SYNTHETIC AND STRUCTURAL STUDIES RELATED TO THE CHEMISTRY OF HEPARIN

By

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ABSTRACT

Heparin of types A and B were subjected to deaminative degradation and fragments containing their minor constituent sugars were isolated. Structures are proposed for these fragments based primarily on n.m.r. spectroscopy. The results obtained contribute to a knowledge of the detailed structure of the biopolymer, and have a bearing on questions related to the biosynthetic pathways of heparin. Support is provided for the view that the α-L-idopyranosyluronic acid moiety in heparin exists preponderantly in the $^1C(L)$ conformation.

A novel synthesis of L-idopyranose has been developed starting with readily available L-sorbose. The sequence of reactions involves the reduction of 1,3,4,5-tetra-O-benzyl-L-sorbopyranose, and subsequent oxidation of the tetra-O-benzyl-L-iditol formed, to give 2,3,4,6-tetra-O-benzyl-L-idopyranose. The latter constitutes a derivatized form suitable for the synthesis of higher saccharides related to heparin.

R E S U M E

La dégradation déaminante d'héparines de types A et B a permis d'isoler des fragments contenant les sucres mineurs pour lesquels une structure est proposée, en se basant surtout sur les spectres r.m.n. Les résultats obtenus contribuent à la connaissance de la structure détaillée du biopolymère et ont des implications sur les questions relatives à la biosynthèse de l'héparine. Nous apportons des preuves à l'effet que la partie de l'héparine contenant l'acide α-L-idopyranosyluronique existerait d'une manière prépondérante dans sa conformation $^1C(L)$.

Nous avons développé une nouvelle synthèse de l'L-idopyranose à partir de l'L-sorbose. La séquence de réactions comprend la réduction de la 1,3,4,5-tétra-O-benzyle-L-sorbopyranose et l'oxidation subséquente du tétra-O-benzyle-L-iditol formé, pour produire la 2,3,4,6-tétra-O-benzyle-L-idopyranose. Ce dernier dérivé pourra servir à la synthèse de saccharides plus complexes, reliés à l'héparine.

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LIST OF ABBREVIATIONS

Bn	:	benzyl
c	:	concentration
c.m.r.	:	carbon-13 magnetic resonance
d	:	doublet
F.T.	:	fourier transform
h	:	hour
Hz	:	Hertz
I.R.	:	infra-red
J	:	coupling constant in Hz
lit	:	literature
m	:	multiplet
M	:	molar
mCi	:	millicurie
MHz	:	megaHertz
mM	:	millimole
m.p.	:	melting point
n.m.r.	:	nuclear magnetic resonance
p.m.r.	:	proton magnetic resonance
p.p.m.	:	part per million
q	:	quartet
r.t.	:	room temperature
s	:	singlet
t	:	triplet
TBDMS	:	tert - butyldimethylsilyl

t.l.c.	:	thin layer chromatography
T.M.S.	:	tetramethylsilane
Ts	:	p - toluenesulfonyl
u.v.	:	ultra-violet
$\sim$	:	approximately
δ	:	chemical shift in p.p.m. downfield from TMS
Δ	:	heat

CHAPTER 1

INTRODUCTION

1.1 Heparin

The term "heparin" has been, and still is, used with four different meanings, depending on the author (1): 1. any heat-stable, naturally-occurring substance that exhibits anticoagulant activity (neutralized by protamine, etc.); 2. a sulfated mucopolysaccharide present in tissues, highly metachromatic, with a high critical electrolyte concentration; 3. a commercial drug of varying composition and activity; 4. a sulfated mucopolysaccharide with distinctive chemical and biological properties.

This glycosaminoglycan (mucopolysaccharide), first discovered in 1916, and usually isolated from lung and mucosa, is probably the only polymer of the proteoglycan class which is not commonly present in connective tissues. Moreover, it possesses a wide variety of biological activities, of which the anticoagulant and antilipaemic effects are best known. In its anticoagulant role, this highly anionic polysaccharide specifically inhibits protein molecules involved in blood clotting.

The relationship between the chemical structure of heparin and its biological activity has been actively investigated for many years (1,2). Presumably, there is a precise relation between function and the shape of the heparin macromolecule, and the location and nature of active sites within the heparin chain is becoming a subject of increasing interest. Also, the biosynthesis of heparin is receiving particular attention (3). From a chemical viewpoint there is a need to fully characterize heparin and to differentiate it from heparin-like biopolymers such as heparan sulfate and dermatan sulfate, whereas until now anticoagulant activity has been the main criterion to dis-

tinguish them.

It was the goal of the present study to more fully characterize the composition and chemical structure of heparin and, particularly, to investigate the nature of minor components known to be present. Since structural studies on biopolymers are greatly facilitated by the availability of reference compounds, another objective of this study was to investigate the synthesis of molecules that are constituents of heparin. Furthermore, such compounds could be useful in biological studies for the synthesis of oligosaccharides related to heparin.

1.2 The chemistry of heparin

A number of excellent reviews are available on the chemistry of heparin; for example see ref 1,3,4,5 & 6. Therefore, this subject will be dealt with only briefly here.

Heparin is a linear, highly sulfated glycosaminoglycan made up largely of disaccharide repeating units, one sugar of which is a hexosamine, the other a uronic acid. Much work has been done over the years to elucidate the nature of its constituent residues and their mode of linkage, and to locate the sulfate ester groups along the chain.

