYDNEY

ABSTRACT

Heparan sulphate is a glycosaminoglycan, closely associated with heparin and isolable from the mother liquor of commercial heparin preparations. The complete structure of this polymer is still unknown, and the studies described here are accordingly concerned with the question of its chemical constitution. Fractions of a heparan sulphate from beef lung have been isolated by precipitation and high speed centrifugation, and examined intensively by proton and carbon-13 n.m.r. spectroscopy, as well as by chemical and enzymic methods. The data obtained indicate that the heparan sulphate consists of polymer I, the spectra of which show an absence of N-acetyl groups and are virtually indistinguishable from those of heparin in many ways; polymer II, with an intermediate amount of N-acetyl; and polymer III, with the hexosamine almost completely N-acetylated. Polymer III consits mainly of β-D-glucuronic acid and 2-acetamido-2-deoxy-α-D-glucose. Chemical shifts, coupling constants, ^{13}C relaxation properties, and enzymic results obtained for the D-glucuronic acid residues both of heparan and of a disaccharide prepared from it, are consistent with assignment of the β-configuration and the $^4C_1$ (D) conformation. ^{13}C.m.r. data support earlier evidence that the hexosamine unit possesses the α-configuration and has the $^4C_1$ (D) conformation.

RESUME

Le sulfate d'héparane est un glycosaminoglycane apparenté à l'héparine et pouvant être isolé des eaux mères des préparations commerciales de celle-ci. La structure complète de ce polymère étant toujours inconnue, les études décrites ici concernent sa constitution chimique. Des fractions de sulfate d'héparane provenant de poumons de boeuf ont été isolées par précipitation et centrifugation à vitesse élevée, et étudiées ensuite intensément par spectroscopie r.m.n (proton et carbone-13), ainsi que par des méthodes chimiques et enzymatiques. Les données obtenues montrent que le sulfate d'héparane est composé de trois polymères: les spectres du polymère I démontrent l'absence de groupes N - acétyles et sont en bien des points pratiquement indiscernables de ceux de l'héparine; le polymère II comprend une quantité moyenne de groupes N - acétyles; l'hexosamine du polymère III est presque complètement N - acétylée. Le polymère III est composé principalement d'acide β-D-glucuronique et de 2-acétamido-2-deoxy-α-D-glucose. Les déplacements chimiques, constantes de couplage, propriétés de relaxation du carbone-13 et les résultats enzymatiques obtenus pour les résidus de l'acide D-glucuronique de l'héparane et d'un disaccharide qui en est dérivé, sont compatibles avec l'assignation d'une configuration β et de la conformation $^4C_1$(D). Les données ^{13}C.m.r. corroborent les preuves antérieures en ce sens que l'unité hexosamine possède la configuration et ainsi que la conformation $^4C_1$ (D).

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LIST OF ABBREVIATIONS

∿	-	approximately
< , >	-	less than, greater than
Me	-	CH_3-
Et	-	CH_3-CH_2-
A_c	-	CH_3-CO-
GGS	-	glycosaminoglycan sulphate
PG	-	proteoglycan
GP	-	glycoprotein
Ch 4-S	-	chondroitin sulphate A
CH 6-S	-	chondroitin sulphate C
DS	-	chondroitin sulphate B
KS	-	Kerato sulphate
HA	-	hyaluronic acid
Cl	-	chondroitin
H	-	heparin
HS	-	heparan sulphate
RNA	-	ribonucleic acid
mRNA	-	messenger RNA
PL	-	phospholipid
OP	-	oxidative phosphorylation
LPL	-	lipoprotein lipase
MI	-	myocardial infarction
DEAE	-	diethylamino ethyl ether of dextran
CPC	-	cetyl pyridinium chloride
CTAB	-	cetyltrimethyl ammonium chloride

HSLC = HPLC = high speed (pressure) liquid chromatography

T.l.c.	-	thin layer chromatography
r.p.m	-	rounds per minute
m.p.	-	melting point
NaOH	-	sodium hydroxide
Na^+	-	sodium ion
Ba^{2+}, Ca^{2+}	-	barium ion, calcium ion
Hg^{2+}	-	mercury ion
Gd^{3+}	-	gadolinium ion
DMSO	-	methyl sulphoxide
w	-	weight
v	-	volume
g	-	gm, gram
h	-	hr, hour
k	-	10^3, kilo
M	-	10^6, mega
n.m.r.	-	nuclear magnetic resonance
p.m.r.	-	^{1}H.m.r., proton magnetic resonance
c.m.r.	-	^{13}C.m.r., carbon-13 magnetic resonance
^{2}H, D	-	deuterium
D_2O	-	deuterium oxide
pH	-	negative logarithm (to the base 10) of the hydrogen ion concentration, $(H^+_{(aq)})$
pD	-	negative logarithm (to the base 10) of the deuterium ion concentration, $[D^+]$
Hz	-	Hertz, cycle per second
p.p.m. or δ	-	parts per million

C.A.T.	-	computer averaged transient
(P.) FT	-	(pulsed) Fourier transform
F.I.D.	-	free-induction decay
R.F.	-	radio frequency
N.O.E.	-	nuclear Overhauser enhancement
J	-	coupling constant (Hz)
T.M.S. or TMS	-	tetramethyl silane
T.M.T. or TMT	-	tetramethyl tin
CS_2	-	carbon disulphide
μ	-	magnetic moment of the nucleus
H_o	-	magnetic field strength
H_e	-	local magnetic field
#	-	number
ν	-	frequency of exciting radiation
U and UH	-	carbon and hydrogen, respectively of a uronic acid
G	-	U of D-glucuronic acid
I	-	U of L-iduronic acid
A and AH	-	carbon and hydrogen, respectively, of acetamido-deoxyhexose
S and SH	-	carbon and hydrogen, respectively, of deoxy sulphaminohexose
M	-	carbon of 2,5-anhydromannose

LIST OF COMPOUNDS

Compound

1. β-D-glucuronic acid
2. α-L-iduronic acid 2-sulphate
3. D-Glucose
   - 3a. 2-Deoxy-2-sulphamino-D-glucopyranose 6-sulphate
   - 3b. 2-Acetamido-2-deoxy-D-glucopyranose
   - 3c 2-Amino-2-deoxy-D-glucopyranose 6-sulphate
4. D-Galactose
   - 4a. 2-Acetamido-2-deoxy-D-galactopyranose
5. D-Xylose
6. Constituent disaccharide of heparan
7. Disaccharide obtained from heparan by nitrous acid deamination
8. 2,5-Anhydro-D-mannose
9. D-glucuronolactone
10. 1,2-O-Isopropylidene-α-D-glucurono-6,3-lactone

TERMINOLOGY

Glycosaminoglycan	-	acidic mucopolysaccharide
Chondroitin 4-sulphate	-	chondroitin sulphate A
Chondroitin 6-sulphate	-	chondroitin sulphate C
Dermatan sulphate	-	chondroitin sulphate B
Keratan sulphate	-	Kerato sulphate
Heparan sulphate	-	heparitin monosulphate
Polysaccharide	-	condensation polymers of monosaccharides resulting from the formation of glycosidic linkages by elimination of water
Mucopolysaccharide	-	aminosugar and uronic acid containing polysaccharide
Heteroglycan	-	polysaccharide containing two or more types of monosaccharides
Homoglycan	-	polysaccharide containing one monosacharide component
Glycoprotein	-	monosaccharides or oligosaccharides covalently bound to one or more protein chains. The protein moiety is the dominant feature of the macromolecule
Proteoglycan	-	macromolecule in which one or more polysaccharide chains are covalently bound to one or more protein chains

CHAPTER 1

GENERAL BACKGROUND

The physiological importance of glycosaminoglycans cannot be overemphasized[1,2]. Small wonder numerous investigators and research enthusiasts are engaged in studies pertaining to these heteropolymers. As such, the biochemistry, physiology and structure of these polymers, have been subjected to intensive studies in the past two decades. In spite of all of these efforts, structural and stereochemical characterization continue to evade complete clarification.

Glycosaminoglycans are proteoglycans which belong to a category of glycoproteins. They occur in a wide variety of organisms. Several of these polymers, such as chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate, hyaluronic acid, heparin, heparan sulphate and keratan sulphate, have been recognized in mammalian tissue. They are constituted primarily of uronic acid and hexosamine residues, which may have O-sulphate, N-sulphate and/or N-acetyl substituents, and exhibit considerable heterogeneity. Also, variations occur in their attachment to protein, molecular size and charge distribution. In this report, heparan sulphate, previously known as heparitin monosulphate and which is less sulphated than heparin, with which it has commonly been associated, will be identified simply as "heparan".

Difficulties in separating glycosaminoglycans in a native state from protein attachment and from other constituents of connective tissue, and the complexity of their structures, are the primary problems. Since, according to various reports, heparan is the most heterogeneous glycosaminoglycan, the study of its structure is beset by these problems to a greater extent.

Heparan has been isolated from beef-lung, α-aorta, human intestine and amyloid liver, and the urine of patients with mucopolysaccharidosis. Unlike heparin, heparan has been found in virtually all tissues examined to date.

The heparan used in this study is a by-product from a commercial preparation of heparin.

This heteropolymer is of obvious biological interest owing to its involvement in disease processes such as amyloidosis[4-6], atherosclerosis[7], mucopolysaccharidoses[1,8-9,27], its presence in blood-vessel walls and cell membranes, and its close structural resemblance to, as well as differences from, heparin the anti-coagulant and antilipaemic agent. The anti-coagulant activity of heparan, however, is very low compared to that of heparin. Such varied physiological roles for heparan necessitate extensive fractionation and detailed characterization of the polymer.

It is of interest to know if the reported macro and micro heterogeneity of heparan is apparent or real. In addition, it is desirable, (a) to establish if this glycan is truly a block heteropolymer, (b) to identify precisely the constituent subunits, and (c) to arrive at a definite pattern of arrangement of these units. The specific distribution and orientation of charged groups within a polymer chain affect the interaction of the polysaccharide with charged molecules in its biological environment. Therefore, such chemical studies on composition and structure of this anionic mucopolysaccharide are necessary supplements to the acquisition of biochemical knowledge about pathways of anabolism and catabolism in the mammalian systems, and particularly in human beings[10-12].

Despite the complexities of isolation and purification, a number of structural features have been established. Heparan contains N-acetyl glucosamine (2-acetamido-2-deoxy-D-glucose), glucosamine N-sulphate (2-deoxy-2-sulphamino-D-glucose), glucosamine N-, O-6-disulphate (2-deoxy-2-sulphamino-D-glucose 6-sulphate), D-glucuronic acid, L-iduronic acid[13], and probably N-acetyl glucosamine 6-sulphate (2-acetamido-2-deoxy-D-glucose 6-sulphate)

residues. From studies of the heparans isolated from patients with Hurler's disease, it was concluded that heparan is a block heteropolymer with at least two units. One unit is covalently linked to serine and contains mostly N-acetylated glucosamine residues. The other unit, which is smaller in molecular size contains no significant amounts of serine and N-acetyl groups, has mostly N-sulphated glucosamine residues[14]. The favored view is that heparan is a block heteropolymer[15,16] with some units free of sulphate groups and containing N-acetyl glucosamine; other units are intermediate in O-sulphate content and contain N-sulphated-D-glucosamine; while still others are high in both N- and O- sulphate groups.

However, detailed structures are not available. Seemingly contradictory reports on the constituent residues and their conformation, and the configuration of the glycosidic bonds abound.

Both two[17] and three[18] sulphate groups per disaccharide have been reported. The bond between glucuronic acid and hexosamine residues has been reported to be α by some[19] and β by others[20] with concomitant high and low optical rotations[13,21-25]. Measurements of the total N-acetyl and N-sulphate molar ratios to hexosamine residues are claimed to be higher than one[13,23]. The presence of L-iduronic acid has been reported[13,23]. Molecular sizes ranging from as small as 3×10^3 to as large as 4×10^4 have been measured for heparan. Although it is known[13] that uronic acids differ in their susceptibility to degradation under conditions of acid hydrolysis, ratios of glucuronic/iduronic acids have been reported without allowing for such differences.

These discrepancies necessitate a clearer definition of heparan and also a specification of the source, the method of extraction, fractionation, purification and analysis.

An important aspect of the problem concerns the possible relationship of heparan to heparin. That is, heparan is commonly obtained from crude preparations of heparin, and the two polymers are considered to have some structural features in common. Nevertheless, heparin is chiefly found in mast cells, whereas heparan can be isolated from various connective tissues which have few mast cells. Some suggestions[2] have been advanced in attempts to relate these two glycosaminoglycans. Heparan comes from circulating heparin-like mucopolysaccharide synthesized by mast cells and discharged into the blood or extracellular spaces to be picked up and stored in a variety of tissues[2]. Alternatively, the two polymers are part of a continuum of polysaccharides that varies over a wide range in their N-sulphate and total sulphate contents[2].

The polydispersity and high charge density of heparan are held responsible[13] for difficulties experienced in separating it from other glycosaminoglycans and in subsequent fractionation. Thus, anomalous behaviour may be expected in using precipitation with reagents such as cetyltrimethyl ammonium bromide or cetyl pyridinium chloride, which depend on charge distribution. Analyses of composition based on acid hydrolysis are unreliable, as noted above, because of the sensitivity of uronic acid residues, and colorimetric analysis of these residues is limited by the empirical nature of the carbazole[37] and orcinol[38] methods. The most detailed structural information about heparan has come from investigations on the products obtained by degradation, either with nitrous acid[23,26], or with eliminase enzymes from Flavobacteria[19,28-30].

In principle, an ideal approach to the chemistry of heparan should involve as little modification of the polymer as possible. N.m.r. spectroscopy is a non-destructive method that has been used extensively in structural studies of glycosaminoglycans[32,33], such as heparins[33,34] and chondroitins A, B. and C[35].

It can readily differentiate between the heparin types of structure (A and B) and the heparan type, and hence is an effective technique for monitoring the fractionation of heparan from heparins[36]. Differences in the intensity of the methyl signal associated with variations in the acetamidodeoxyhexose content of these polymers can be followed by p.m.r. spectroscopy even at 100 MHz, and greater detail is revealed at a higher, e.g. 220MHz, resonance frequency[33]. These heteropolymers commonly give well-resolved ^{13}C.m.r. spectra, which should facilitate an even more complete characterization, and detection of fine structural features. Furthermore, coupling the spectroscopic technique to classical chemical and enzymic methods should enhance the reliability of this information. With this approach, a re-examination of the structural features of heparan has been undertaken, and forms the subject-matter of this thesis. As will be shown, it has been possible to clarify some discrepancies that existed before, and to add materially to our knowledge of the structure of heparan.

CHAPTER II

HISTORICAL INTRODUCTION

An attempt will be made to deal with heparan, in an introductory fashion, in terms of historical landmarks, biological significance, clinical implications and methods used to elucidate its structure.

II. 1 Historical Note

The isolation of heparan was achieved in 1948 by Jorpes and Gardell[25]. The large quantities of this dextrorotatory polymer isolated from urine of patients suffering from mucopolysaccharidosis fanned the interest in heparan. Hunter provided the first clinical description of a mucopolysaccharide storage disease and two years later, another example was described by Hurler[40]. These two forms of disease were later known as Hunter Syndrome and Hurler Syndrome. However, nearly forty years passed before the connection between these diseases and heparan was established. Brante[41,42] coined the term "mucopolysaccharidosis", in 1952, when he reported the presence in large amount of a sulphated mucopolysaccharide of, apparently, simple constitution and related to chondroitin sulphate, in a liver specimen of a patient with Hurler Syndrome.

These findings initiated vigorous attempts to isolate and study the structure of the compounds from organs of mucopolysaccharidosis patients. In 1957, Brown examined the mucopolysaccharide isolated from patients with Hurler's disease[43]. Fractions it of contained glucosamine, glucuronic acid, both N-acetyl and N-sulphate groups in a ratio of approximately 2:1, and O-sulphate in varying amounts. They exhibited high positive optical rotations, indicating the presence of residues with α-configurations, and had low molecular weights. It was concluded that this is heparan, and a principal storage compound characteristic of the liver of patients with Hurler's disease. The glycosaminoglycan constituted 0.5-1.5% of the wet weight of the organ of these patients. In the same year Dorfman et al. reported the presence of

heparan in the urines of these patients[44]. This was confirmed by the findings of Meyer and his associates in subsequent years[11]. They found that from 70% to over 90% of the total mucopolysaccharide fraction from livers of these patients was heparan and the rest was dermatan. Encounters with heparan were not confined to the field of mucopolysaccharidosis, but occurred in the study of heparin. Heparin was discovered in 1916[45], but it was long after that heparan was recognized as a material accompanying heparin isolated from various tissues, e.g., lung and liver[25,46].

Comparison of the molecular sizes of heparan from mucopolysaccharidosis patients and from normal tissues led to the conclusion that the accumulation of heparan in mucopolysaccharidosis syndromes is a defect in degradation rather than a defect in synthesis[27]. Dorfman et al. concluded that two chains of Hurler heparan, different in molecular size, are formed by partial enzymatic hydrolysis of a parent heparan molecule[14]. Furthermore, it was hypothesized that heparan exists in the tissues as a proteoglycan. This idea arose from the observation that, when isolated after proteolysis of the tissues, heparan contained serine as the main residual amino acid[47] as well as galactose and xylose[14]. The interest in heparan naturally shifted to the biosynthesis and occurrence of this polymer in tissue cells.

Kraemer and others propounded that heparan is synthesized by cells other than mast cells. This heteropolymer was isolated, for example, from Chinese hamster cells grown in suspension culture[48], and a variety of established lines of cells grown in culture were used to study the production of heparan[8,49,51]. Much of the heparan was located in the external membrane and shown to be discharged into the growth medium as the proteoglycan[50]. It was concluded, therefore, that heparan may be present on the external surface of all cells[8]. With the tentative establishment of its synthesis and localization,

speculation moved to the function of heparan in vivo.

In 1973, it was pointed out that heparan has a possible primary role in protecting the cell and immediate environment from coagulation and fibrinolysis mechanisms[52]. The part played in fibrinolysis inhibition as a check on the spread of normal cells, as opposed to fibrinolysis by malignant cells, was considered to be of special interest. Other possible roles of heparan in lipolysis and atherogenesis cannot at this juncture, be ruled out. Findings of great importance in such major subjects as clotting, atherogenesis and cancer may emerge from studies in these directions. As such, it is worth while to review the clinical implications of the glycosaminoglycan.