Some of the basic properties of the polymer have been well-established for a number of years. Other chemical features have been discovered only recently. Perhaps the main reason for the relatively slow progress in arriving at the detailed structure of heparin has been the extreme resistance of this polysaccharide to depolymerization by acid. The glucosamine residues are N-sulfated and in the course of acid hydrolysis the labile sulfamino group is hydrolysed, producing a free, protonated amino group at C-2. As a result, most of the glycosaminyl bonds are rendered stable towards attack by protons of the acid and,

therefore, the severe conditions needed to effect cleavage of the polymeric units result in undue destruction of individual residues, especially the uronic acids. The validity of determinations of the composition of acid hydrolyzates was for years one of the outstanding problems in the study of heparin.

Nevertheless, Wolfrom et al (7) isolated a reducing disaccharide, "heparosinsulfuric acid" (i.e., 4-O-α-D-glucosaminyl-hexuronate) in 20% yield from a hydrolyzate of heparin. The α-D configuration of the glucosaminyl residue was inferred from the optical rotation of the compound. Also, evidence for the presence of D-glucuronic acid was put forward by Wolfrom and Rice (8) when D-glucaric acid, formed (in low yield) by a combined oxidation-acid hydrolysis procedure, was isolated. The identity of the 2-amino-2-deoxy-D-glucose component was established either by direct isolation (9), degradation of the heparin polymer with ninhydrin (10), or oxidation of the residue to 2-amino-2-deoxy-D-gluconic acid (8).

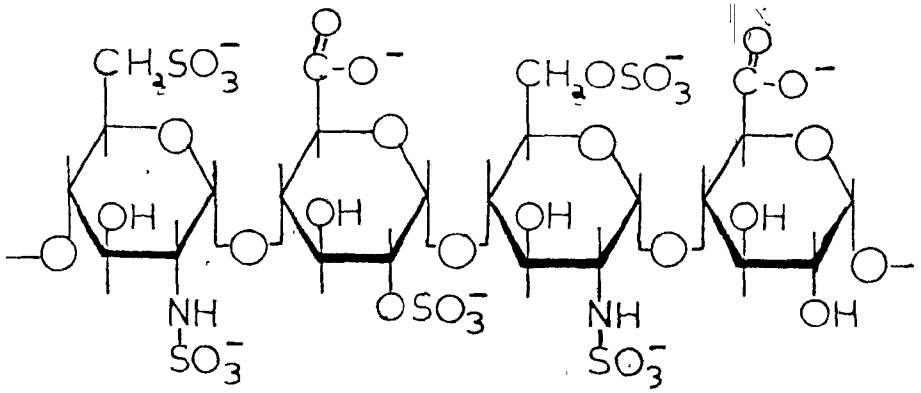
Though substantial progress had been made in elucidating the structure by direct acid hydrolysis and oxidation procedures, modified methods for easier depolymerization had to be found. Thus Wolfrom et al (11) subjected heparin successively to de-N-sulfation, partial de-O-sulfation, N-acetylation and reduction of carboxyl groups. The N--acetylation step thus afforded 2-acetamido-2-deoxy-D-glucopyranosyl residues, which have been shown (12,13) to hydrolyse about 100 times as rapidly as 2-amino-2-deoxy-D-glucopyranosyl residues. Furthermore, the neutral type of glycosyl residue formed in the carboxyl-reduction step, was more prone to hydrolytic cleavage than the uronosyl structure. On graded acid hydrolysis of this modified heparin, two crystalline

amino sugar-containing disaccharides were obtained (in 20% yield); namely, 4-O-(α-D-glucopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranose and 4-O-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-α-D-glucopyranose. It was thought that these structures represented the alternating sequence of units in heparin, and that the polymer possesses an α-D-(1→4) linked backbone.

Further evidence for the α-configuration of glycosidic linkages of the glucosamine units was reported again by Wolfrom et al (14) when 2-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)-1,3-di-O-acetylglycerol was isolated from modified heparin.

Methylation (15) and periodate oxidation studies (16, 17) provided more evidence for a regular (1→4) sequence of linkages in heparin and for the presence of D-glucuronic acid residues. Further identification of D-glucuronic acid was obtained by other procedures (18). Studies on the location of the O-sulfate ester group of heparin (17, 19) showed that these substituents are attached to C-6 of the 2-deoxy-2-sulfamino-D-glucopyranose residues, and to half of the glucuronic acid residues at C-2.

Until the late 1960's, therefore, it was logical to presume that heparin is represented basically by the following partial structure:



However, our view of the structure of heparin has undergone a major change over the past ten years. A degree of uncertainty over the proposed structure (above) developed, basically because the yield of D-glucuronic acid or products derived from it could only account for a very minor portion of the total uronic acid content of heparin. In addition, the observed optical rotation of heparin was significantly lower than would be expected for an entirely α-D-linked polymer. Therefore the nature of the uronic acid component of heparin was re-investigated.