II 2 Clinical Implication and Significance of Heparan

II 2.1 Introduction

No direct evidence for the biological role of heparan is available. The obvious interest in this heteropolymer is due to some structural features it has in common with heparin or, indeed, to its possible occurrence in heparin preparations and also its involvement in mucopolysaccharidosis syndromes.

A-type heparin, seemingly contains heparan in proportion to its content of acetamidodeoxy glucose and glucuronic acid. Although B-type heparin has a much lower content of such residues, the clinical use of both A-type and B-type heparins indiscriminately, raises interesting questions relevant to heparan. It is not known, however, if this polymer contributes to the properties and functions attributed to heparin. Nevertheless, because of the widespread view that the two mucopolysaccharides are closely associated, it is worthwhile, in working with heparan, to be familiar with the biochemistry of heparin as well. Extensive, reviews of this latter subject are available in the literature[2,53].

II. 2.2 Mucopolysaccharidoses and Heparan.

The clinical significance of heparan not only is derived indirectly from the action of heparin, but also is implied directly, as in mucopolysaccharidoses.

Mucopolysaccharidoses consist of five or more syndromes. These disorders are genetically distinct and are characterized by a wide range of phenotypic expression. The most common mucopolysaccharidoses are the Hunter, Hurler, Scheie and Sanfilippo Syndrome.

Recognition and description of other mucopolysaccharidoses seems to be an on-going process. Among the recently described mucopolysaccharidoses are Morquio, Maroteaux-Lamey, and Sly Syndromes[7,25]. Their discovery is so recent that not much is known about these disorders. The patients with Morquio Syndrome are strikingly dwarfed, develop a characteristic stature, severe neurological systems, corneal clouding and may excrete excessive amounts of mucopolysaccharide. This particular syndrome is also known as Mucopolysaccharidosis IV. Patents with Maroteaux-Lamy Syndrome (Mucopolysaccharidosis VI) show characteristics that resemble those of patients with Hurler's disease in skeletal abnormalities and the presence of corneal opacity. These patients excrete large amounts of dermatan sulphate.

Sly Syndrome patients show profound hepatosplenomegaly, the leukocytes are deficient in β-glucuronidase activity and the skin fibroblasts have just 2% of the β-glucuronidase activity of normal cells. The mucopolysaccharides isolated appear to be chondroitin sulphates A and B.

The only known mucopolysaccharidosis transmitted as an x-linked recessive trait was first described by Hunter in 1917[1]. The patients with this syndrome (Mucopolysaccharidosis II) have dwarfed stature, protruberant abdomens, grotesque facial features and deformed hands. They store and excrete

10-40 times as much dermatan sulphate and heparan as that excreted by normal people.

Mucopolysaccharidosis III was first described by Hurler in 1919[40]. The patients show deformed features, clouding of the cornea, mental retardation, and conspicuous gibbus. The last three features are absent in Hunter Syndrome. Ten to forty times the amount of dermatan sulphate and heparan are excreted by these patients as by normal beings.

Sanfilippo Syndrome is also known as Mucopolysaccharidosis III A and IIIB . Patients with this syndrome show relatively mild skeletal abnormalities, severe disturbance of central nervous system function and severe mental deterioration. The typical characteristic is the urinary excretion of a large amount of heparan as the only mucopolysaccharide. The two types of this syndrome are phenotypically indistinguishable[1]. The degradative enzymes are N-sulpho-D-glucosamine sulphatase[54] for type A and α-N-acetylglucosaminidase[55] (EC 3.2.1.50) for type B.

II. 2.3 Correlation between structural parameters and biological activity.

Numerous attempts to correlate the chemical and structural parameters of heparin and heparan with biological activity have been made. They provide an interesting example of how chemical modifications of a polymer can affect, sometimes in a dramatic way, the normal functioning of the macromolecule.

Charge density is considered to be the dominant property. The charge on these mucopolysaccharides comes from the following units: N-SO_4^-, O-SO_3^- and COO^-, i.e., sulphamino, ester sulphate and carboxyl groups. Anticoagulant activity is reported to be related to the degree of sulphation as well as molecular conformation[56]. Foster and others reported that the N-SO_4^- of heparin is critical for its anticoagulation[57,58]. The ability of heparin to inhibit

translation of a natural mammalian mRNA in a mammalian cell-free system was found to be related to the number of N-SO_4^- and O-SO_3^- groups in the molecule[2,61]. The role played by the degree of sulphation has been examined by removing sulphate groups. Thus, mild acid treatment of heparin results in progressive loss of sulphate groups, especially N-SO_3^-[59,60]. During the first few hours only N-SO_3^- is removed[59], but substantial hydrolysis is found after 24 hours of treatment[61]. Fifty percent of the anticoagulant activity is lost during the first hour of treatment, and 90% in three hours[60]. By contrast, the ability to inhibit protein synthesis is unaffected after four hours' treatment with acid. However, it should be added that heparin which is less sulphated than a highly substituted dextran sulphate, has greater anticoagulant activity[62]. Furthermore, inhibition of protein synthesis initiation is specific to heparin. Hence, the degree of sulphation per se is obviously not the critical factor.

The importance of the carboxylic acid group has also been evaluated[4]. For example, Danishefsky studied the effect of esterifying the carboxyl group, and suggested that the free carboxyl groups of heparin are critical for all the inhibitory sites[63].

A look at the contents of sulphate, N-acetyl and carboxyl of these and other mucopolysaccharides suggests an interesting perspective. It has been stated above that there are two types of heparins, A and B, with the hexosamine component of the former being more N-acetylated. Thus the decrease in the sulphation is of the order heparin B, heparin A and heparan. The level of uronic acid content is about the same in all, although differences are found in their conformation, i.e., between iduronic and glucuronic acids. The strongly anionic nature and associated degree of hydration of these polymers is probably at a relatively uniform level. Now the effect of long term and high dose administration of these mucopolysaccharides is osteopenia,

i.e., demineralization of bones. However, inhibition of protein synthesis is specific to heparin which also has high anticoagulant activity. The other mucopolysaccharides are, to all intents and purposes, considered to display no such activity.

In view of these considerations, one is prompted to correlate osteopenia with chemical and structural parameters common to all the mucopolysaccharides, such as, for example, their carboxyl groups. That is, presumably, the carboxyl groups efficiently sequester calcium ions from bone. Inhibition of the initiation of protein synthesis and anticoagulant activity could be related to parameters, such as the sulphamino group, present in heparins but not so prominent in the other glycosaminoglycans.

However, if the sulphamino group were the major determinant of the above stated activities, the order of potency should follow the degree of N-sulphation. In fact, the anticoagulant activity of type B heparin should be much higher than that of type A which, in turn should not vary as dramatically as is observed from that of heparan. Yet heparan has no anticoagulant activity of any significance, whereas both heparins are clinically used indiscriminately. Therefore, a closer look at the chemical, structural and, indeed, conformational differences among heparin A, heparin B and heparan, and also at the correlation between these parameters and biological activities are imperative.

II.3 The Relationship among the glycosaminoglycans (GGS)

These high molecular weight condensation polymers of amino and acidic sugar monosaccharide derivatives are somewhat related. Glycosminoglycans are proteoglycans, a category of glycoproteins. In proteoglycans the dominant feature of the macromolecules is the polysaccharide, whereas in other glycoproteins it is the protein moiety[3,64].

There are several features common to the seven known different types of GGS. They occur in vertebrate tissues, and are linear polymeric chains of alternating hexosaminyl and hexosyluronic acid residues. This is the predominant sequence. The only exception to this sequence is in keratan sulphate where galactose replaces hexuronic acid. Structural studies of these compounds are beset with difficulty in determining purity, insufficiency of material, lack of procedures that permit selective and high yield degradation of the polymers, or better still, study in the native state. Consequently, these heteropolymers have only been partially characterized.

The dissimilarities among the GGS are equally extensive. The variations occur in chain length, configuration of residues, mode of glycosidic linkages, location of O-sulphate groups, contents of the acetamido group and sulphamino group. Charge density varies from one to four per disaccharide. Some degree of dissimilarity exists in association with amino acids and proteins. Table I is a summary of the composition and structure of acidic GGS.

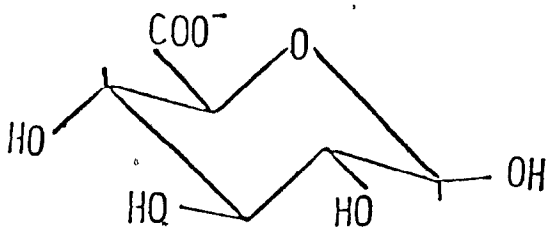
Although there is a multitude of stereoisomeric monosaccharides, only a few are found in natural polysaccharides, and even fewer monosaccharide derivatives are encountered. There seems to be some naturally imposed simplification.

Uronic acids or their sulphate derivatives are present in over 85% of the known GGS and are in the pyranoid form. D-glucuronic and L-iduronic acids are the only uronic acids isolated from acidic GGS to date. The former is present in all, but the latter in less than 50% of the acidic GGS. There is no direct evidence that the uronic acid carboxyl group is esterified with either a primary alcohol or a hydroxyl group of another polysaccharide molecule. These acids occur both as interchain and reducing units, see Table I and Fig. I.

TABLE I

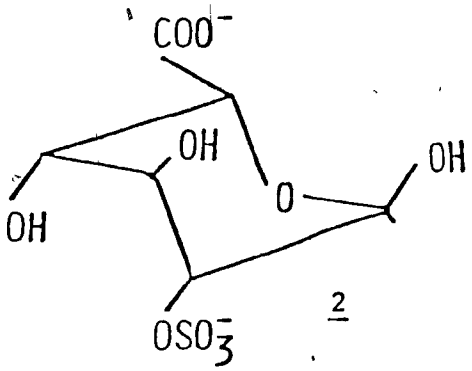
Summary of composition and structure of acidic Glycosaminoglycuronans

Properties	Heparin	Heparan	Hyaluronic Acid	Chondroitin 4-Sulphate	Chondroitin 6-Sulphate	Dermatan Sulphate
Uronic acid component						
D-Glucuronic acid	+	+	+	+	+	+
L-Iduronic acid	+	+				+
Configuration α-L or β-D	+	+	+	+	+	+
Substituent:						
Sulphate at C-2 of L iduronic acid	+	+				+
Glycosyl residue at C-4	+	+	+	+	+	+
Amino sugar component						
2-Amino-2-deoxy-D-glucose	+	+	+			
2-Amino-2-deoxy-D-galactose				+	+	+
Configuration α-D	+	+				
β-D			+	+	+	+
Substituents:						
N-Acetyl	+	+	+	+	+	+
N-Sulphate	+	+				
O-Sulphate	+	+		+	+	+
O-4				+		+
O-6	+	+			+	
Glycosyl residues at:						
O-3			+	+	+	+
O-4	+	+				
Other sugar residues including those in the linkage region						
D-xylose, D-galactose	+	+	?	+	+	+



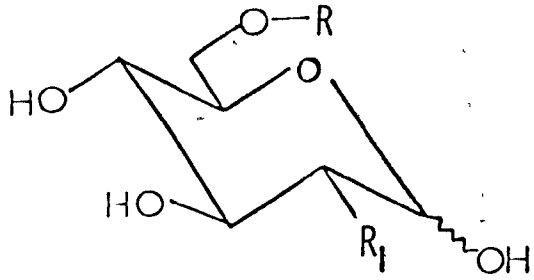
1

β-D-glucuronic acid



2

α-L-iduronic acid 2-sulphate



3, R = H, R_1 = OH

D-glucose

3a, R = OSO_3^-, R_1 = $NHSO_3^-$

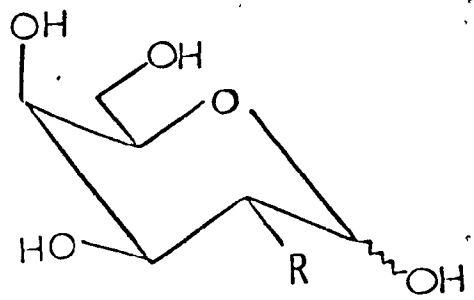
2-deoxy-2-sulphamino-D-glucopyranose-6-sulfate

3b, R = H, R_1 = $NHCOCH_3$

2-acetamido-2-deoxy-D-glucopyranose

3c, R = OSO_3^-, R_1 = NH_2

2-amino-2-deoxy-D-glucopyranose-6-sulfate

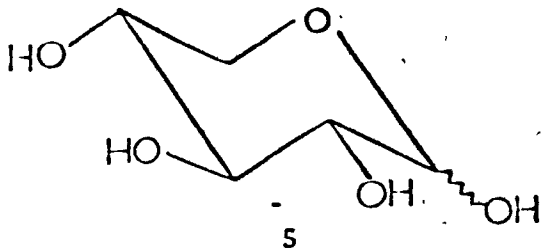


4

R = OH, D-galactose

R = $NHCOCH_3$,

2-acetamido-2-deoxy-D-galactopyranose



5

D-xylose

FIG. 1 Monosaccharides and their derivatives found in glycosaminoglycans.

At present there is no evidence that more than two hexosamine residues occur in GGS. The hexosamines isolated to date are glucosamine and galactosamine. As shown in Fig. I, the amino groups of the hexosamine residues are generally N-acetylated. The 3-,4- and 6- OH groups can be free, O-esterified with sulphate or bound to a hydroxyl group of another sugar residue.

The acidic GGS are bonded to a protein chain by a characteristic trisaccharide unit of galactose, xylose and serine[14,64-66]. The xylose residue is glycosidically linked to serine[67].

II.4 Methods.

II.4.1 Introduction.

Several different types of methods are used in structural studies of GGS. The isolation and degradative procedures constitute the primary process. In both procedures, physical methods may or may not be involved.

The isolation procedure involves alkaline extraction from the protein of the vertebrate tissue, and purification of the mixture of polysaccharides so obtained. The three main extractive procedures in use are disruptive, dissociative and sequential extraction[68]. GGS free of protein is isolated from the intact proteoglycan by base-cleavage of the xylosyl-serine linkage, which is accomplished by a β-carbonyl-elimination reaction[69]. Deproteinization can also be achieved by digestion with proteolytic enzymes. Such enzymes are pepsin, trypsin, papain and pronase[70]. The mixture of amino acids and peptides is separated from the polysaccharide by dialysis, adsorbents or by organic solvent precipitation[71].

A variety of rather similar methods for the isolation and purification of GGS from one another is reported. Separation of acidic polysaccharides from each other and also from neutral polysaccharides can be achieved by precipitation with quaternary ammonium salts[72] such as CTAB and CPC[68].

Ion-exchange chromatography on columns of DEAE-cellulose, and DEAE-Sephadex, or OCTEOLA-cellulose or resins may also be used[73]. Acidic polysaccharides are adsorbed on the anion exchangers at pH 6. Elution is achieved by increasing the buffer concentration or pH of the eluent. Separation according to molecular weight differences can be done by passing through columns of various types of Sephadex or poly(acrylamide) gels. Use of the electrophoretic methods to separate and purify polysaccharides is also reported[74].

Color reactions for some of the component residues in the polysaccharides have been developed. These reactions are useful for preliminary studies. In some instances, colorimetry has been used for following the purity of the isolated polymer[38,39].

Degradative procedures may be only partial, or may involve complete depolymerization to monosaccharides. These processes can be achieved by chemical and/or enzymic means, and depend on the nature of the linkages in the biopolymer.

II.4.2 Chemical Methods.

The general classical procedures utilized (see ref. 24,31,72) to obtain data on the type and sequence of units, and linkages, configuration and conformation are, Barry degradation, deaminative hydrolysis, methylation, immunochemical methods, periodate and lead tetraacetate oxidation. In this study nitrous acid deaminative degradation was found most useful and, indeed, was employed extensively. Hence the supposed mechanism of this reaction will be described, though briefly .

II.4.2.1 Nitrous Acid Deamination.

Deamination of hexosamines by nitrous acid has been used for the estimation of aminosugars for many years[75]. This technique is useful for structural studies of hexosamines with free amino or sulphamino groups. When

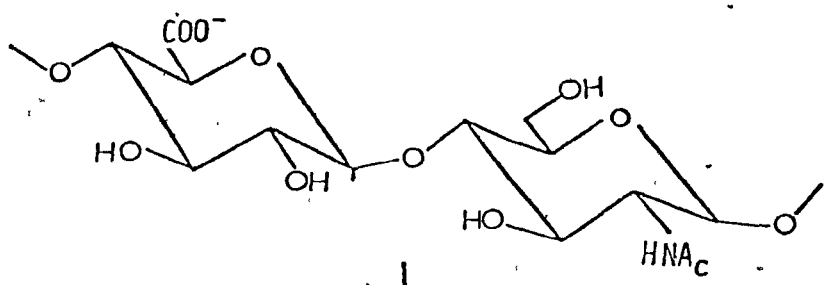
acetamido groups are present it is necessary to N-deacetylate prior to carrying out the deamination (Fig. 2).

The mechanism of deamination is proposed on the basis of observations made in related studies. Deamination of primary amines involves a diazonium intermediate[76]. This intermediate forms a carbonium ion and substitution with inversion occurs. Using cyclohexanyl, monosaccharide and disaccharide[77] derivatives, several features of this reaction have been formulated. The course of the reaction depends on the group which is antiparallel to the nitrogen atom and also trans to the C1-C2 bond. When the amino group is equatorial, the reaction forms a five-membered ring. This applies to both 2-amino-2-deoxy-α-, and β-D-glucopyranose. However, the β form, with an equatorial glycosidic group, reacts faster than the α[78]. In these residues, the moieties antiparallel to the equatorial amino group are the ring oxygen atom and C4. Rupture of the oxygen ring is preferred to that of the C4-C3 bond and the main end product is 2,5-anhydro-D-mannose (chitose)[78]. The reaction is not hindered by blockage of the glycosidic group.

Although this reaction proceeds in good yield, it is not quantitative and an alternative ring contraction with the formation of methyl 2-deoxy-3-C-formyl-D-ribofuranosides as minor products has been reported[77]. With axially oriented 2-amino-2-deoxyglycosides, this reaction, in contrast to the mechanism followed by equatorial amines, proceeds with the formation of aldose residues with altered configuration at C-2. The glycosidic linkage remains unaffected[79].

II.4.2.2 Electrostatic Shielding of Glycosidic Bonds (see, e.g., ref. 24)

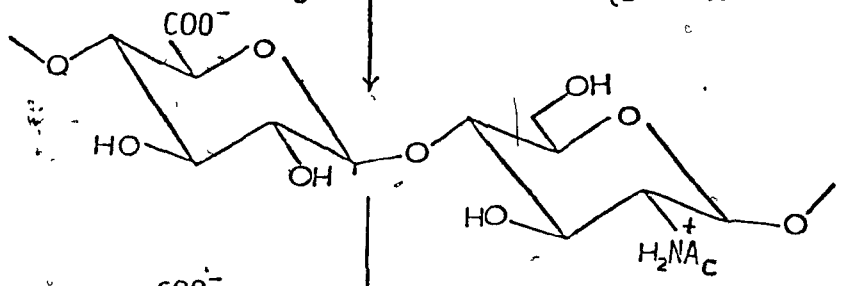
Preferential partial hydrolysis of GGS depends on the polar substituents. D-Glucopyranosyluronic acid residues are not readily hydrolysed from the chain residue to which they are glycosidically bound. The stability of the glycosidic bond to acid-catalyzed hydrolysis is so great that extensive destruction of

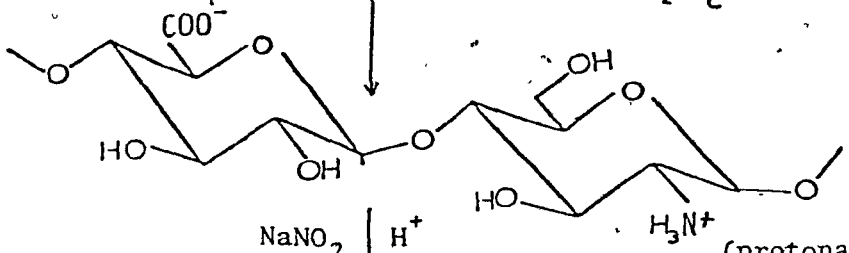


6

(2-acetamido-2-deoxyhexosamine residue)

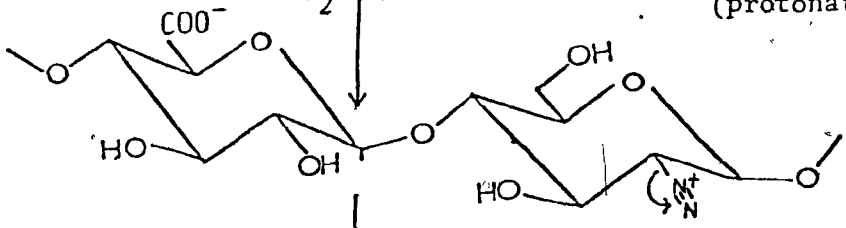
H_3O^+



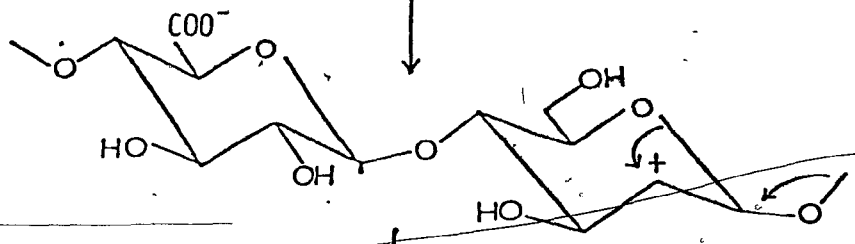


(protonated amine)

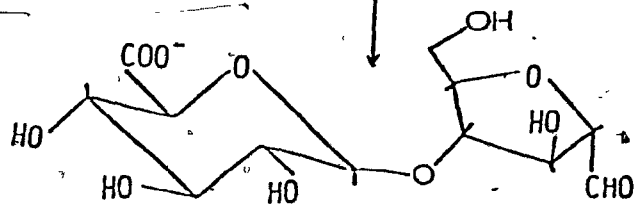
$NaNO_2$ H^+



(diazonium intermediate)



(carbonium ion)



7

(2-5-anhydro-D-mannose residue)

FIG. 2 The possible mechanisms of acidic N-deacetylation/desulphination and HNO_2 deamination of a glycosaminoglycan.

the uronic units occurs on attempts to force hydrolysis of the chain to completion. Cleavage of this bond by acid-hydrolysis can only be easily achieved after the reduction of uronic acid residue to normal hexose units. In N-acetylated hexosamine residues, the relative resistance of glycosiduronic acid linkages to hydrolysis leads to the isolation of aldobiuronic acids. The reverse situation obtains if free animo groups are present, or are readily formed, when protonated amino groups provide an electrostatic shielding of hexosaminidic linkages.

Explanation for the high acid stability of the D-glucopyranurosyl-oxy bond is postulated as follows: the ease with which the electron pair constituting the bond can be removed entirely from the influence of the sugar residue determines the rate of decomposition of the conjugate acid. Secondly, an inductive effect, arising from the presence of an electronegative group, e.g. carboxyl, at position C-5 of the sugar molecule, opposes the withdrawal of the electron-pair. The same argument can be used to explain preferential hydrolysis of a glucosyl→uronic acid bond.

Generation of an electronegative center on C-2 of a glycosamine unit by acidic N-desulphation/deacetylation of this unit, will strengthen the glycosaminidic bond to acidic hydrolysis.

Such electrostatic shielding of the glycosidic linkages has been utilized to great advantage in this study. Thus acid N-desulphation and N-deacetylation are used without any fear of depolymerizing heparan. The acid hydrolyzes the N-sulphate or N-acetate substituent and exposes the amino group which subsequently protects the hexosamine linkage from being hydrolyzed by the acid. The carboxyl group protects the uronosyl linkage. Application of nitrous acid deamination, then, selectively hydrolyses the hexosamine linkage, leaving the uronosyl bond intact. Thus nitrous acid deamination, one of the few reactions which results in the selective cleavage of glycosidic

linkages under mild conditions, was invaluable to this study.

II.4.3 Enzymic Methods.

Various enzymes may also be employed to degrade polysaccharides. There are two aspects of applications of enzymes. They are employed for graded hydrolysis to specific fragments. When the specificity is known, they can be used to determine the configuration of glycosidic linkages.

Most of the enzymic applications in GGS chemistry are of the first type. This limitation is due to the considerable difficulty encountered in obtaining enzyme extracts of sufficient purity for specificity studies. Examples of this type are furnished by hyaluronidases, chondroitinases, heparinases and heparanases[36].

An enzyme system that degrades heparin and heparan is induced from Flavobacterium heparinum: This is a preparation obtained from a soil bacterium grown on a medium containing N-acetyl heparin[80]. The crude extracts contain α- and β-eliminases[30,81-83], sulphatases[84] and glycuronidases[81] which degrade these polymers to unsaturated oligosaccharides, disaccharides and finally to monosaccharides, respectively. However, the presence of a hydrolase in these same extracts is reported[85]. This discrepancy is interpreted by Hovingh to be due to differences in purity and enzyme/substrate ratios used[30].

Sufficient information about D-glucuronic acid hydrolases or β-D-glucosiduronases [E.C. 3.2.1.31] (also known by the obsolete trivial name "glucuronidase") is available[86,87]. This warrants the use of this enzyme in specificity studies. These enzymes are localized in subcellular particles and hydrolyze the glycosides of D-glucuronic acids. That the linkage broken is that between the C1 of the acid and the oxygen bridge was confirmed for β-D-glucosiduronases[89]. Other examples of hydrolyases are 2-acetamido-2-deoxy-α- or -β-D-hexosidases also known by the obsolete trivial name "N-acetyl hexosaminidases"[88].

The activity of E.C. 3.2.1.31 is generally measured by the colorimetric method of Fishman[86], which uses phenolphthalein β-D-glucopyranosiduronic acid as a substrate. Studies of the aglycon specificity have revealed that β-D-glucosiduronases from different sources show similar rates of hydrolysis. This enzyme has no action either on glycosides of α-D-glucopyranosiduronic[86] acid or on α- or -β-D-glucopyranosides. It can be activated by deoxyribonucleic acid or albumin[86,87]. The hydrolysis by this enzyme is strongly and competitively inhibited by D-glucaric-1,4-lactone, various organic peroxides, to a lesser extent by D-glucuronic acid, several hydroxylated carboxylic acids and heparin, and heavy metal ions e.g., Hg^{++}[87]. The optinum pH is about 5 and temperature is 37°. The enzyme is markedly stable in the cold. When lyophilized and refrigerated it keeps for 6-12 months[86]. The initial velocity of hydrolysis bears a linear relation to substrate concentration at low substrate concentration. At high substrate concentration some inhibition is observed[86].

II.4.4 Physical Methods.

II.4.4.1 Introduction.

A good number of physical methods are employed in the characterization of GGS. Most of the physical methods aim to obtain parameters on molecular size, weight and distribution in a given preparation. Some of the methods used are electron microscopy, X-ray diffraction, viscosity and osmotic pressure measurements, ultracentrifugation, infra-red, optical rotatory dispersion, and chromatography. The main limiting factor for physical methods is resolution. N.M.R. is gaining in popularity since it yields invaluable information in a satisfactory non-destructive style.

II.4.4.2 N.M.R. (see ref. 90-97).

The intention here is not to present a theoretical treatment of N.M.R. spectroscopy; but to describe the advantages and some salient points relevant to the present study.

N.M.R. involves the transition of a nucleus from one spin state, say E_2 to another, say E_1. The energy, ΔE, absorbed during the transition or which would induce the transition if imposed is given by

$$\Delta E = E_1 - E_2 = h\nu = 2\mu(H_o \pm H_e)$$

where μ = magnetic moment of the nucleus

H_o = magnetic field strength

H_e = local magnetic field

h = Planck's constant

and ν = frequency of exciting radiation.

In reality, the interactions among neighbouring nuclear magnets, the inter-nuclear distances, the orientations of the nuclei and the phase state affect the local magnetic field experienced by the nucleus under consideration. The result is fluctuation and inhomogeneity of the local magnetic field.

Since various nuclei have different values of μ, they undergo spin transitions at different frequencies, in a given applied field. For instance, a field strength of 23 kG would permit ^1H spectra to be observed at 100 MHz and ^{13}C spectra to be recorded at 25 MHz. Introduction of superconducting magnet systems has made measurements of p.m.r. spectra at radiofrequencies in excess of 200 MHz possible. Applied fields commonly available are in the range 10-25 kG and hence resonance frequencies of most nuclei lie within the 50-100 megahertz margin. At such fields the Larmor frequencies are detectable by radio techniques. The experimental strategy consists of varying one of the two potential variable ν and H_o in the above equation, while maintaining the other constant. Thus, if H_o is kept constant, the oscillating radio frequency can be varied to detect the absorption of the energy by a particular nucleus. Alternately, fixing the frequency, ν, of the exciting energy, absorption would be observed by sweeping the magnetic field, H_o.

The use of n.m.r. spectroscopy on the study of biopolymers has picked up momentum progressively. This is due to the dramatic improvement in techniques, such as the introduction of the Fourier transform (FT). FT involves exciting all the spins of the nuclei under consideration by irradiating the sample by a series of short, powerful radio frequency pulses. The order of the pulse lengths is of μsecs. A computer Fourier transforms the accumulated resultant free induction decay (FID) signals from the time domain to the frequency domain. The result is a considerable improvement in signal to noise ratio for a given accumulation time. Pulse spectra and FT techniques make it possible to overcome the low sensitivity for detecting ^{13}C resonance signals, a consequence of the low, 1.1%, natural abundance.

What makes ^{13}C.m.r. so attractive is the large spread of chemical shifts about 200 p.p.m. mostly downfield from the TMS reference signal. In addition, the use of broad band proton decoupling, its concomitant nuclear Overhauser enhancement, (N.O.E.) and off-resonance partial decoupling, enhance ^{13}C signals and facilitate signal assignments. N.m.r. spectroscopy allows for direct detection and reasonably quantitative measurements of structural features, and also, is, on the whole, nondestructive and rapid. P.m.r. spectroscopy has the additional advantage of higher sensitivity and hence requires smaller quantities of sample. Despite all these attractions, there are some inherent difficulties in n.m.r., and limitations to its application in studies of biopolymers.

Low solubility and high viscosity of polymer solution augment, unfavourably, the inadequate level of detection of natural abundance ^{13}C resonance signals and line broadening, respectively. Therefore, larger quantities of material are required and, in some cases, elevated temperatures

have to be used. Relaxation times of the various carbons constituting a polymer chain are, among other conditions, dependent on the position of the respective carbons in the molecule. Hence, ^{13}C.m.r. integrated spectra do not provide as consistent measurements as those of proton spectra, and the experimental time needed to acquire ^{13}C.m.r. spectra is longer than that for p.m.r. spectra.

Stabilization of the field/frequency ratio depends on the lock signal whose strength and stability, in turn, limit the resolution. Usually the ^{2}H signal of heavy water or any appropriate deuterated solvent is used as the lock signal. Current practice is to use internal TMS as the reference signal rather than the once popular $^{13}CS_2$. The chemical shift of TMS is

$$\delta_{TMS} = \delta_{CS_2} - \text{(from 192 to 193) p.p.m.}$$

depending on the solvent.

^{13}C.m.r. is probably more useful than p.m.r. in the characterization of complex polysaccharides. The strategies employed in characterizing structures are by direct observation of the polymer itself and/or by studying the monomers, oligomers and pertinent model components of the polymer. Many general features of chemical shifts and coupling constants of carbohydrates are well established.

Carboxyl carbons are observed between 190 and 170 p.p.m., the respective shifts of $-C(=O)-O^-$ and $-C(=O)-H$. This is due to sp^2 hydridization and the highly deshielding effect of the oxygen nuclei attached to these carbons. The same deshielding effect is responsible for the display of the anomeric carbon signals between 100 and 85 p.p.m., the lowest field position for the ring carbons. The anomeric carbon bearing an axial proton is found at lower field than that bearing an equatorial one. The other ring carbons come between 80 to 70 p.p.m. Equatorial substituents on pyranose rings produce

slightly higher shifts. Primary alcohol carbons appear between 62 to 55 p.p.m.

O-Alkyl substituents on any ring carbon produce deshielding effects of 7 to 10 p.p.m. on the carbon to which they are bonded and about a 1 p.p.m. shielding effect on the adjacent carbons. The orientation of the -OH group, too, leads to substantial shielding effects. Steric crowding appears to be a dominant factor because, in general, axial substituents are associated with more shielded ^{13}C nuclei. Thus because of the shielding effects of a C-1 axial substituent at the α, β and γ positions, all the ring carbons other than C-4, appear at lower field in the β anomer relative to their positions for the α anomer. The C-6 carbons are almost equivalent in both anomers and appear at higher field.

Supplementing chemical shifts with coupling constants, allows for elucidation of configuration and conformation.

Couplings in general consideration are the direct, geminal and vicinal, i.e., couplings across 1,2 and 3 bonds, respectively. The J_{C-H} couplings are large and range from 140 to 180 Hz, values for anomeric centers being the largest. Anomeric carbons bearing equatorial protons give a J value ~10 Hz larger than do their axial counterparts. Long range couplings are generally much smaller, 0-10 Hz. There are several factors which influence coupling. Orientation of substituents on the carbon plays an important role on the size of geminal couplings. The gauche conformation is associated with large J values whereas the anti-conformation with values close to zero. Large vicinal $^{13}C-^{1}$couplings of up to 6 Hz are associated with the anti, or eclipsed, conformation, whereas smaller couplings characterize gauche conformations, i.e., there is a close analogy to the dependence of $^{1}H-^{1}H$ couplings on the dihedral angle.

These, and other, well established features of n.m.r. spectra facilitated this study.

CHAPTER III

RESULTS AND DISCUSSION

III.1 Isolation of Heparan Preparations.

Barium heparan salt (34 gm) from beef-lung heparin by-product, was fractionated as follows. A 5% (v/v) water solution was made and clarified by centrifuging at 10 k r.p.m. (temperature, 8° and pH, 7). The residue obtained was fraction 1. The centrifugate was resubjected to high speed centrifugation at 20 k r.p.m., and fraction 2 recovered. Several other fractions were obtained by alternately adding ethanol to incipient turbidity and centrifuging at high speed. The total volume handled was decreased by maintaining the concentration at 5% and lowering the temperature at which fractionation was performed.

Plotting the weight of precipitated material versus percent ethanol concentration, produced a precipitation curve for this mixture, Figure 3. Solubility differences of the components in the mixture cause the vertical inflections, which indicate the number of components possibly present in solution. The weights of precipitated material, the optical rotations, the percent ethanol concentration and the respective fraction numbers are given in Table 2. A low temperature was necessary to minimize enzymic activity as well as chemical hydrolysis. The pH was kept at or near 7, where the polymer was expected to remain stable during the long periods required to complete the preparations.

The barium salt of heparan was found to precipitate and centrifuge down much better than the sodium salt. In the presence of inorganic cations, the uronic acid units are normally ionized and highly hydrated, giving the polymer good solubility and solution stability over a wide range of hydrogen ion concentration. Most of the hydration is lost and coulombic repulsion between the molecules is diminished when the acidity of the solution is lowered to such an extent that ionization of carboxylic acid groups is repressed.