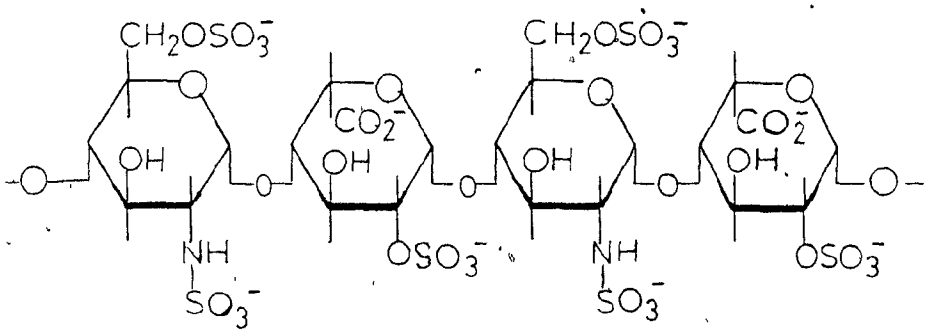
As far back as 1962, Cifonelli and Dorfman (20) claimed that L-iduronic acid was present in a hydrolyzate of heparin. It appears that this report was met with skepticism by many workers in the field. However, a number of years later its validity was confirmed by the isolation of L-idose (after carboxyl reduction) and, with supporting data from p.m.r. spectroscopy, it was shown that the L-ido isomer is the major uronic acid in heparin, whereas the D-glucuronic acid is a minor component. These, and other studies (below), utilized a de-polymerization technique which involves deaminative cleavage of the glycosaminyl bond with nitrous acid. In this reaction glucosamine residues of heparin are converted, in an degradation process, primarily into 2,5-anhydro-D-mannose units, with cleavage of the glycosamindic linkages* (21). The uronic acids remain intact. Thus, after deaminative degradation

* Although the major degradation product is expected to be a uronosyl-anhydromannose, a second product may be formed by deamination of the D-glucosamine residue without bond cleavage. Shively et al (22) identified such a product, which contained a hydroxymethylpentose sulfate residue.

of heparin, oligosaccharides were isolated (23) from which both D-glucuronic and L-iduronic acids were released by acid hydrolysis. Other supporting evidence for the presence of iduronic acid residue in heparin has been obtained by methanolysis of the polymer (24) and by deaminative degradation studies on carboxyl-reduced (25) and N-acetylated heparin (26). Gas-liquid chromatographic analysis of such degradation products (26) has shown that L-iduronic acid accounts for approx. 70% of the total uronic acid content in some heparins, whereas highfield p.m.r. spectral studies of beef lung heparin (27) require that iduronic acid constitute ≈80% of the uronide portion. ^{13}C.m.r. spectra of heparin and its major deaminative degradation product (28) have likewise shown that L-iduronic acid is the major acid moiety in heparin.

The L-iduronic acid residues were shown to be 4-linked and to have the α-configuration, by the isolation of an elimination product, i.e., 4-O-(4-deoxy-α-L-threo-hex-4-eno-pyranosyl-uronic acid 2-sulfate)-2-deoxy-2-sulfamino-D-glucopyranose 6-sulfate (29, 30) formed by enzymatic degradation of heparin. From these studies and from periodate oxidation of heparin (19, 23) it has been shown that the 2-position of L-iduronic acid is sulfated.

Thus, the major structural alternating unit of heparin consists of 1→4 linked residues of α-L-idopyranosyluronic acid 2-sulfate and 2-deoxy-2-sulfamino-α-D-glycopyranosyl 6-sulfate as illustrated below:



However, other details of structure have not been fully established. Heparin composed entirely of N- and O-sulfated disaccharide units (1) has not been encountered. Instead, the presence of significant proportions of N-acetyl glucosamine and non-O-sulfated residues (19, 31, 32) must be accomodated, as well as the glucuronic acid residues known to exist in heparin and reported to have the β-configuration (33).

The presence of α-L-iduronic acid 2-sulfate as a major component of heparin has stimulated interest in the conformation of this residue, because α-idopyranose itself has for long been regarded as a species having a relatively high conformational free energy. Although the functional significance of the L-iduronic acid residues is largely unknown, conformational aspects of this type of residue are important in determining the overall macromoleclar shape and properties of heparin (e.g., the ability to bind certain cations). This question is considered further below.

There appears to be at least two types of heparin marketed currently for medicinal purposes. This has been suggested by n.m.r. studies (34), the major difference being the presence (type-A) or virtual absence (type-B) of N-acetyl-hexosamine residues. The present

study has examined both types of heparin and has used n.m.r. spectroscopy extensively to deduce structure, with an emphasis being placed on the minor components.

1.3 Synthetic studies on heparin saccharides

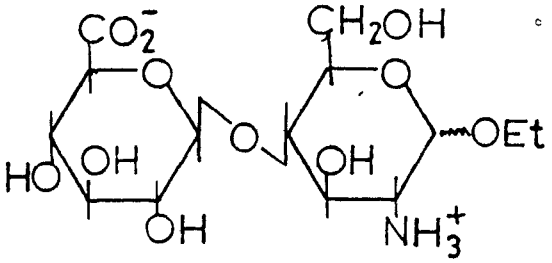
Much of the information available on the constituent monosaccharides of heparin has been obtained by characterizing the saccharides released by chemical and enzymatic degradation of heparin, or modified heparins. It would be beneficial to the overall study of this polymer to synthesize structurally-related saccharides, because model compounds would be useful as reference substances in chemical and spectroscopic investigations, as well as biological studies.

In recent decades, many attempts have been made to develop inexpensive synthetic products possessing heparin-like activity. For this purpose, a number of sulfuric acid esters of various degraded polysaccharides (cellulose, amylose, chitin, dextran, etc.) have been prepared (35, 36, 37). However, most of these heparinoids showed low anticoagulant activity and/or were found to be toxic for clinical use.