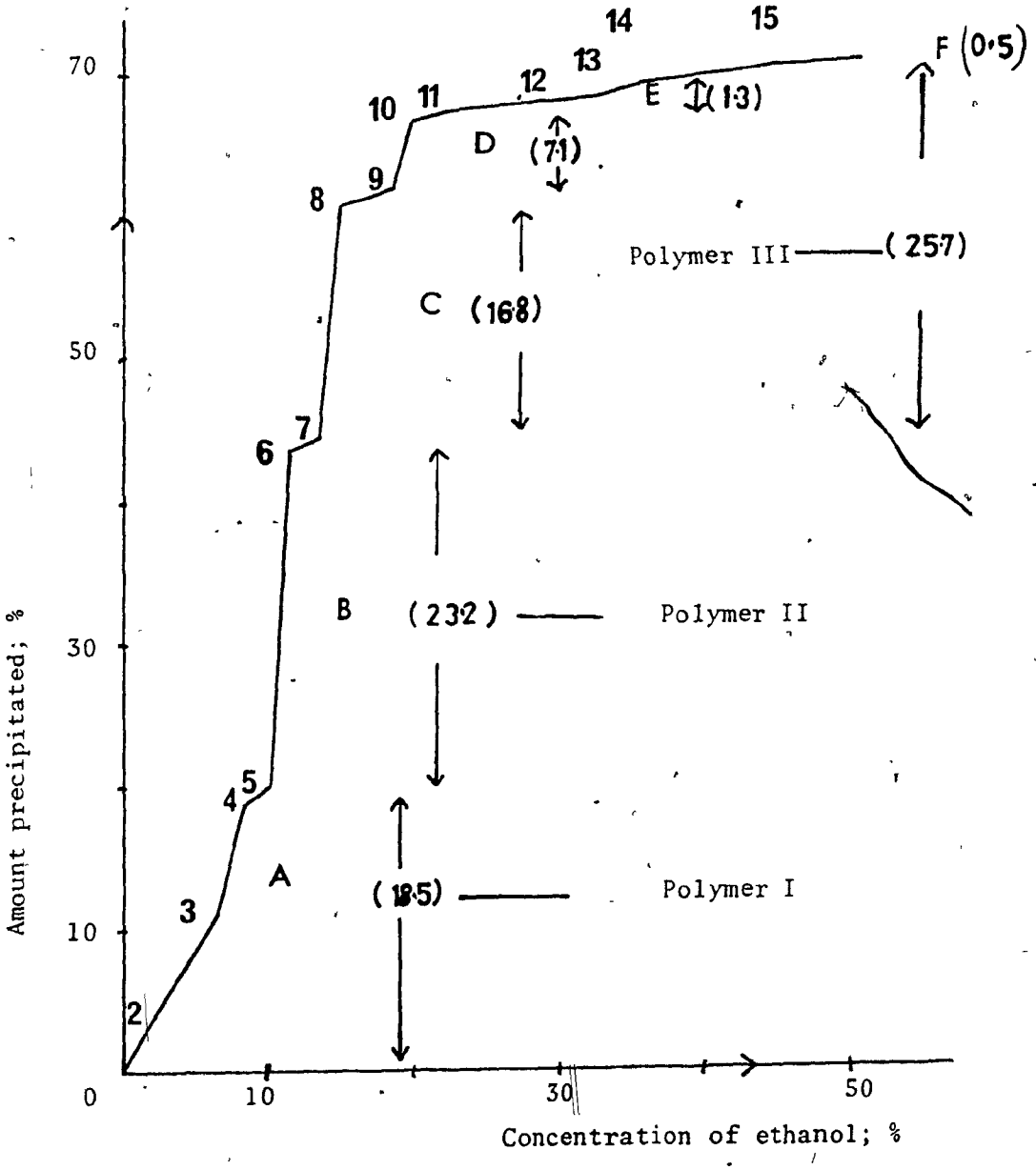


FIG. 3 Fractional precipitation curve for barium heparan salt at 5% concentration and pH ~7.

Numbers above curve = fraction numbers

Numbers in brackets = % of material between arrows

Letters A,B, ... = components distinguishable from curve

TABLE 2

Percentage of weight of barium heparan salt precipitated and ethanol added, optical rotations and fraction number.

Fraction #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Precipitated Wt.	1.9	0.14	3.6	2.53	0.51	7.9	0.5	5.2	0.51	1.8	0.12	0.2	0.21	0.31	0.3
Σ Wt.	1.9	0.14	3.74	6.27	6.78	14.68	5.18	20.38	20.89	22.69	22.81	23.01	23.22	23.53	23.87
% Wt.	/	0.4	11	18.5	20	43.2	44.7	60	61.5	66.7	67.1	67.7	68.3	69.2	70.1
ml EtOH added	0	0	55	18	7	15.5	11	14.2	26	16	15	45	36	219.3	76
Σ EtOH	/	0	55	73	80	95.5	106.5	120.7	146.7	162.7	177.7	222.7	258.7		
% EtOH	/	0	6.9	9.1	10	11.9	13.3	15.1	18.4	20.4	22.4	29	32.4	35.4	44.9
O.R. $[\alpha_o]_D^{25}$ (H_2O)	/	61.1	47.9	60.97	59.3	49.56	43.99	49.86	43.9	43.52	26.37	/	25.16	25.12	26.41

Between ethanol concentrations of 0 and 9.5%, about 18.5% of the material precipitated out. This component (A) consists of fractions 2 to 5. Between ethanol concentrations of 10 and 11.5%, component (B) separated out, accompanied by a small proportion of component (A) amd somewhat more of component (C). Between ethanol concentrations of 14 and 50%, components (C) to (F) separated out (Fig. 3). This latter group accounted for about 26% of the starting material.

III.2 Monitoring of the Fractionation.

The fractionation was monitored by 100 MHz p.m.r. spectroscopy. Each fraction was examined spectroscopically, and major differences among them were demonstrated. From the spectra of these fractions it was found that at one extreme (fractions 1 and possibly up to 5) the material closely resembled a B-type heparin, whereas fractions 7 and higher gave spectra similar

to those reported for heparans[98].

Components (A) to (F) were combined into three groups according to the prominence of their N-acetyl signal at ∿2 p.p.m. Polymer I consisted of fractions 2 to 5 (component A) and showed a very weak if not insignificant N-acetyl signal. Polymer II consisted of fraction 6 (component B) with a relatively strong signal at ∿2 p.p.m. Finally, polymer III (components C to F) consisted of fractions 7 and higher. This group exhibited the strongest N-acetyl signal (Fig. 4). Further, fractional precipitation of components (C) to (F), and high-speed centrifugation at 35 k r.p.m. did not reveal any significant differences spectroscopically. This was taken to be a reasonable criterion for combining components (C) to (F) into a single group.

Since the p.m.r. signal patterns of polymer I and III tallied closely with those previously obtained[33] for heparin and heparan, respectively, more attention was paid to polymer III which was subjected to high speed liquid chromatography for further separation.

III.3 HSLC Fractionation of Heparan.

Although a number of chromatographic and electrophoretic methods have been applied to the separation and characterization of acid mucopolysaccharide, application of HSLC to the study of aqueous solution of GGS is virtually unknown in the literature.

Whereas there is no compelling evidence for the superiority of the commonly applied methods, the new methodology is of great interest to the important problem of purification and characterization of heparan. This new technique is a powerful method for molecular size analysis and separation, and is superior in decreasing the time needed for analysis. As little as 5 mg of heparan can be detected and, preparatively, the system can handle as much as 20 mg.

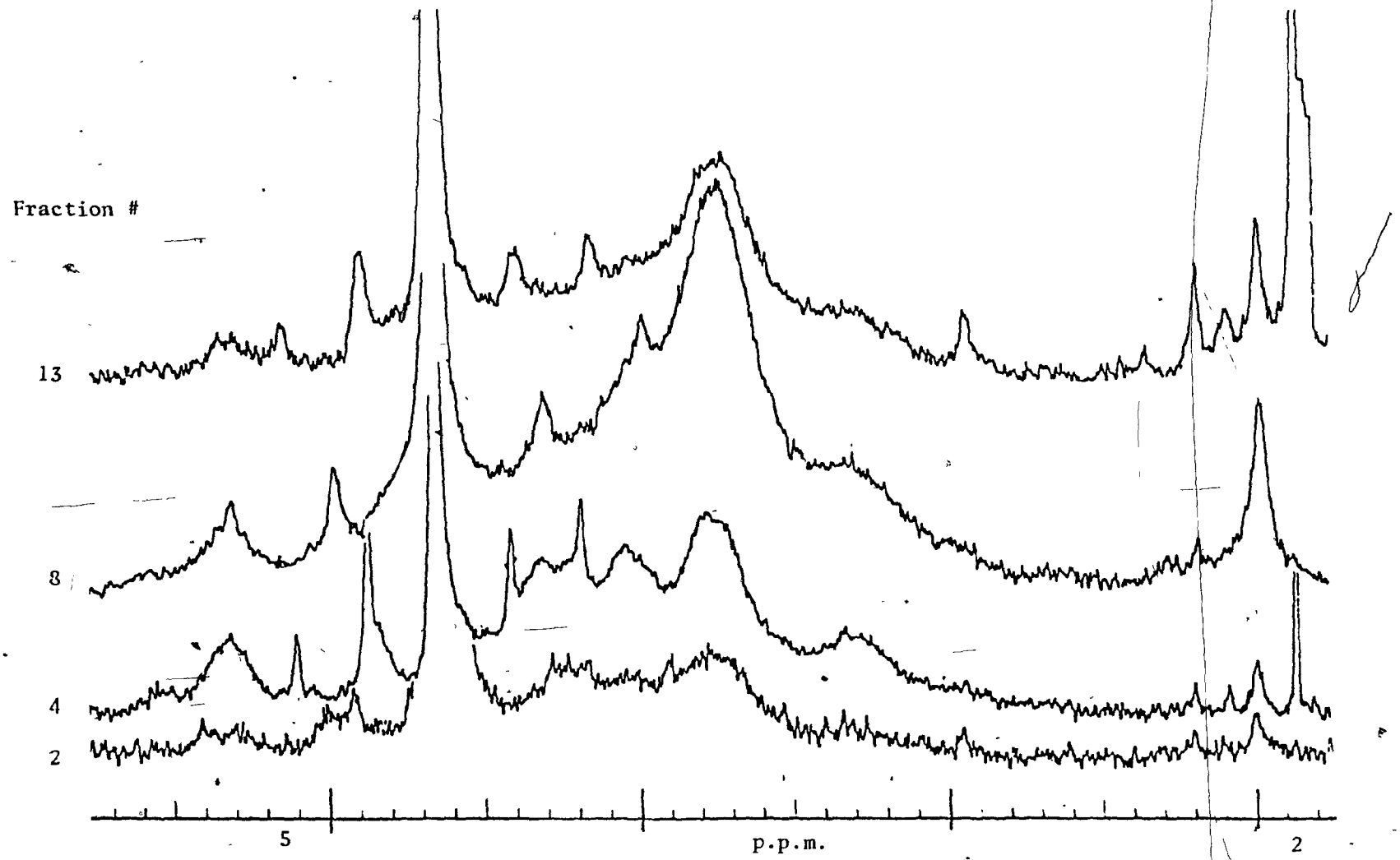


FIG. 4 100 MHz p.m.r. spectra of heparan fractions 2,4,8 and 13 (solvent D_2O, temp. 30°C).

When polymer III was subjected to HSLC, it give two sub-fractions (Fig.5a). No noticeable differences were observed - both by 220 MHz p.m.r. and c.m.r. - spectroscopically between these two separated samples and the original sample injected. However, gel-filtration on Sephadex G-75 indicated molecular size differences. The molecular weight of the injected sample ranged from 17,500 to 12,500 daltons. The molecular size of the sample which was eluted first was 17,500 and that of the second sample was 16,500 daltons. The overlap region of the elution chromatogram gave a sample with a molecular size distribution of 16,500 to 12,500 daltons (Fig. 5b).

From the HSLC separation data, in conjunction with the spectroscopic evidence, it can be concluded that these fractions obtained at relatively high ethanol concentration were of reasonable purity. Furthermore, it is deduced that component (A) and possibly part of (B) is of a molecular size higher than 18,000 daltons, and is heparin-like material and barely N-acetylated. Components (C) to (F), and possibly a major portion of component (B), are highly N-acetylated and have molecular weights of less than 17,500. Since their spectra are all significantly similar, the differences among components (C) to (F) can be attributed to molecular size.

III.4 220 MHz p.m.r. Spectral Study of Heparan.

The differences among these fractions, evident from their precipitation characteristics as well as their 100 MHz p.m.r. spectra, are even more graphically demonstrated by 220 MHz p.m.r. spectroscopy. In Fig. 6 are spectra of four fractions, selected to illustrate specific features and differences. These spectra are of the crude commercial sample (a), fractions 2-4 (b), fraction 8 (c) and fraction 14 (d). The latter correspond to the high (I), intermediate (II) and low (III) electrophoretic mobility materials of ref. 98. The two extremes are dramatically different from each other, i.e. the material virtually

Fig. 5 (a) HSLC chromatogram of heparan fraction (7-14).

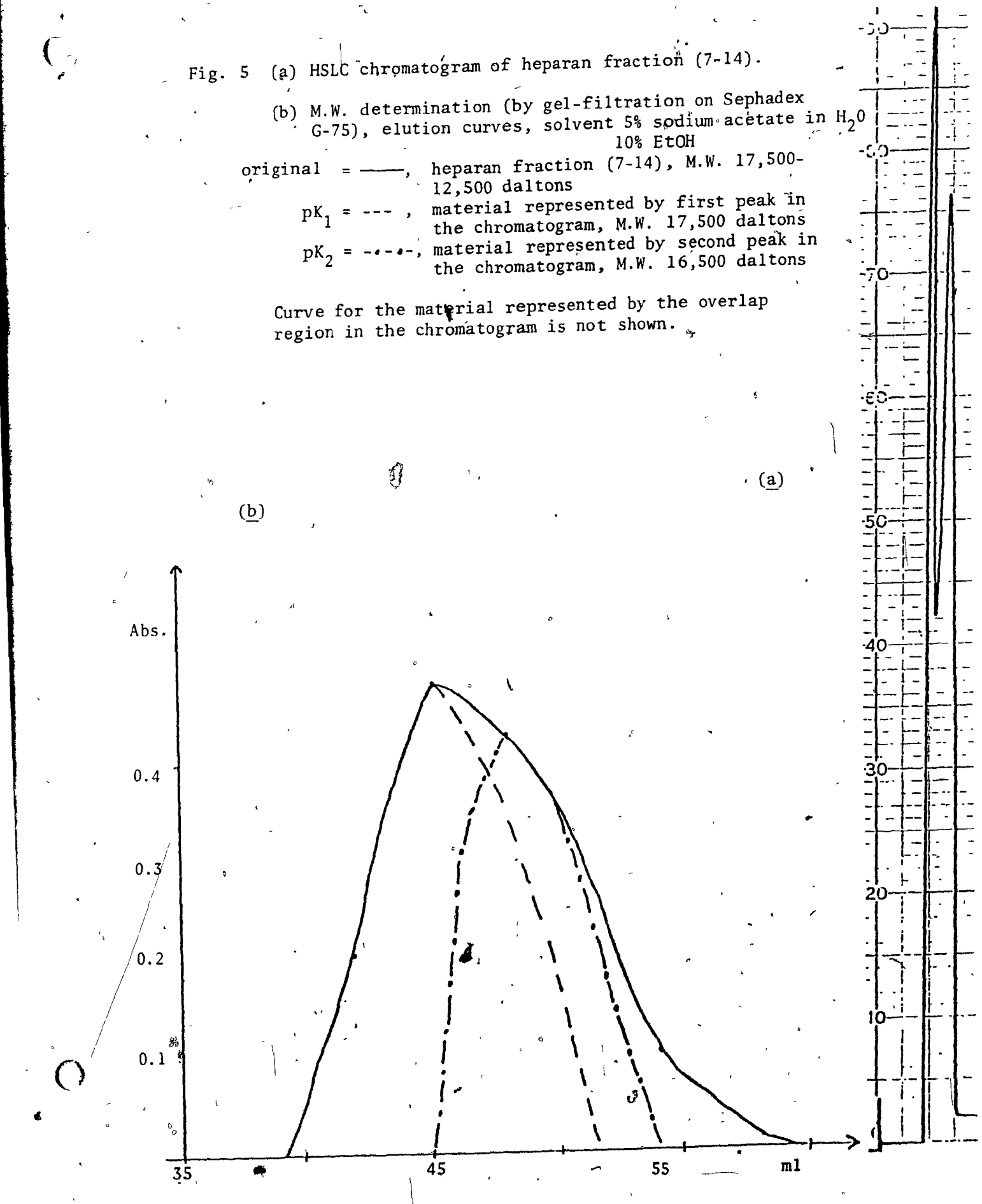
(b) M.W. determination (by gel-filtration on Sephadex G-75), elution curves, solvent 5% sodium acetate in H_2O 10% EtOH

original = ——, heparan fraction (7-14), M.W. 17,500-12,500 daltons

pK_1 = --- , material represented by first peak in the chromatogram, M.W. 17,500 daltons

pK_2 = -·-·-, material represented by second peak in the chromatogram, M.W. 16,500 daltons

Curve for the material represented by the overlap region in the chromatogram is not shown.



indistinguishable from heparin (Fig. 6b) and that which can be designated as heparan (Fig. 6c). There are intermediate fractions, of course.

Spectrum 6b is closely similar in all respects to that of A-type heparin[33]. The various spectral features used to characterize the 2-deoxy-2-sulphamino-α-D-glucose 6-sulphate (3d) and α-L-iduronic acid 2-sulphate (2) residues of A-type as well as B-type are evident[33]. In addition to the prominent B-type heparin signals at 3.3, 3.8, 4.1, 4.4, 4.8, 5.2 and 5.4 p.p.m., ascribed to SH-2, AH-4 and -3, IH-4, -3 and -2, 2SH-6, IH-5, IH-1, and AH-1, respectively[33], typical A-type heparin minor signals are found at 2.1, 3.5, 3.7, 4.6, 5.0 p.p.m., ascribable to protons of the acetamidodeoxyhexose and glucuronic acid residues. A signal at 5.7 p.p.m. has yet to be identified. Interestingly enough, the integral trace ratios appear to be 6:5 for AH-1: IH-1, 1:1 for IH-1:IH-5, 6:1 for AH-1:N-A_c, and 1:1 for IH-1:SH-2, which seem to account for the presence though in minor proportions, of a residue other than those stated above. From these spectral observations and earlier data[33,98], polymer I, (i.e. fraction 2-5) is designated heparin.