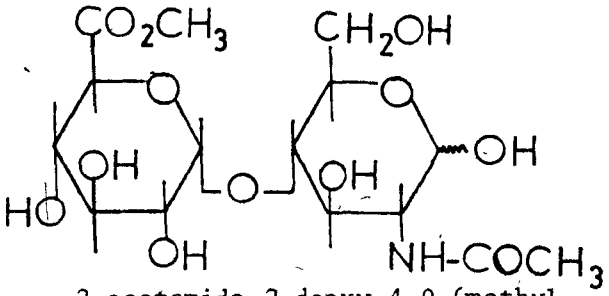
A substantial amount of work has been done on the synthesis of low molecular weight compounds structurally related to heparin. But, because the importance of L-iduronic acid had not been recognized until recently, starting materials for the synthesis of reference compounds have involved mainly D-glucuronic acid; Fig. 1 illustrates most of these saccharides that have been synthesized. As a consequence, in the current studies some attention has been paid to synthesis in the L-ido series.

L-Idose does not occur naturally, and L-iduronic acid is

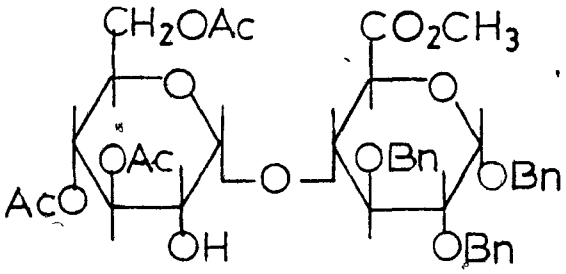
Fig.1. Synthetic saccharides related to heparin



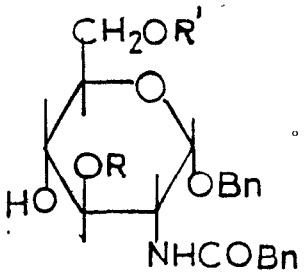
Ethyl 2-amino-2-deoxy-4-O-(β-D-glucopyranuronsyl)-D-glucopyranoside (38)



2-acetamido-2-deoxy-4-O-(methyl ∝-D-glucopyranosyluronate)-D-glucose (39)

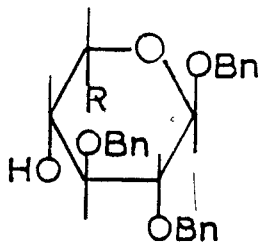


Methyl(benzyl 2,3-di-O-benzyl-4-(3,4,6-tri-O--acetyl-∝-D-glucopyranosyl)-∝-D-glucopyranoside) uronate(40)



R=Bn, R'=Tosyl
R= " R'= Benzoyl
R =Benzoyl, R'= "

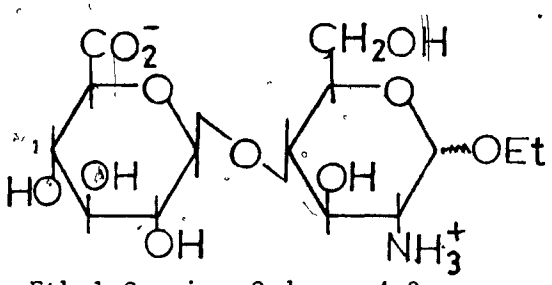
Derivatives of D-glucosamine(40)



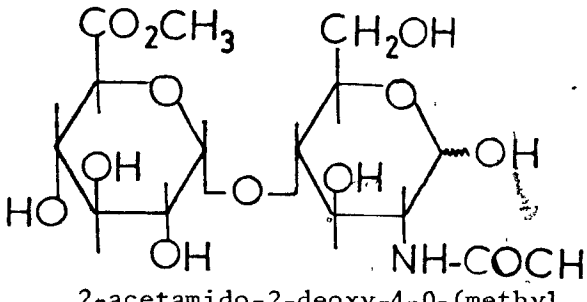
R= -CO_2CH_3

Methyl(benzyl 2,3-di-O-benzyl--∝-L-idopyranosid)uronate (42)

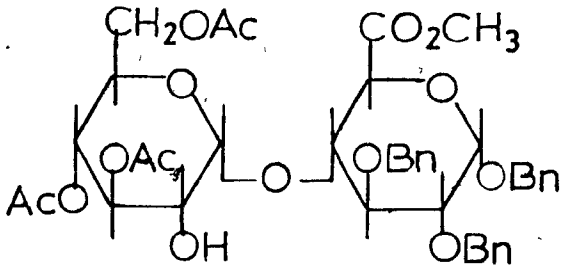
Fig.1. Synthetic saccharides related to heparin



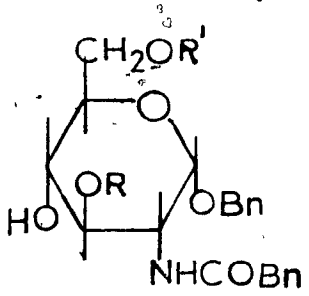
Ethyl 2-amino-2-deoxy-4-O-(β-D-glucopyranuronsyl)-D-glucopyranoside (38)



2-acetamido-2-deoxy-4-O-(methyl α-D-glucopyranosyluronate)-D-glucos (39)

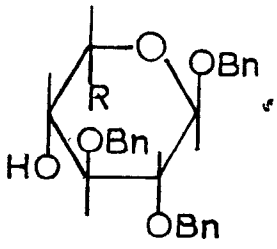


Methyl(benzyl 2,3-di-O-benzyl-4-O-(3,4,6-tri-O-acetyl-α-D-glucopyranosyl)-α-D-glucopyranoside) uronate(40)



R=Bn, R'=Tosyl
R=" , R'=Benzoyl
R=Benzoyl, R'="

Derivatives of D-glucosamine(40)



R= $-CO_2CH_3$

Methyl(benzyl 2,3-di-O-benzyl-α-L-idopyranosid)uronate (42)

CHAPTER 2

STRUCTURAL STUDIES ON HEPARIN

2.1 Heparin (B-type)

2.1-1 Deaminative degradation of heparin (B-type)

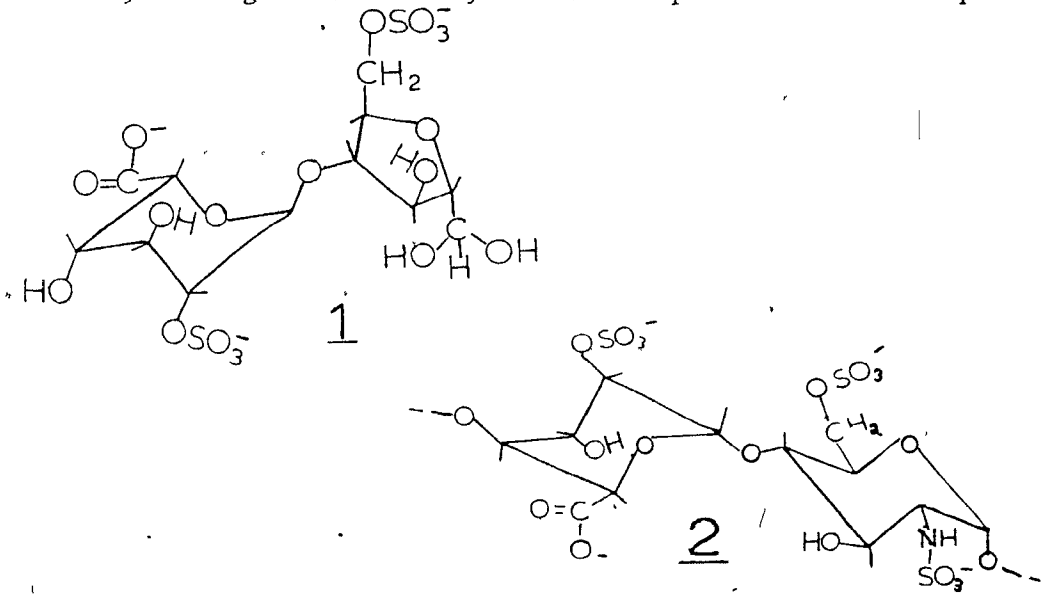
This section of the thesis deals with the products formed by deaminative degradation of heparin with nitrous acid. Although such products had been examined in earlier studies (23, 28), it was of interest to achieve a fuller characterization than previously available of fragments that contain minor constituents of heparin. In this way, it was proposed to provide details about the structural significance of these constituents in the polymer. As will be shown, n.m.r. spectroscopy proved to be especially useful in pursuing this objective.

Beef lung heparin (B-type) was analysed first because of its relative purity (containing only a small proportion of acetamidodeoxyhexose residues). An exhaustive deaminative degradation of this polymer should produce disaccharide 1, i.e., 4-O-(α-L-idopyranosyluronic acid 2-sulfate)-2,5-anhydro-D-mannose 6-sulfate as the major product (28). Minor degradation products (which have not been characterized) would likely contain residues of D-glucuronic acid, 2,5-anhydro-D-mannose and acetamidodeoxyhexose and might include saccharides in which a low degree of O-sulfation in the original heparin, or partial de-O-sulfation during the deamination reaction could be represented.

An examination of the minor constituents of heparin should be more feasible with the more plentiful products to be obtained from A-type heparin. However, a detailed n.m.r. analysis of disaccharide 1 and any other products isolated from type B should provide useful data for conformational studies, and furnish an additional array of chemical shift information for comparative purposes. Furthermore,

a pure preparation of disaccharide 1 was required for use in biochemical assays, as noted below.