Differences between the spectrum of fraction 14 (Fig. 6d) and that of the heparin-like fraction (Fig. 6b) are strikingly obvious. Features such as the highly prominent signals in the latter at 3.3, 4.1, 4.8 and 5.2 p.p.m., most typical of the heparin type of spectrum, are now either absent in 6d, or barely detectable. The intensity of the signal at 2.1 p.p.m. ascribable to CH_3, needs no emphasis for fraction 14, and the prominence of its signals at 3.5 and 5.4 p.p.m. that are ascribed to AH-2 and AH-1, respectively, of the 2-acetamido-2-deoxy-α-D-glucopyranose moiety (3b) is notable. Signals, probably produced by H-1 and H-5 of the uronic acid components of heparan, are found at 5.0, and 4.6 p.p.m., their prominence is somewhat obscured by spinning side bands in Fig. 6d. A characteristic feature of spectrum 6d, which further serves to

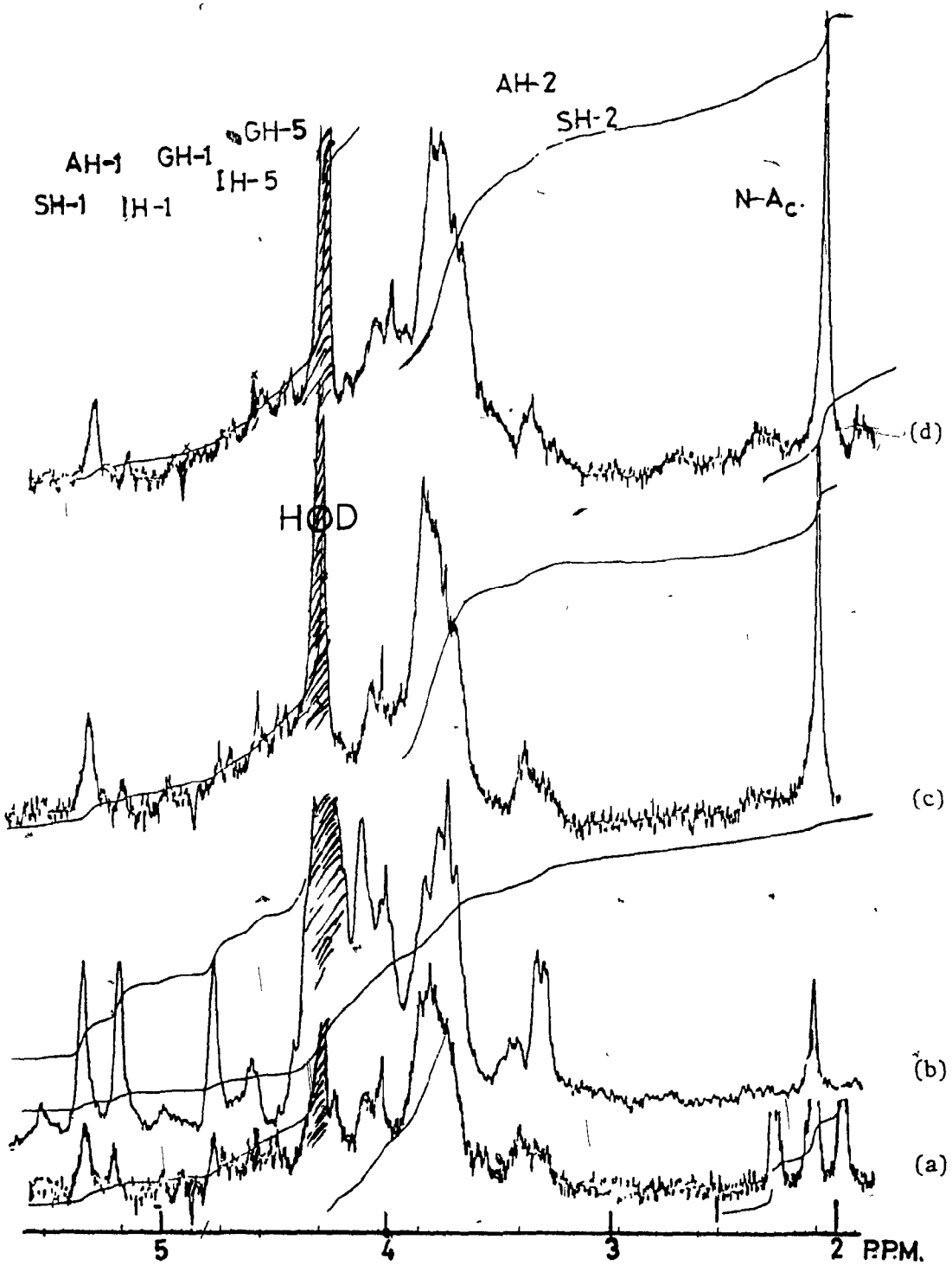


FIG. 6 Proton magnetic resonance spectra at 220 MHz (solvent D_2O; temp. 70°) of fractions of heparan from beef-lung tissue. [a, the crude commercial heparan; b, fraction 2-5; c, fraction 8; d, fraction 14. AH, signal of acetamidodeoxyhexose; SH, signal of deoxysulphaminohexose residue; IH, signal of iduronic acid residue; GH, signal of glucuronic acid residues (tentative); N-A_c, acetamido CH_3 signal.]

dramatize spectral differences between it and 6b, is the fact that there is a much stronger group of signals in the region 3.6-3.9 p.p.m. than at 4.1-4.3 p.p.m. Conversely, the latter are relatively the stronger group in spectrum 6b. This difference, i.e., the upfield position of most of the heparan protons is in accord with the evidence that this polymer has a low content, if any, of O-sulphate groups, whereas the presence of such groups in heparin causes deshielding of primary and secondary protons. Furthermore, the presence of a large number of signals of differing relative intensities in the heparan spectra (6c,6d) indicates that this polymer is structurally more heterogeneous than heparin.

III.5 22.63 MHz ^{13}C.m.r. Spectral Study of Heparan.

III.5.1 Introduction.

Some support for the structure of heparan deduced from chemical and enzymic studies has been provided by p.m.r. spectroscopy (vide supra). However, because heparan solutions give rise to broad proton resonances, attempts at detailed analyses of these spectra are interfered with by signal overlap. Better resolution of the ^{13}C spectra of heparan, than of their ^{1}H spectra is favored by the relatively narrow ^{13}C-signal line-widths. Indeed, examination of the heparan fractions by ^{13}C.m.r. spectroscopy reveals striking differences between these materials.

III.5.2 General Spectral Comparison of the Main Fractions.

Spectra representing the two extremes, selected to emphasize featuristic differences, are reproduced in Fig. 7a,b. These materials correspond to I and III of the precipitation curve. At one extreme (fractions 2-5), the spectrum (Fig. 7a) is virtually indistinguishable from that of heparin type-A, which parallels the p.m.r. spectral observations. The various major signals that have been correlated[34] with the 2-deoxy-2-sulphamino-α-D-glucose 6-sulphate and α-L-iduronic acid 2-sulphate residues are vividly evident.

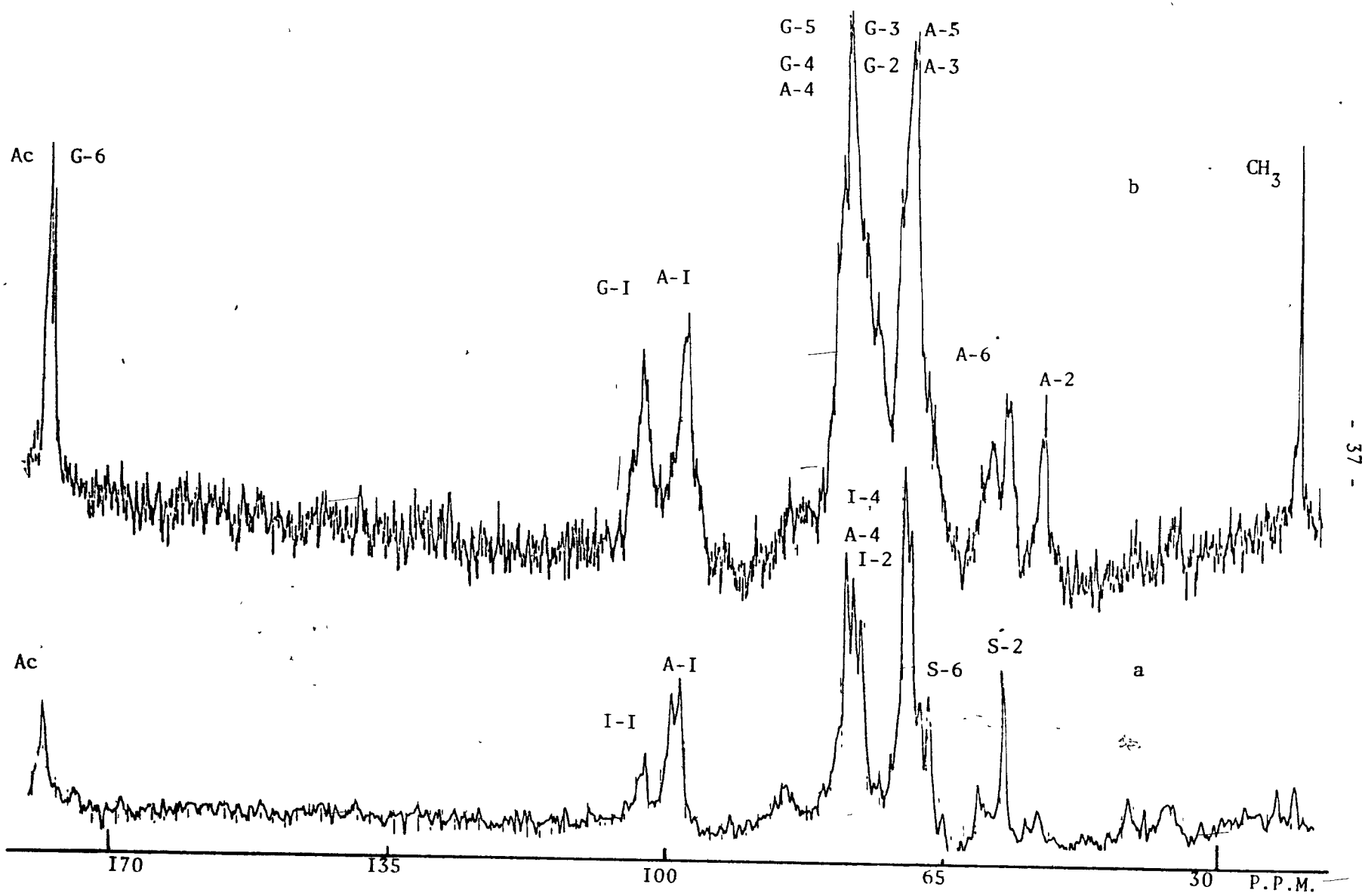


FIG. 7 ^{13}C.m.r. Spectra of I and II.

In addition to these major signals, a close examination of Fig. 7a, reveals minor signals at 103.4, 61.1, 54.6 and 23.4 p.p.m., barely distinguishable from the noise.

The spectrum of III (Fig. 7b) illustrates the other extreme. Features most typical of the polymer I spectrum are virtually absent. By contrast, signals that are of minor importance in I are now prominently present at 103.4, 77.4, 61.1, 54.6 and 23.4 p.p.m. The fact that there is a large number of signals of differing relative intensities, indicates that III has a more heterogeneous type of structure than I. A detailed spectral analysis throws some light on the possible identities of the primary moieties constituting the polymer, as illustrated by spectrum 7b.

III.5.3 ^{13}C Spectral Analysis.

In the spectrum of III (sodium salt, Fig. 8a), 13 major signals are clearly observable. The relative intensities of the signals (moving progressively upfield from 175 to 23 p.p.m.) are 0.8:1.0:1:0:1.8:3.0:1:0:1.0:1.0:2.7:0.9: 0.9:0.8. These data correspond to a repeating formula based on a minimum of 18 carbon atoms involving the disaccharide moiety 6 (Fig. 2 Sect. II.3) and an extra monosaccharide unit, most probably 3c (Fig. 1), i.e., residues of β-D-glucopyranosyl uronic acid (non-O-sulphated), 2-acetamido-2-deoxy-α-D-glucopyranose, and 2-amino-2-deoxy-α-D-glucopyranose 6-sulphate (Fig. 1).

The most readily designated signals are those due to carbonyl carbons (A-c and G-6, overlapping) at 175.6 p.p.m., the anomeric carbons (G-1 and A-1) at 103.4 and 98.2 p.p.m., respectively, the C-6 of the hexosamine (A-6) at 61.1 p.p.m., the carbon (A-2) bearing the acetamido group at 54.6 p.p.m., and the acetamido methyl (CH_3). The specific assignments have

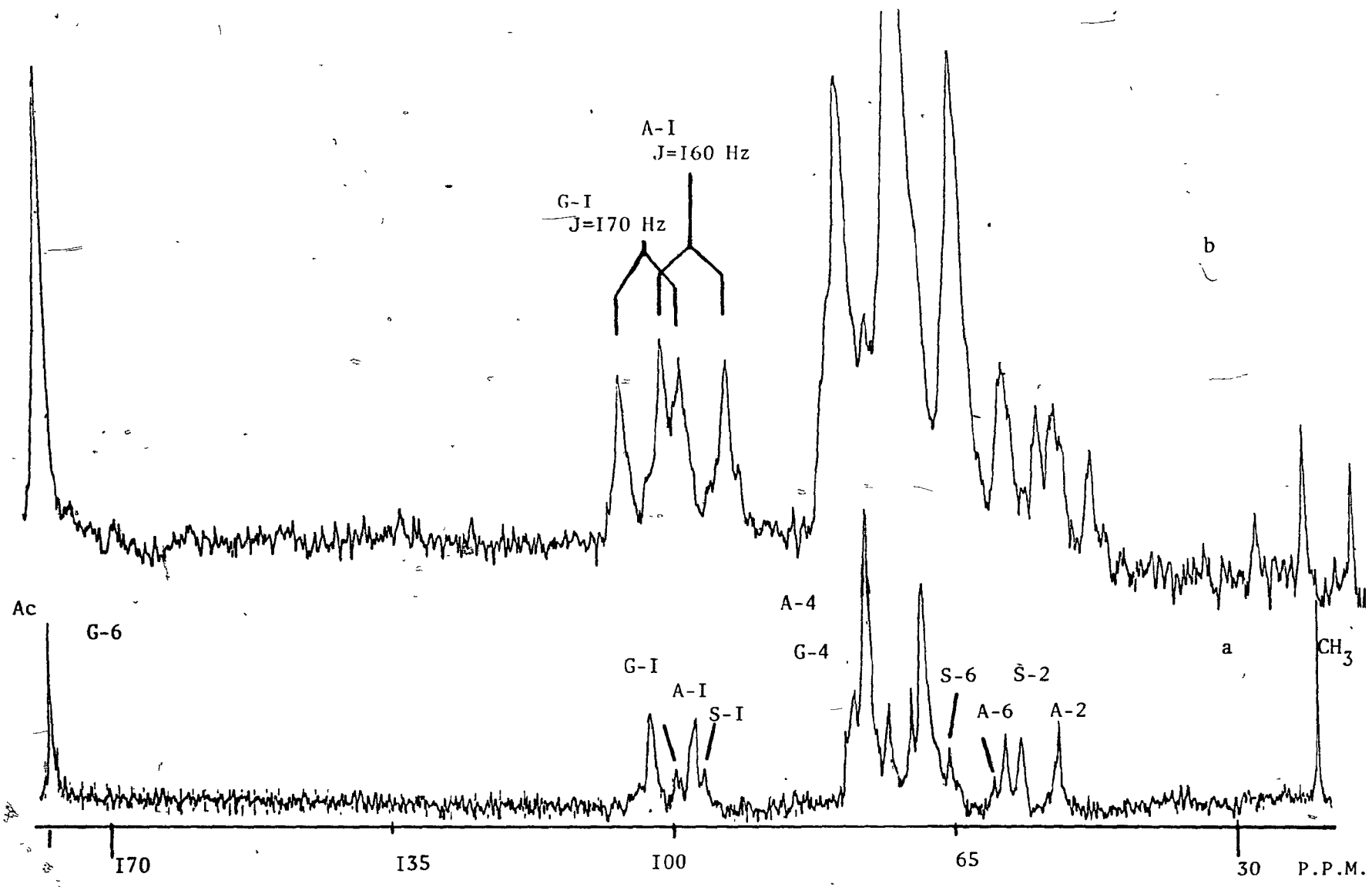


FIG. 8 ^{13}C.m.r. spectra of III

(a) sodium salt,

(b) gated.

been made by reference to spectral data for appropriate compounds (Tables 3,4) and similar glycosaminoglycans[34-36] and supplemented by coupling constant measurements, gadolinium nitrate tests, enzymic tests, selective and non-selective desulphation, and deaminative degradation (sect. III.5 and III.6).

^{13}C chemical-shifts for the acetamidodeoxyhexose residue of heparan were calculated from reported[99] values for 2-acetamido-2-deoxy-α-D-gluco-pyranose. Allowance is made for the strong, deshielding change, +9[100] and +7 p.p.m., expected of A-4 and A-1, respectively, due to glycosidic bond formation[97] and for small increases (-1 to -2) in shielding at adjacent carbons. These calculated values coincide favorably with the chemical shifts of designated signals (Tables 3 and 4) (Fig. 8). The table shows that signals A-3, -4, -5 of the model compound are closely grouped within the range 71.2-71.8 which coincides with the broad signal at 71.9 p.p.m. However, A-4, which appears to be glycosidically bonded in heparan, should be one of the signals in the second broad group 6 p.p.m. further downfield. This possibility is comfortably within the 5-9 p.p.m.[100] shift expected due to glycosidic bonding. The Table further shows that A-6 of the model compound, of heparan itself and of its deamination disaccharide, all coincide within a 1 p.p.m. margin. Considering the relatively greater line-broadening of this type of signal due to a shorter T_1 (inversely proportional to the number of attached protons)[101], and more effective spin-spin relaxation- an even shorter T_2[34] -, the correspondence of calculated and observed values of these A-6 signals is more than reasonable.