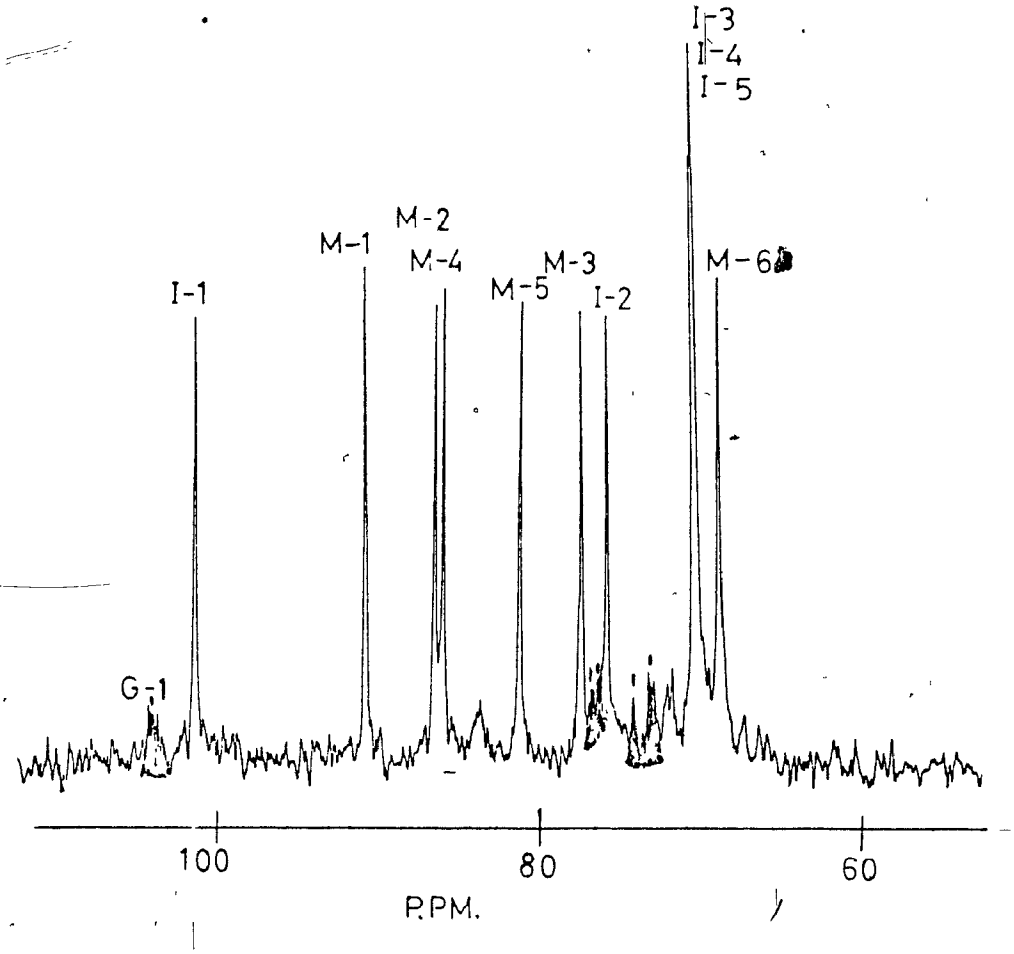
When treated with nitrous acid ($NaNO_2$-HCl), heparin B was degraded extensively into compound 1. The proton-decoupled spectrum of the total reaction product was similar to that of the spectrum published by Perlin et al (28) for compound 1; signals other than those of 1 were minor (Fig. 2). Fractionation by gel permeation chromatography on Sephadex produced a product in 60% yield, the ^{13}C.m.r. spectrum of which was practically identical to that of 1. It has been shown (28) that the observed chemical shifts of 1 are generally consistent with the major biose repeating sequence 2 of heparin, (see Introduction) although a full analysis of its spectrum was not reported.

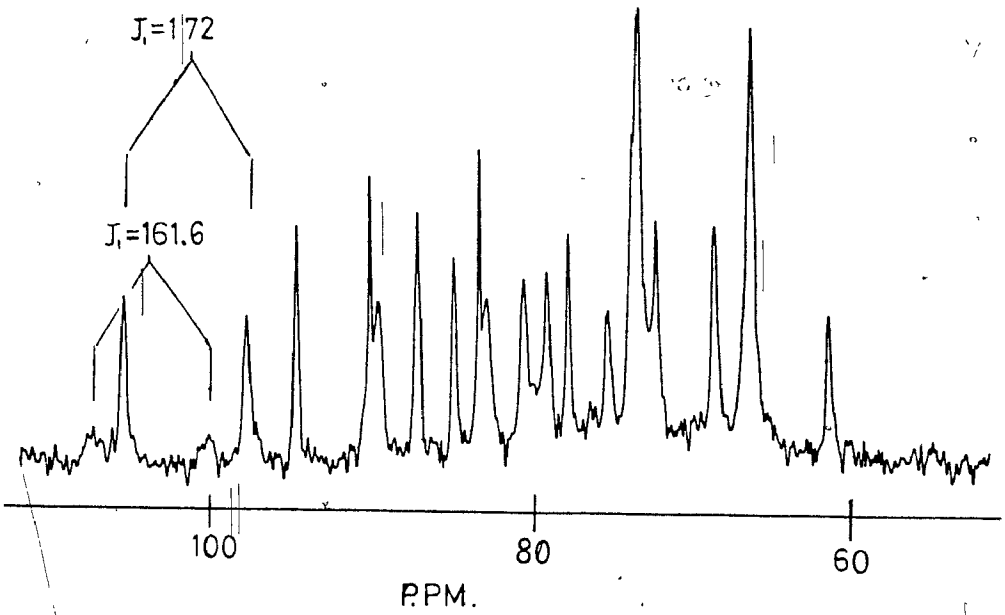


Disaccharide 1 unequivocally produces the twelve resonance lines required by the compound itself and sequence 2 (Fig. 2). Several important ingredients of structure 2 are derivable directly from this spectrum. Thus, the three-carbon signal at 70.9 p.p.m. is coincident with I-2 and I-5 of the heparin spectrum (I is ascribed to the α-L-idosyluronic acid residue). I-4 of 1 should now account for the remaining

Fig.2. Partial ^{13}C-n.m.r. spectrum of the deaminative products of B-type heparin.

Fig.3. Partial ^{13}C-n.m.r. proton — coupled spectrum of the deaminative products of B-type heparin


I-3
I-4
I-5
M-2
M-1
M-4
M-3
M-5
M-6
I-1
I-2
G-1
100
80
60
P.PM.