Similarly, reference to the ^{13}C chemical shifts of the uronic acids, D-glucopyranuronic acid and α-L-idopyranuronic acids (Table 3,4), provided a basis for assigning the individual uronic acid signals in Fig. 8a[34,36]. The

TABLE 3: Observed and calculated ^{13}C chemical shifts of major signals in the spectra of heparan fractions and related materials

Carbon	β-D-glucuronic acid[a]	D-glucosyluronic in heparan			α-L-idosyluronic 2-sulphate in heparin[d]	L-iduronic acid		
	Obs.	Increment[b]	Calc.	Obs.	Obs.	Increment	Calc.	Obs. in heparin[e]
U-1	97.4	+7	104.4	103.6	101.5	+1	102.5	100.5
U-2	75.5	-1	74.5	74.7	77.5	0,-7	70.5	77.4
U-3	77.1	-1	76.1	74.7	71.5	+1	72.5	73.4
U-4	73.3	+9	82.3	78.9	77.5	0	77.5	77.4
U-5	77.5	-1	76.5	77.4	71.5	0	71.5	69.1
U-6	177.5	0	177.5	176.0	175.5	0	175.5(177.2)[c]	176.1

	2-acetamido-2-deoxy-α-D-glucopyranose	acetamidodeoxyhexose in heparan		
	Obs.[d]	Increment[b]	Calc.	Obs.
A-1	92.1	+7	99.1	98.3[f]
A-2	55.3	-1	54.3	54.6
A-3	72.0	-1	71.0	70.4
A-4	71.4	-1,+9	79.4	77.4
A-5	72.8	-1	71.8	72.0
A-6	61.9	0	61.9	61.1
CH_3	23.3	0	23.3	23.2
A_c	175.7	0	175.7	175.5

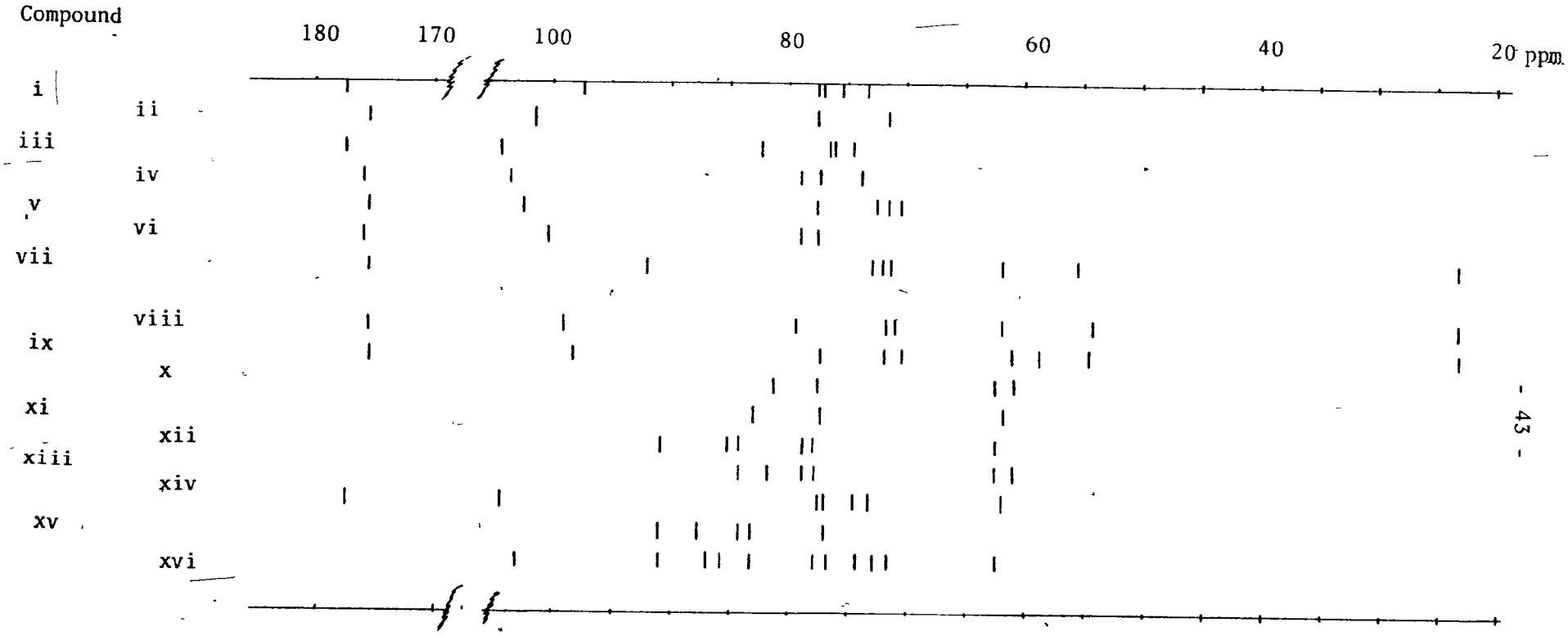
a) Sodium salt. b) Approximate change in chemical shift expected relative to the corresponding carbon of the model compound: on formation of glycosidic bond at anomeric (+7 p.p.m.) or at a secondary C (+9 p.p.m.)[34,97]; on introduction of sulphate group (+7 p.p.m.), or its removal (-7 p.p.m.)[34]; due to bond or sulphate group introduced at adjacent position (-1 p.p.m.), or removed (+1 p.p.m.)[34,35,97,100]. c) Chemical shifts for sodium (methyl α-D-idopyranosid)uronate[35] are closely similar: U-1, 102.0; U-2 to U-5 (not individually assigned), 73.5, 72.0, 71.0 and 71.0 p.p.m. One of these (U-4) should be displaced downfield by glycosidic bond formation. The chemical shift of U-6 for β-L-idopyranuronic acid is 177.2 p.p.m. d) Ref. 99, e) Ref. 109, f) A-2 of α-D-glucosamine HCl observed at 55.4 p.p.m. g) Ref. 111.

TABLE 3 (continued)

Carbon	2,5-anhydro-mannitol[g]	2,5-anhydro-mannose	2,5-anhydro-D-mannose in disaccharide 7				2,5-anhydro-glucitol[g]	2,5-anhydro-iditol[g]	2,5-anhydro-D-mannose 6-sulphate[e] in heparin disaccharide		
	Obs.	Obs.	Increment[b]	Calc.	Obs.	$^1J_{C-H}$ Obs.[h]			Increment	Calc.	Obs.
M-1	61.8	90.9	0	90.9	90.8	164.3	61.0	60.9	0	90.9	90.3
M-2	83.1	85.2	0	85.2	85.8	148.3	81.8	81.2	0	85.2	85.7
M-3	77.2	78.0	-1	77.0	77.9	149	77.8	77.5	-1	77	77.2
M-4	77.2	78.7	+9	87.7	87.0	152	78.9	77.5	+9	87.7	86.2
M-5	83.1	84.2	-1	83.2	83.3	148.3	85.9	81.2	-2	82.2	81.1
M-6	61.8	62.5	0	62.5	62.5	143	62.5	60.9	+7	69.5	68.8

[h] $^1J_{C-H}$ in Hz; observed $^1J_{C-H}$ values for the D-glucuronic acid moiety in 7 are 145, 146.4, 148.3 and 145.7 Hz for G-5, G-3, G-2 and G-4, respectively.

TABLE 4. Stick diagram of observed and calculated ^{13}C-chemical shifts of major signals in heparan fractions, disaccharide 7 and related materials.



i, β-D-glucuronic acid; ii, α-L-iduronic acid 2-sulphate; iii, D-glucosyluronic acid in heparan, calculated, iv, observed; v, L-iduronic acid in heparin, calculated, vi, observed; vii, 2-acetamido-2-deoxy-α-D-glucopyranose, viii, in heparan, calculated, ix, observed; x, 2,5-anhydro-iditol, xi, -mannitol, xii, -mannose, xiii, -glucitol; xiv, D-glucuronic acid; vx, β-D-glucuronic acid and 2,5-anhydro-D-mannose in disaccharide 7, calculated, xvi, observed.

Table shows that signals of U-2 to U-5 are closely grouped within the range 71.6-75.5 p.p.m., which probably correspond to the two broad signals at 70.5 and 77.4 in Fig. 8. Accepting that U-4 is glycosidically bonded, it should resonate downfield[105] and, like A-4, probably gives rise to one of the signals at 77.4 p.p.m.

The presence of relatively weak signals at 100.5, 97.1, 67.6, 62.5, and 58.9 (arrow) p.p.m. attests, again, to the structural heterogeneity of heparan. Their occurrence indicates that the sample contains, in addition to material constituted as in Fig. 2, 15% of material that produces resonance signals akin to those that are prominent with the heparin-like fractions. This would fall in line with the evidence from chemical analysis that heparan contains ~15% iduronic acid[13], an N-sulphate:hexosamine ratio of 0.3 to 0.6[102], an O-sulphate:D-glucosamine ratio of 0.2 to 0.8 and a sulphate:disaccharide ratio of 0.46[103]. Hence, the weak signals at 100.5, 97.1, 67.6 and 58.9 p.p.m. (Fig. 8a) correspond in chemical shift to signals I-1, S-1, S-6 and S-2, respectively, in the spectrum (Fig. 7a) of the heparin-like material. Also, as will be seen later (sect. III.6.1) the signal at 58.9 p.p.m., attributed to S-2, is markedly affected by selective N-desulphation, which is consistent as well with the presence of a sulphamino-deoxyhexose residue. Support for this likelihood is found additionally in the presence of a weak (broad) signal at 3.3 p.p.m. in Figs. 6c and 6d, ascribable to SH-2.

There is a weak signal at 62.5 p.p.m., which is in the region commonly associated with C-6 of aldohexoses. Since, the stronger signal at 61.1 p.p.m. is assigned to carbon-6 of the acetamidodeoxyhexose residues, it seems possible that this minor signal is due to a residue such as a sulphaminodeoxyhexose that does not bear a sulphate group at C-6. Detailed spectral analysis and more

structural studies are needed for definitive assignments of these four signals in the 54 to 63 p.p.m. region.

It is not clear from the evidence available as to the relationship between this 15% of material and the remainder of the heparan. Possibly it represents simply some contamination of the heparan by heparin. Alternatively, however, it constitutes a portion of the heparan molecule that contains types of residues in common with the major ones in heparin.

III.5.4 Spectral evidence for Configurational and Conformational Aspects of Heparan.

Information gleaned from the effects of the relaxation agent, gadolinium nitrate, and measurements of coupling constants on the stereochemistry of the polymer is consistent with the results obtained above by chemical shift analyses of the ^{1}H-decoupled ^{13}C spectra. That is, the acetamidodeoxy-D-glucose and D-glucuronic acid residues of heparan, are shown to possess α- and β-anomeric configuations, respectively, and the $^{4}C_{1}$ (D) conformation.

The lanthanide salt, gadolinium nitrate (Gd^{3+}) acts as a ^{1}H[104,105] and ^{13}C[106] relaxation agent in water. Gd^{3+} has a marked effect on T_1 of C-1 and C-6 of α-anomers (axial C-1, O-1 bond) of uronic acids. Addition of Gd^{3+} to a solution of heparan, as well as of its major degradation disaccharide, caused no discernible selective diminution in the apparent intensities of the C-6 and C-1 signals of the uronic acid residues. This lack of interaction with the paramagnetic metallic ion, suggests that the main uronic acid residues of heparan possess the β- (equatorial, C-1, O-1) configuration, a deduction which also is consistent with the β-D-glucosiduronidase studies (Sect. III.6.3.2)

on disaccharide 7 obtained from heparan (sect. III.6.3).

Coupling between anomeric carbons and protons ($^{1}J_{C-H}$) is an invaluable ^{13}C.m.r. parameter. The ^{13}C spectrum shown in Fig. 8b was obtained without proton-decoupling (gated). Measurements on this and other ^{1}H-coupled spectra provided corroborative stereochemical information. The signals attributable to the anomeric carbons are of special interest. Coupling between each anomeric carbon and its appended proton (i.e. $^{1}J_{C-H,H-1}$) is either around 160 Hz or 170 Hz (Table 5). These values are known [35,91,92] to be characteristic of an equatorial or axial anomeric configuration, respectively, i.e., when the C-1, O-1 bond is equatorial $^{1}J_{C-H}$ is ∿160 Hz, and when this bond is oriented axially $^{1}J_{C-H}$ is ∿170 Hz. Consequently, since the C-1, H-1 coupling for the glucuronic acid residue of heparan is 160 Hz (and of 7, the disaccharide degradation product is 160 Hz) (Table 5), the β-D-configuration and $^{4}C_{1}$(D) conformation can confidently be assigned to this residue. By contrast, the acetamidodeoxyhexose residues of heparan give $^{1}J_{C-H}$ of 170 Hz, corresponding to the α-D-configuration and $^{4}C_{1}$(D) conformation.

III.6 Studies on Modified and Depolymerized Heparan.

Heparan was modified by solvolytic and hydrolytic desulphation, and by N-deacetylation/desulphation, and also it was depolymerized by nitrous acid deamination. Both procedures helped to clarify some aspects of the structure of heparan.

III.6.1 Hydrolytic and Solvolytic Desulphation.

Hydrolytic desulphation is known [16,107,108] to be nonselective; i.e., the hydrolysis of the N-sulphate group is accompanied by some cleavage of glycosidic linkages and O-sulphate groups. When heparan was subjected to hydrolytic desulphation a number of spectral changes were observed (Fig. 9a) relative to Fig. 8a. The large upfield shifts expected of A-1, A-2

TABLE 5

Direct-bond coupling $^{1}(J_{C-H})$ between ^{13}C-1 and ^{1}H-1, and anomeric configuration (all uronic acids as sodium salts)

	$^{1}J_{C-H}$ Hz	
	α	β
D-Glucopyranuronic acid	170	162
D-Glucuronic acid residue (chondroitins A and C)		160-162[35]
L-Iduronic acid residue (beef lung heparin)	172	
L-Iduronic acid residue (disaccharide)	172[109]	
D-Glucuronic acid residue (disaccharide)		160[109]
D-Glucuronic acid residue (heparan fraction)		160
2-Acetamido-2-deoxy-D-glucopyranose	173	160
2-Acetamido-2-deoxy-D-galactose residue (chondroitins A and C)		161[35]
2-Deoxy-2-sulphamino-D-glucose residue (beef lung heparin)	170	
2-Acetamido-2-deoxy-D-glucose residue (heparan fraction)	170	

and A-3 (in addition to lesser effects on others) were found. The new signals in the 91-96 p.p.m. region can reasonably be assumed to arise from an effect on A-1 as attested to by the decrease of its relative intensity. Since the anomeric carbon shifts of deoxyhexoses are in this region, it is reasonable to assume that the signals at 92.7, 93.3 and 96.1 p.p.m. are due to the anomeric carbon of deoxyhexoses freed from glycosidic linkage by acid hydrolysis. For example, C-1 of 2-acetamido-2-deoxy-D-glucopyranose resonates[99] at 92.1 and 96.2 p.p.m. (α and β, respectively), and C-1 of D-glucosamine hydrochloride, at 90.2 and 93.8 p.p.m. The uronosyl bond is certainly not hydrolysed, since the signal at 103.4 p.p.m. ascribable to G-1 did not appear to have decreased in intensity.

When heparan was more selectively desulphated by the solvolytic method, the only notable signal shifts occurred in the 54-62 p.p.m. region (Fig. 9b). The signals at 58.9 and 61.1 originally, appear to have shifted to 59.3 and 62.5 p.p.m. This observation is consistent with the suggestion that solvolytic desulphation is selective and causes no glycosidic linkage degradation.

III.6.2 Nitrous Acid Deamination prior to N-Deacetylation/Desulphation.

When unmodified heparan (fractions 7-14) was subjected to treatment with nitrous acid, limited depolymerization was detected spectroscopically (Fig. 9c). It is suggested that some hexosaminyl bonds were cleaved and residues of 2,5-anhydro-D-mannose formed. This is based on the following:

(i) a diminution in the intensity of the A-1 signal, and disappearance of the minor signal at 97.1 p.p.m. attributable to S-1 (Fig. 8a, arrow);

(ii) an increase in the relative intensity of the minor signal at 62.6 p.p.m. attributable to M-6 (Fig. 8a, arrow);

(iii) the signal at 58.9 p.p.m. (Fig. 8a), is no longer there;

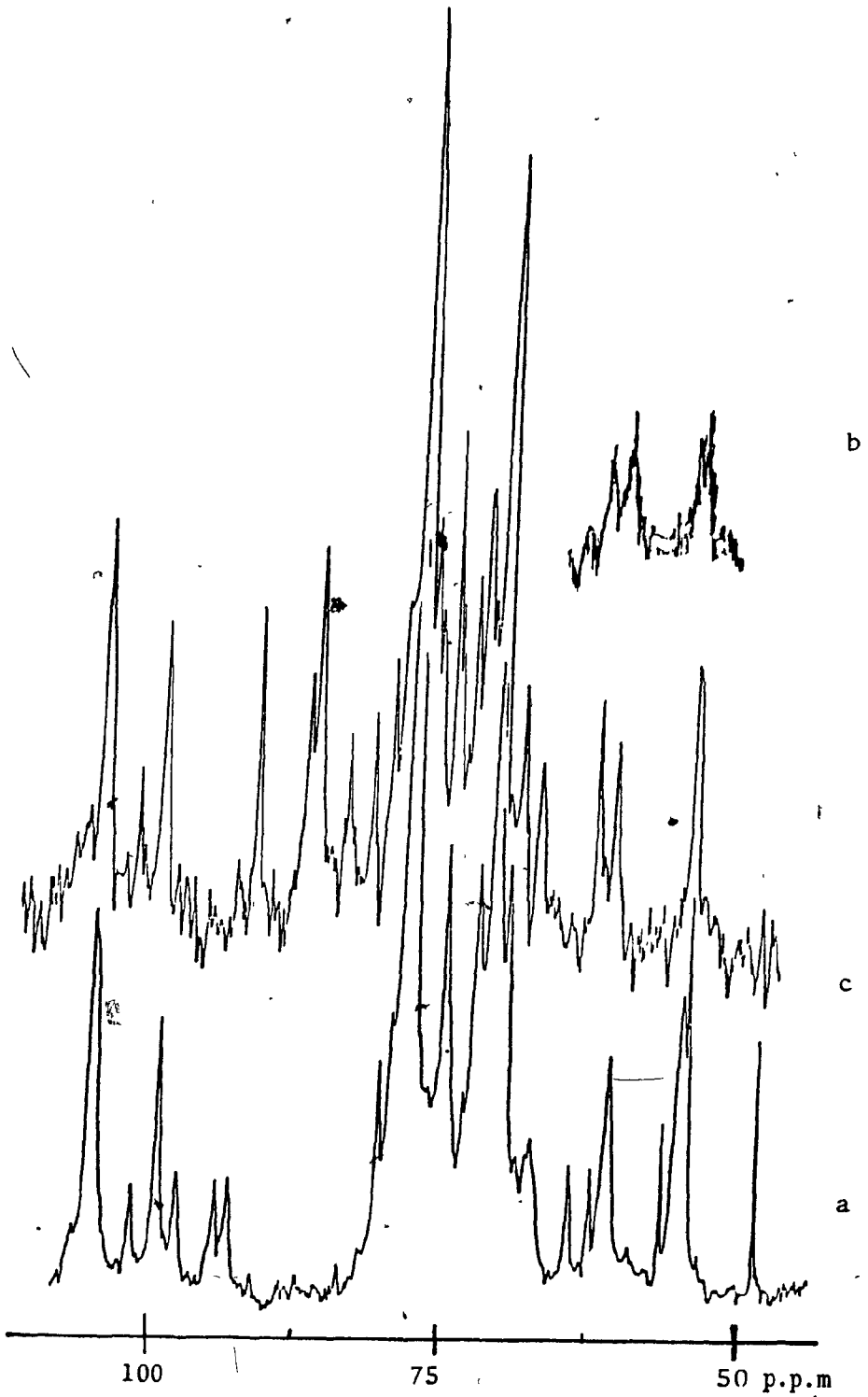


FIG. 9 ^{13}C.m.r. for III, after (a) hydrolytic N-desulphation, (b) solvolytic N-desulphation (insert) and (c) deamination prior to N-deacetylation.

(iv) the signal at 61.1 p.p.m., ascribed to A-6 is considerably diminished in intensity relative to that in Fig. 8a;

(v) four signals at 90.8, 86.2, 83.3 and 62.6 p.p.m. coincide, respectively, with M-1, M-2, M-5 and M-6 of the disaccharide (7) obtained from heparan by deamination (see sect. III.6.3).

(vi) four minor signals at 100.6, 86.9, 77.83 and 68.9 p.p.m. coincide, respectively, with I-1, M-2, M-5 and M-6 of the disaccharide from heparin by deamination[109].

These data suggest the presence, though of only 10 to 15%, of the following residues: L-iduronic acid, and two hexosamine units - one sulphated at C-6 and the other not[107]. Since nitrous acid attacks both sulphamino hexoses and free amines, it is not clear whether the disappearance of the signal at 58.9 p.p.m. is due to deamination of a sulphamino, or amino, hexose. N-Deacetylation/desulphation with trifluoroacetic acid caused the four signals in the 54 to 62 p.p.m. region (Fig. 8a) to merge into two broad signals at 60 and 54.6 p.p.m. (Fig. 9); changing pH did not produce striking shifts of these two signals. Further studies are necessary to allow definitive assignment of these four signals.

III.6.3.1 Nitrous Acid Deamination Depolymerization

To render heparan amenable to extensive, if not complete, depolymerization to saccharide fragments by nitrous acid deamination, preliminary hydrolysis of the N-ester substituents was necessary.

Since the N-acetyl is much more resistant than the N-sulphate group towards acid hydrolysis, total removal of the N-acetyl, as indicated by the absence of the CH_3 peak in ^{13}C.m.r. and p.m.r. spectra, was taken as reasonable evidence of complete hydrolysis to the aminosugar.

After subsequent treatment with nitrous acid, examination of the

the deaminative degradation mixture prior to passage through a Sephadex G-15 column, indicated ~90% depolymerization to disaccharide residues Fig. 10a).

By column chromatography on Sephadex G-15, it was found that the main deamination product was disaccharide 7 (Fig. 11). It was contained in fractions comprising 270-340 ml of the eluate (yield, 60%). Several minor products that were formed in the deamination reaction emerged in the first 270 ml of eluate (yield, 4%) and in the volume comprising 340-600 ml (yield, 25%). Both of these minor fractions gave p.m.r. spectra quite different from that of 7, although each spectrum contained a doublet and triplet (at 4.8 and 3.9 p.p.m. respectively, J, 5.94 Hz) indicative of the presence of a 2,5-anhydro-D-mannose residue.

The ^{13}C.m.r. spectrum of the early fraction (0-270) was not well resolved, although signals which coincided in chemical shift with the G-1, M-1, M-5 and M-6 of 7 were sharp and intense, indicative of the presence of a residue of 2,5-anhydro-D-mannose and one of glucuronic acid. The quantity of this minor fraction was too small to give a satisfactory ^{13}C spectrum.

Disaccharide 7 has been isolated as a chromatographically, electrophoretically and spectroscopically distinct compound in 60% yield. Its ^{13}C spectrum (Fig. 10b) is commensurate with structure 7. The assignments given were made by reference to spectra of related compounds (Tables 3) and also were based on coupling constant measurements (Table 5) and enzymic tests (sect. III.6.3.2).

^{13}C chemical shifts for the 2,5-anhydro-D-mannose residue were calculated from the observed values of 2,5-anhydro-glucitol, -iditol and mannitol. A +9 p.p.m. allowance has been made for the deshielding effect due to glycosidic bond formation[97] and -1 p.p.m. for small increases in shielding

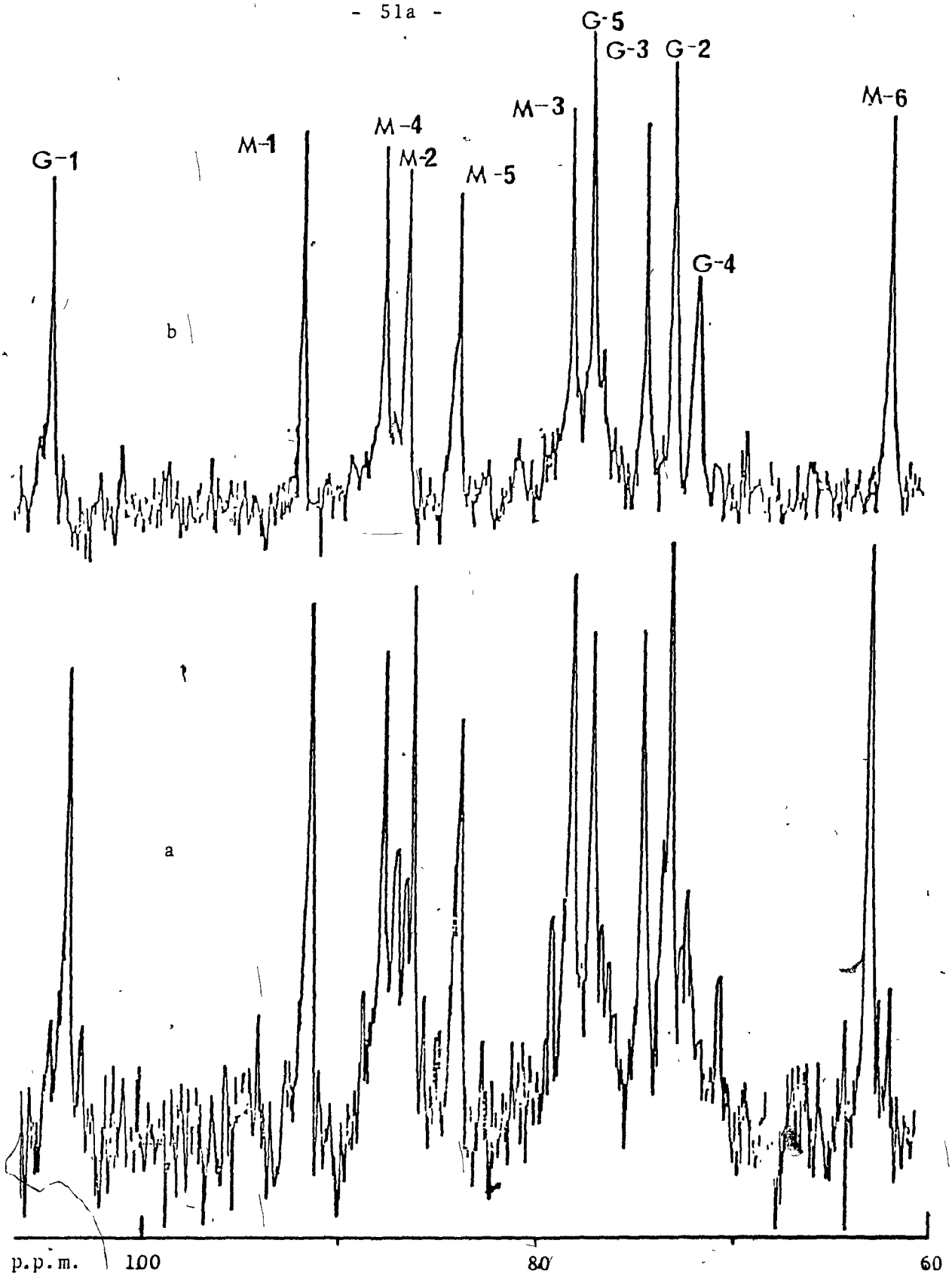


FIG. 10 Product obtained from heparan by nitrous acid deamination.

(a) The mixture of products before passage through a Sephadex G-15 column. (b) Major disaccharide product (7) obtained after passage through the column.

at adjacent carbons. The agreement between the calculated and observed values is within 1 p.p.m. (Tables 3,4). This Table shows that the signals at 90.8, 83.2 and 62.5 p.p.m. are ascribable to M-1, M-5 and M-6. M-1 occurs at lowest field because in the hydrated aldehyde structure the deshielding effect of two oxygens would increase the chemical shift of carbon-1. Also, this carbon shows relatively broad C-H coupling; i.e. J_{C-H} is about 164 Hz, which is noticeably larger than the couplings (140-153 Hz) for the other carbons[110]. The signal furthest upfield is found to be that of a $-CH_2$ group, i.e., it appears as a triplet, in contrast to the doublet structures of the other proton-bearing carbons. It is ascribed, therefore, to M-6. This assignment is consistent with the fact that M-6 is the only primary alcohol carbon in the dimer, and also that a sulphated C-6, as in heparin and its degradation products, resonates at 68.8 p.p.m., 7 p.p.m. downfield of this M-6 signal.

Reference to reported ^{13}C chemical shift values of 2,5-anhydroglucitol and -mannitol[111] (Tables 3,4) analogs, afforded assignments for the signals at 78.0 and 78.7 p.p.m. in the spectrum of free 2,5-anhydro-D-mannose to M-4 and M-3, respectively. Consistent with this is the fact that a counterpart of the signal at 78.7 p.p.m. does not appear in the spectrum of 7, but a signal is found at 87.0 p.p.m., corresponding to a deshielding by 8 p.p.m. when M-4 is glycosidically linked in the disaccharide. In addition M-4 has the largest coupling constant (152 Hz); the other three have (148.3 and 149 Hz). The remaining two signals at 85.8 and 83.3 p.p.m. are assigned to M-2 and M-5, respectively. M-2 is farthest from M-4, and hence will move least when the monomer and dimer are compared. That M-2 and M-5 should appear at lower field as compared to M-3 and M-5 in the 2,5-anhydro-D-mannose, or M-3 in the disaccharide, is consistent with the fact that these two carbons are bonded to the ring oxygen, which has a deshielding effect. In addition, sulphation of the mannose at C-6 in the dimer, will have least effect on M-2 of all the ring

carbons, but an upfield shift of about 1 p.p.m. (β effect) on the adjacent carbon (C-5) (Tables 3,4). Indeed the M-5 signal of the disaccharide obtained from heparin is found upfield of that in disaccharide 7.

Similarly, assignment of the uronic acid signals in Fig. 10b was based on reference to the ^{13}C chemical shifts of β-D-glucopyranuronic acid and residues of this compound in mucopolysaccharides. The signal at lowest field (176 p.p.m.) is undoubtedly ascribable to G-6 due to the strongly deshielding effect of sp^2 hybridization. Also the signal at 103.3 p.p.m. (J_{C-H}, 160 Hz) is undoubtedly attributable to G-1. The assignment of the remaining uronic acid signals (G-2 to G-5) is based on analogy with the corresponding carbons (C-2 to C-5) of β-glucopyranuronic acid (Table 3).

III.6.3.2 Stereochemical Aspects of Disaccharide 7.

Addition of the relaxation agent, gadolinium ion, to disaccharide 7 in solution effected no diminution in the apparent intensity of signals ascribed to G-6 and G-1 of the glucopyranuronic acid residue. However, disaccharide 7 was hydrolysed by the specific β-D-glucosiduronidase enzyme, as noted by the total disappearance of signal G-1 and the commensurate appearance of (free sugar) anomeric carbon signals at 97.4 (β) and 93.6(α) p.p.m., and less striking shifts of several other signals.

These latter data, together with the $^1J_{C-H}$ value of 160 Hz (Table 5), are consistent with disaccharide 7, i.e., a β-D-glucopyranuronic acid linked to a 2,5-anhydro-D-mannose by a 1→4 linkage, and the acid moiety favouring a $^4C_1$(D) conformation.

The high degree of consistency (Tables 3,4) found in comparing appropriate chemical shifts within the series heparan, β-D-glucopyranosiduronic acid and disaccharide 7 indicates that the conformation of D-glucuronic acid residues in heparan is close to that in the smaller molecules.

III.6.4 Isolation of 1,2-O-Isopropylidene-D-glucurono-3,6-lactone from Heparan.

Isolation of a derivative of D-glucuronic acid, i.e., 1,2-O-isopropylidene-D-glucurono-3,6-lactone from the enzymic hydrolysis product of disaccharide 7 unequivocally confirmed the spectroscopic identification of this acid in the polymer. The reaction sequence used is shown below.

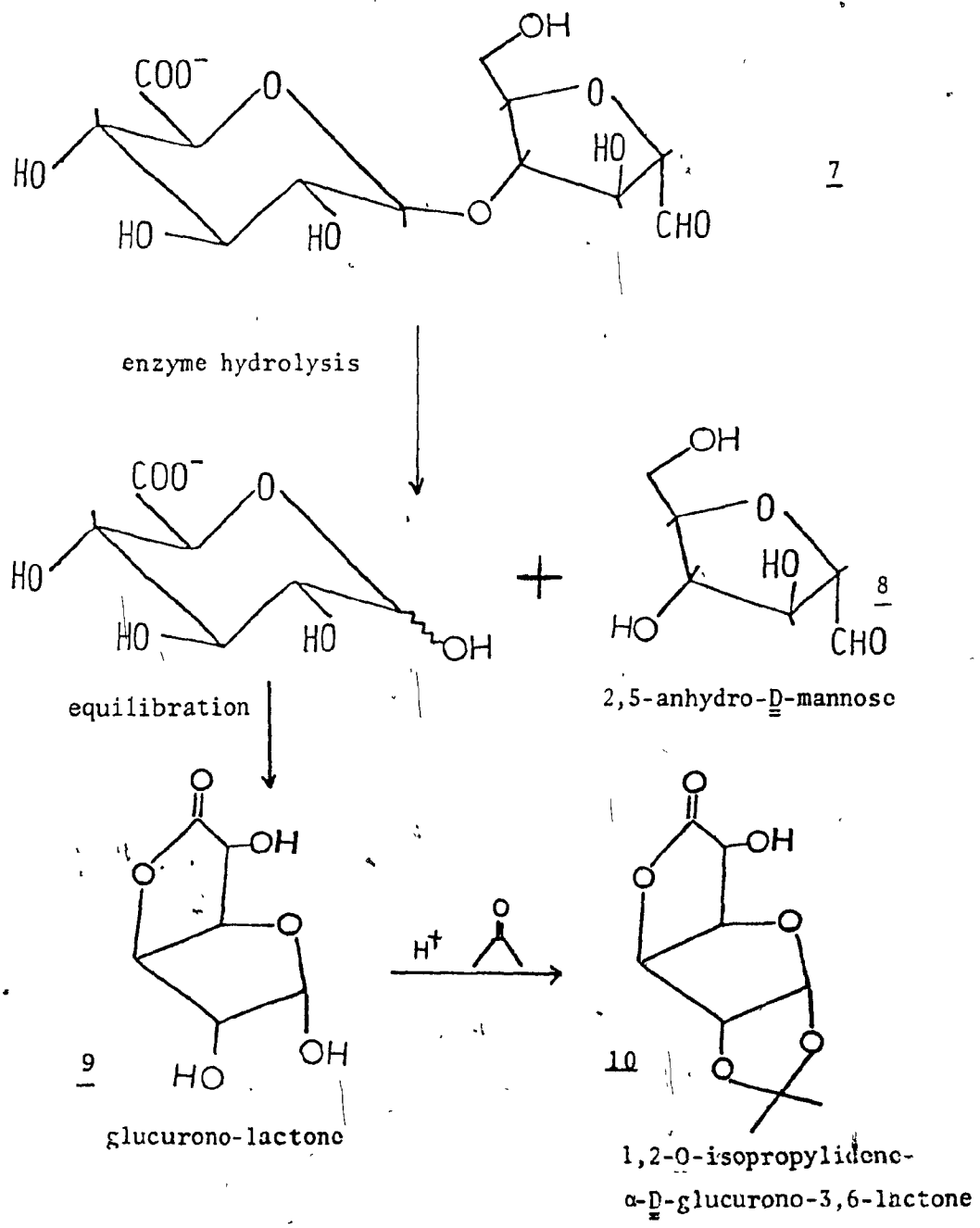


FIG. 11 Isolation of 1,2-O-isopropylidene-D-glucurono-3,6-lactone from disaccharide 7

CHAPTER IV

EXPERIMENTAL

IV EXPERIMENTAL

IV.1 General Procedures

IV.1.1 Analytical Methods.

IV.1.1.1 Melting points and optical rotations.

Melting points were determined with a Fischer-Johns hot plate melting point apparatus. The melting points are uncorrected. Optical rotations were measured with a Carl Zeiss 367732 polarimeter.

IV.1.1.2 Infrared and Visible Spectra

Infrared spectra were recorded with a Unicam S.P. 200G Grating infrared spectrophotometer. For the carbazole measurements a Beckman 91-F UV-VIS spectrophometer was used.

IV.1.1.3 P.m.r. Spectra.

A Varian HA-100 spectrometer (field sweep mode) was used to record the p.m.r. spectra with a sweep width of 1000 Hz. The solvent was D_2O, unless otherwise specified, and the operating temperature was 30°C. The commonly used reference is tetramethyl silane contained in a coaxial capillary tube mounted inside the sample tube. This, however, causes a downward displacement of the signals by 0.5 p.p.m.; accordingly the actual spectra have to be corrected by -0.5 p.p.m. Unless specified, the 100 MHz spectra were obtained with the aid of an internal capillary containing tetramethyl tin (TMT). With TMT, no correction is necessary since the signal positions are virtually equivalent to those obtained with internal TMS.

220 MHz spectra were recorded at the Canadian 220 MHz Centre, Sheridan Park, Ontario, using a temperature of 70° to 75°C. The 220 MHz spectra are not shift corrected, although of precise width (i.e. 1000 Hz) and hence the chemical shifts in p.p.m. are given relative to the 100 MHz spectra.

The samples were subjected to a preliminary deuterium exchange by

repeated (3 to 5 times) treatment with fresh deuterium oxide and then dissolved in 99.95% D_2O. Chemical shifts are in δ p.p.m.

IV.1.1.4 ^{13}C.m.r. spectra.