J₁=172
J₁=161.6
100
80
60
P.PM.

signal in this upfield region because C-4 is no longer engaged in the polymeric linkage. Other signals common to both spectra are those at 101.1, 76.4 and 177.4 p.p.m. ascribable in each instance to I-1, I-2 (O-sulfated) and I-6, the carboxyl ^{13}C, respectively. A more thorough discussion of these ^{13}C assignments can be found in the following section.

The coupling constant $^{1}J_{C-H}$ for I-1 of 1 was found, by means of a proton-coupled spectrum, to be 172 Hz. This $^{1}J_{C-H}$ value is characteristic of α-anomeric carbons (axial C-1-O-1) including those involved in glycosidic linkages (51, 52). That is, coupling between an anomeric carbon and its appended proton is either around 160 Hz or 170 Hz, these values being associated with an equatorial or axial anomeric configuration, respectively.

Reference to the ^{13}C spectrum of sodium methyl α-D-idopyranosiduronate (53) as a possible model for the uronic acid moiety of heparin, shows that I-3, I-4 and I-5 are closely grouped within the range 70.6-72.6 p.p.m. This coincides with the broad four-carbon signal at 71.5 p.p.m. of heparin (which includes I-3, I-5).

Signals (designated M) of the 2,5-anhydro-D-mannose residue of 1, with the exception of that of the primary sulfated carbon (M-6) at 69.4 p.p.m., are seen to be markedly downfield (region of 77.8 to 90.0 p.p.m.) relative to those signals of the uronic acid moiety and to those of the hexosamine residue in heparin from which it was derived. This downfield shift effect is consistent with most ^{13}C signals of furanose derivatives (34,54). M-1, the hydrated aldehydic carbon, resonates at 91.0 p.p.m.

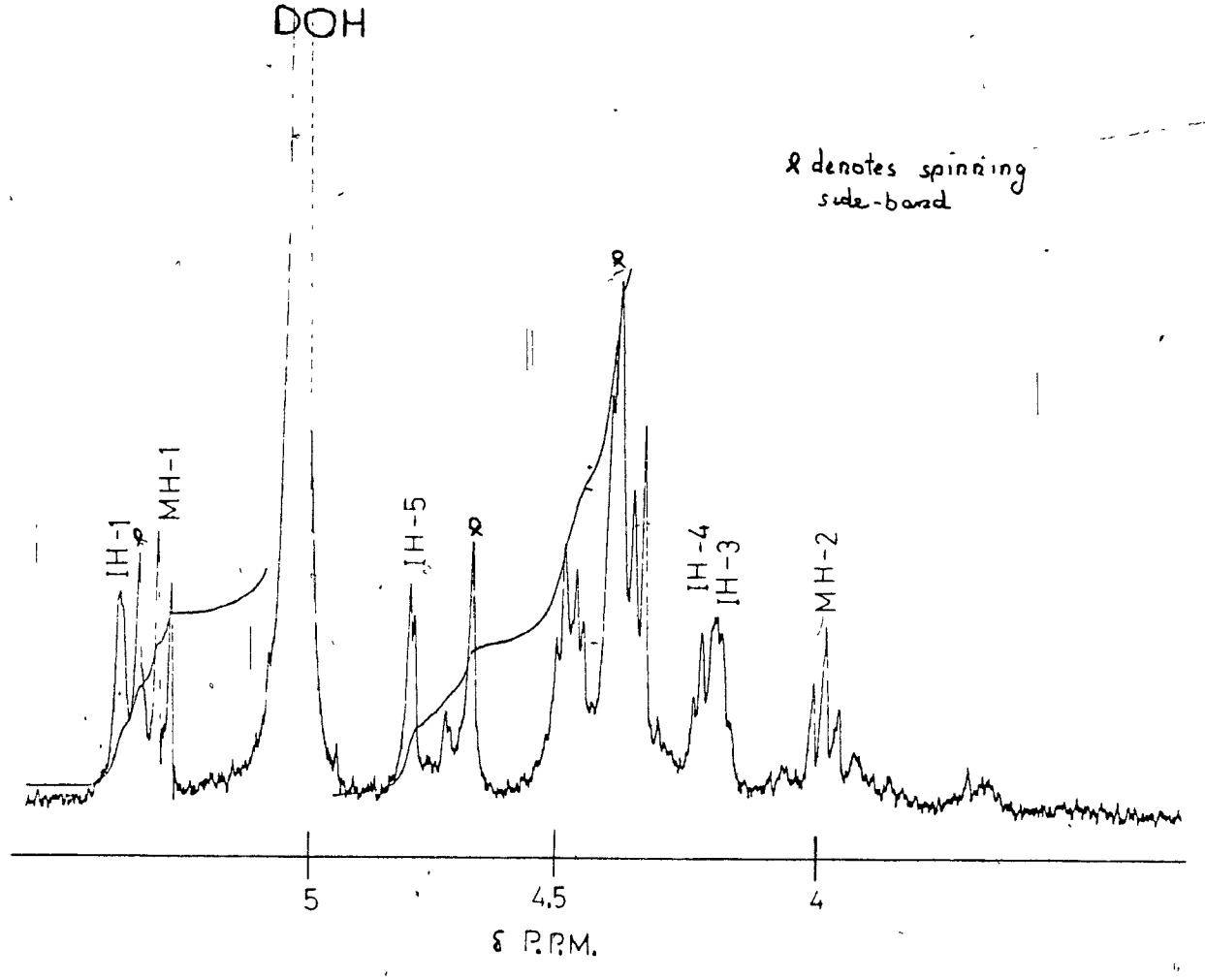
P.m.r. spectroscopy afforded additional supporting evidence of the structure of 1 and its relevance to the heparin biose repeating sequence 2. The 100 MHz p.m.r. spectrum of the isolated disaccharide was almost indistinguishable from that of the deamination product presented in ref. 50, Fig. 10. At 220 MHz and 270 MHz, the spectra more clearly show IH-1 and IH-5 of the L-idosyluronic acid residue to resonate at 5.34 and 4.67 p.p.m., respectively (Fig. 4). IH-5 is strongly influenced by pH and will shift within a 0.3 p.p.m. range. These signals coincide in shift with the corresponding resonance of heparin (27). The 270 MHz spectrum* (Fig. 5) better resolves these two signals and affords a coupling constant of 2.5 Hz for IH-1 and 2.0 Hz for IH-5. These results are consistent with the α-L-ido configuration and $^1C(L)$ assignments proposed earlier for the uronic acid residues (30,53). The hydrated aldehydic proton (MH-1) resonates at 5.25 p.p.m., giving a doublet with a coupling constant of 5.5 Hz (measured at 220 MHz).