Proton-decoupled ^{13}C.m.r. spectra were recorded with a Bruker WH-90 spectrometer operating at a frequency of 22.63 MHz using deuterium oxide as solvent and source of the lock signal. The 8K memory B. NC 13 computer was used to acquire the spectral accumulations and perform the Fourier transformation. The proton-decoupled FT spectra were measured at 0.6 sec. repetition time, 18μ sec (70°) pulse width, 4K real data points, unless specified, a sweep width of 4,000 Hz and 50-120,000 scans.

The "gated decoupling" technique was employed to obtain the proton coupled spectra. The commonly employed parameters for the ^{1}H-coupled spectra were a repetition time of 1.2 sec, decouple time of 0.5 sec, and pulse width of 24 μsec (90°).

The heparan samples in D_2O solution were examined without deuterium exchange. Typical concentrations were 150-400 mg/ml. Over this range, a slight variation in the relative intensities of signals was observed, although there was no observable change in chemical shift. D_2O solutions of heparan salts had a pD of ∿6.5; in some instances, HCl was introduced to lower the pH to ∿2.

For the solutions of heparan a probe temperature of 60-70°C was used to reduce viscosity and, hence, decrease line widths. For less viscous solutions a probe temperature of 35-45°C was used. Differences in chemical shifts of samples recorded at high temperature and those at low temperature were < 0.2 p.p.m.

The chemical shifts are in the δ p.p.m. scale with respect to external TMS using methanol as an internal reference. The accuracy of the measurements

is considered to be 0.05 p.p.m. The chemical shift of methanol, in deuterium oxide relative to that of TMS contained in a coaxial capillary mounted inside the sample tube, was found to be 50.7 ± 0.1 p.p.m. at temperature range 45°-60°C.

Measurement of the frequency difference between the mid-points of the two peaks of the C-1 "doublet" gave the direct-bond coupling ($^1J_{C-H}$). Half-line widths were 10-13 Hz, and the error in ^1J values was estimated at ±2 Hz.

IV.1.2 Chromatographic Techniques

IV.1.2.1 High Speed Liquid Chromatography.

High speed liquid chromatographic separation was carried out with an Altex Model 300 liquid chromatograph. The chromatographic conditions were:

detector:	Altex model 153 UV.
column :	10 x 500 mm stainless steel packed with Poragel PN particle size 37-75μ.
flow rate:	2 ml/min.
pressure:	230 psi, nitrogen.
temperature:	ambient.
solvent :	H_2O twice distilled.
sample concentration :	20 mg/ml.
injection loop :	500 μl.

IV.1.2.2 Paper Chromatography.

Descending paper-chromatography was effected on Whatman No. 1 paper. The commonly used solvents were:

(A) acetic acid - 98% formic acid-H_2O = 5:1:4, (V/V/V).

(B) ethyl acetate-acetic acid-H_2O = 9:2:2, (V/V/V).

(C) t-butanol-ethanol-H_2O = 4:1:2. (V/V/V).

IV.1.2.3 Paper Electrophoresis.

Paper electrophoresis was performed on Whatman No. 3. Unless otherwise stated, the electrophoretic conditons used were;

potential : 500 V for 2 hrs.

buffers (A): high resolution (Gelman, pH=8.8)

(B): pyridine-acetic acid (pH=6.5)

In both the paper-chromatographic and electrophoretic techniques, visualization was effected by the Smith's[112] silver dip method. The silver nitrate reagent was,

(A) $AgNO_3$ saturated solution in H_2O (0.1 vol.)

Acetone (20 vol. or 100 vol.)

(B) NaOH, 0.5 per cent, in ethanol

The background was fixed with 2N ammonia.

IV.1.2.4 Thin Layer Chromatography.

T.l.c. (ascending) was carried out on silica gel G (Mackerey-Nagel) adsorbent on glass plates. The solvents commonly used were,

(A) ethyl acetate-methanol = 8:5:1.5, (V/V).

(B) ethyl acetate-ethanol = 3:2, (V/V).

(C) pyridine-ethyl acetate-acetic acid = 5:5:1:3, (V/V/V/V).

The plates were developed by spraying with concentrated H_2SO_4 followed by heating the plate with a flameless drier.

IV.1.2.5 Gel Permeation Chromatography.

Gel permeation was effected with neutral Sephadex (G-15) contained in a glass column (2.5 x 105 cm). The Sephadex powder was allowed to swell overnight in water, heated in a steam bath for 3 h, and then packed into the column. The packing was washed with water for 3 h for stabliization; the flow rate was 35 ml/h.

Molecular weights were determined by gel-filtration on Sephadex (G-75); 5% Na acetate in H_2O and 10% ethanol were used as the solvents.

IV.1.2.6 Ion-Exchange Chromatography.

Amberlite IR-120, $RSO_3^-H^+$, Amberlite IR-400 C.P., $RH(CH_3)_3^+Cl^-$ Dowex 50 W ($X-8,H^+$) resins were commonly used for ion-exchange. The resin was suspended in water and stirred. The fine particles which did not settle rapidly on standing were decanted off. The resin was suspended in sufficient 4N HCl at 100°C for 1 h. The yellow supernatant was decanted and the process repeated until the supernatant was colourless. This washing process was repeated with 2N NaOH. It was then washed with water and stored either in this Na^+ form or regenerated to the H^+ form, washed and stored.

Weak acid resins were purified at room temperature, and the anion exchange resins at a temperature of 30°C. The anion exchange resins were similarly treated with carbonate-free 2N NaOH and HCl.

Columns were fitted with an outlet tip above the level of the upper glass wool plug to prevent the column from running dry.

IV.1.3 Evaporation and Lyophilization

Evaporations were carried out under reduced pressure (about 20 mm Hg) and at a temperature of 40°C or less. Lyophilization was performed at < 0.5 mm Hg in flasks with a total volume of < 2 liters connected to a vacuum-pump through a Dewar flask trap containing acetone-dry ice.

IV.1.4. Centrifugation

High speed centrifugation was effected with a preparative Spinco Model ultracentrifuge equipped with 21 k and 40 k r.p.m. rotors. Speeds of up to 20 k and 35 k with respective rotors were used.

IV.1.5 Materials.

IV.1.5.1 Enzyme.

Bovine liver β-D-glucosiduronase; EC 3.2.1.31, type B-3, lot 104C-85101 was purchased from Sigma Chemical Co. The conditions used were: temperature 37°C in sodium acetate buffer, pH 5.

IV.1.5.2 Heparan.

Crude barium heparan salt, lot number 5-RTS-16, was obtained from Upjohn Co., Kalamazoo, Michigan.

IV.2 Fractional Precipitation and High Speed Centrifugation.

A 5% (W/V) aqueous solution (680 ml) of crude barium heparan salt was centrifuged in a preparative model ultracentrifuge at 10 k r.p.m. for three hours at low temperature to clarify the solution. The supernatant was then recentrifuged at 20 k r.p.m. giving about 5% of the material as a sedment (fraction 1). Ethanol was added dropwise, with vigorous mechanical stirring, to the supernatant solution at pH 7 and 25°, to incipient turbidity. The dispersion was centrifuged. The precipitated material was washed with 95% ethanol, then with absolute ethanol several times, redissolved in water, reprecipitated with ethanol, centrifuged, dissolved and finally lyophilized (fraction 2). The mother liquor was treated with more ethanol to yield another precipitate, which was recovered by centrifugation and workup, as just mentioned, giving fraction 3. This procedure was repeated until no further precipitation with ethanol could be effected. This point was reached after addition of about 60% (V/V) of absolute ethanol.

All in all, fifteen fractions were obtained by ethanol precipitation. A precipitation curve (Fig. 3) giving the weight of material precipitated out versus percent ethanol added, suggested that these fractions represented six distinguishable components. The fractionation process was followed by

p.m.r. spectroscopy for all fractions, and by ^{13}C.m.r. for some, as a guide to the composition of these materials.

Some fractions were further recentrifuged at higher speed, 35 k r.m.p. after addition of ethanol to incipient turbidity. However, re-examination by n.m.r. spectroscopy did not reveal any significant improvement in separation. Furthermore, other fractions were subjected to HSLC separation. Although differences in molecular size were observed (Fig. 5), the c.m.r. spectra of the sub-fractions were substantially similar.

IV.3 Desulphation of Heparan

Desulphation of heparan was effected by the procedures described by Inoue and Nagasawa[108]:

IV.3.1 Hydrolytic Desulphation.

The sodium salt of heparan (1.0 g) was dissolved in 0.04 M hydrochloric acid (86 ml) and heated at 100°C for 2 h. The solution was neutralized, dialyzed (by the same method as described below, and lyophilized (yield 76%). The product was examined by n.m.r. spectroscopy (Fig. 9).

IV.3.2 Solvolytic Desulphation.

The barium salt of heparan (460 mg) was ion-exchanged to the acid form with a column of Dowex 50W (X-8, H^+, 20-50 mesh, regenerated - (vide supra) at ~5°C. The effluent was neutralized with pyridine, evaporated to dryness, dissolved in dimethyl sulphoxide containing 5% of water (45 ml) and heated at 50°C for 1.5 h. The solution was diluted with an equal volume of water, adjusted with 0.1M NaOH to pH ~9.2, dialyzed against distilled water in batches (8x) for 72 h, and finally lyophilized (yield, 50%). The material recovered was examined by n.m.r. spectroscopy (Fig. 9).

IV.4 N-Deacetylation of Heparan.

Two methods of N-deacetylation were used, i.e. those described by Erbing, et al.[113] and Höök et al.[103]

IV.4.1 N-Deacetylation with NaOH and Methyl Sulphoxide[113].

Methylsulphinyl anion was synthesized as described by Sandford and Conrad[105]. In a dry, three-necked, round-bottomed flask (250 ml) fitted with a rubber serum cap and containing a magnetic stirring bar was weighed 1 g of sodium hydride (57%, coated with mineral oil). The hydride was washed (3x) by stirring with 30 ml portions of n-pentane. After the third wash, the flask was fitted with a thermometer and stoppered condensor, and residual n-pentane was removed by evacuation, the flask then being filled with nitrogen. Dimethyl sulphoxide (10 ml, reagent grade, dried over molecular sieves) was introduced, and the reaction mixture heated with stirring (magnetic stirrer) at 50°C until the solution became green and evolution of hydrogen gas ceased (50 min). The concentration of the anion in DMSO solution was determined by withdrawing an aliquot for titration with 0.1M HCl in aqueous solution, and the solution was then stored under nitrogen at 5° in a septum-capped flask.

Heparan (10 mg) and sodium thiophenolate (100 mg) were dissolved in water (ml), 2.13 M sodium methylsulphinyl methanide in methyl sulphoxide (8 ml) was added, and the mixture was heated at 98° in a sealed tube for 4 days, and lyophilized. Yield, 60%. The degree of N-deacetylation was examined by p.m.r. spectroscopy by noting the disappearance of the N-acetyl signal at 2.06 p.p.m.

IV.4.2 N-Deacetylation with Trifluoroacetic Acid.

Deacetylation with trifluoroacetic acid was carried out, with some modification, as described by Höök et al.[103]

Heparan (5 g) was dissolved in 2M trifluoroacetic acid (500 ml), and the solution was heated at 100°C in 2 separate sealed glass tubes for 3 h. The combined hydrolysate was evaporated to dryness and examined by n.m.r. spectroscopy for completeness of the reaction, as noted by diminution of the N-acetyl signal.

IV.5 Nitrous Acid Deamination.

Nitrous acid deamination was carried out according to Perlin et al.[34]

The N-deacetylated/desulphated heparan (4.7 g, neutral and dry) was dissolved in 1.1M acetic acid (53.3 ml), sodium nitrite (2.6 g) was added; vigorous effervescence occurred with evolution of a brownish orange gas. The solution was stirred for 24 h at 20°C, then nitrogen was bubbled through it for 1 h to remove the excess of nitrous acid. The volume was reduced to 4 ml, and this concentrate was applied in two batches to a column of Sephadex G-15 (2.5 x 105 cm). The column was irrigated with distilled water and the eluant was collected in 10 ml fractions at a rate of ~35 ml/h. Each fraction was examined chromatographically, electrophoretically, colorimetrically (carbazole) and, after evaporation to dryness and dissolution in deuterium oxide, by FT-90 MHz p.m.r. Pooling, where appropriate, was then performed.

IV.6 Carbazole Reaction.

The carbazole test was carried out as described by Dische[37,38].

Concentrated sulphuric acid (ACS grade, 96%) was diluted to 87%. The carbazole reagent was prepared as a 0.1% ethanolic solution of diphenylenimine (dibenzopyrole, commercial preparation twice recrystallized from benzene, and sublimed). Test materials used were D-glucuronic and D-galacturonic acids (Baker Chemicals). A standard curve was prepared with a commercial preparation of glucuronolactone (Anachemia Chemicals).

To 1 ml of the solution (0.3 mg/ml of hexuronic acid) in a stoppered tube equilibrated in an ice bath, was added sulphuric acid (87%, 6 ml). The reaction mixture was immersed in tap water, then in a water bath at 60°C for 90 sec., cooled immediately in tap water, and carbazole (0.2 ml) was introduced. The purple colour, which developed slowly, was measured (at 530 nm) after 1 h,

readings for the corresponding standard and blank being taken at the same time. The results were used qualitatively.

IV.7 Relaxation Effects of Gadolinium Ions.

The gadolinium test was performed as described by Casu et al.[104] Gadolinium nitrate solution (1 ml of 5.94 mg/ml) was added dropwise, to the test heparan or heparan derivative solution, up to 50 times in excess of the appropriate ($1:1x10^{-4}$) sugar:cation ratio. No significant diminution was observed in the apparent intensity of the C-6 and C-1 signals of the uronic acid moiety. However, general line-broadening due to the paramagnetic Gd^{3+} was observed at high concentrations of the ion.

IV.8 Hydrolysis of the Deamination Product.

Two techniques were employed to cleave the deamination product of heparan to monosaccharides:

IV.8.1 Hydrolysis by acid.

Acid hydrolysis was carried out according to the procedure described by Höök et al.[103] The disaccharide (400 mg) obtained from heparan by deaminative degradation was hydrolyzed with 2M trifluoroacetic acid (100 ml) at 100°C for 4 h. The solution was evaporated to dryness made up to a volume of 1 ml, and passed through a Sephadex G-15 (2.5 x 105 ml) column. The effluent was pooled where appropriate, concentrated and lyophilized (yield, 10%) and examined by n.m.r. spectroscopy and paper chromatography, solvent C.

IV.8.2 Enzymic hydrolysis.

A typical enzyme digestion was carried out as follows. Disaccharide 7, (1 g) from the heparan deamination reaction was dissolved in sodium acetate buffer (15 ml, pH 5). β-D-Glucosiduronase (EC. 3.2.1.31, bovine liver, 20 mg) in the acetate buffer (5 ml) was introduced, and the mixture stored at

37° for 24 h. Thereafter, three further 10 mg portions of enzyme in buffer solution (5 ml) were added successively at 12 h intervals, and 36 h later, when the incubation period was deemed to be over, the enzyme was denatured by heating at 100°C for 15 min. The resulting solution was dialyzed against distilled water (4x50) for 48 h. The dialyzate was concentrated, freeze-dried, and examined by n.m.r. spectroscopy and paper chromatography.

IV.9 Synthesis of Model Compounds.

Two model compounds 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone and 2,5-anhydro-D-mannose were synthesized.

IV.9.1 Synthesis of 2,5-anhydro-D mannose[78].

2,5-Anhydro-D-mannose was prepared in the following manner as described by Horton and Philips[114].

A solution of 2-amino-2-deoxy-α-D-glucopyranose hydrochloride (glucosamine HCl, 1.387 g) in water (16 ml) was stirred and allowed to attain mutarorational equilibrium (∿ 6 h at 25°C). The solution was evaporated to dryness and the residue was dissolved in 1.1M acetic acid (13.2 ml). Sodium nitrite (0.661 g) was added, causing a vigorous effervescence and evolution of brownish orange gas, and after 26 h at room temperature t.l.c. indicated disappearance of glucosamine. Nitrogen was bubbled through the solution for 30 min to remove excess nitrous acid, and the reaction mixture was evaporated to dryness. ^{13}C.m.r. spectroscopy indicated complete conversion into 2,5-anhydromannose. The resultant solid was dispersed in methanol (20 ml), and the methanol was evaporated off, and this treatment was repeated until the salts were granular and easily removed by filtration. The residue was washed twice with methanol, the filtrate and washings were combined and evaporated to yield a yellow, viscous, syrupy product. (This syrup darkens readily upon storage at room temperature, but can be stored frozen without decomposition for up

to 15 days). Acetonation of the 2,5-anhydromannose was attempted as described (sect. IV.9.2) but t.l.c. (solvent C) indicated that no reaction had occurred.

IV.9.2 Synthesis of 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone.

Using the procedure described by Owen et al.[106], D-glucuronolactone (240 mg) was introduced with mechanical stirring into a closed flask containing dry acetone (15 ml) and concentrated sulphuric acid (0.1 ml). After 25 h, the resulting clear solution was neutralized with barium carbonate, filtered, and the filtrate was evaporated to dryness. Dry ethyl ether was introduced, the suspension was filtered and petroleum ether (light) was added to the filtrate till incipient cloudiness. On standing at room temperature, colourless, needle-shaped crystals (yield, 180 mg, 60%), m.p. 119-120°, were obtained.

IV.10 Isolation of 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone from enzymic digest of the isolated disaccharide.

The disaccharide enzyme hydrolysate (300 mg) was acetonated (sect. IV.9.2) giving crystals (yield, 108 mg, 48%). Recrystallized from ether/light petroleum ether, the crystals had m.p. 118.5-120°C, undepressed by admixture with authentic 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone, $[\alpha]_D^{20}$ + 68 (C, 0.52, H_2O); literature[106] m.p. 120°, $[\alpha]_D^{18}$ + 70 (C, 1.0 H_2O).

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CLAIMS TO ORIGINAL RESEARCH

1. Fractionation of heparan by precipitation with ethanol and high speed centrifugation, has been monitored by n.m.r. spectroscopic (^{13}C and ^{1}H) techniques.
2. The p.m.r. and ^{13}C.m.r. spectra of the main fractions, and also of a disaccharide obtained as a major product of heparan, have been analysed in some detail.
3. The configuration and conformation of the constituent residues of the heparan polymer have been determined. The results are consistent with those obtained by other techniques.
4. Glucuronic acid, released from the major disaccharide product of heparan by enzymic hydrolysis, has been characterized as a crystalline derivative (i.e., 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone) of this acid.
5. High pressure liquid chromatography has been applied in examining the homogeneity and molecular weight of heparan fractions.