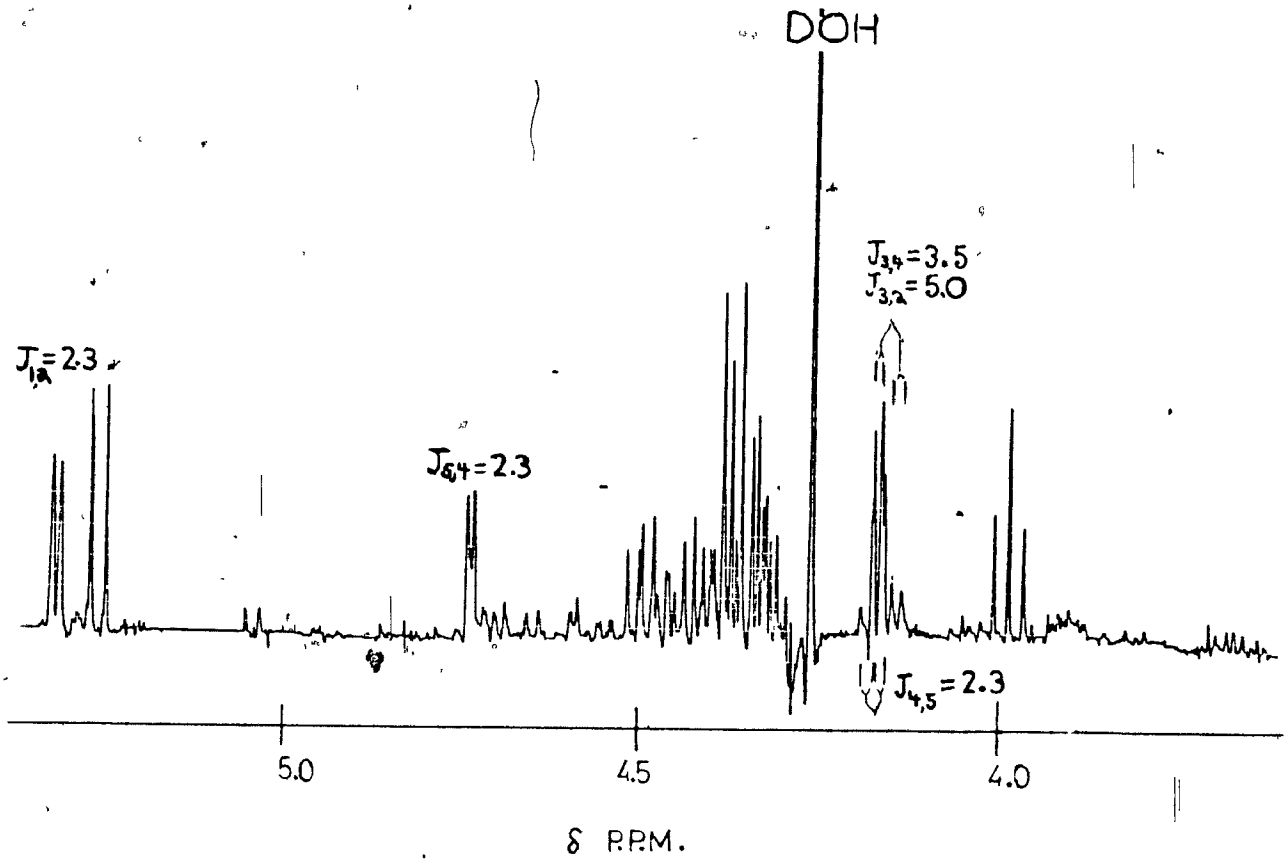
All other proton signals, except MH-2 which, at 3.97 p.p.m., appears as a triplet ($^3J_{H-H}$ of 5.5 Hz at 220 MHz), resonate downfield of 4.0 p.p.m. For IH-2, this observation is in accord with the expected strong deshielding effect of the O-sulfate on carbon-2, without which IH-2 would resonate at approx. 3.6 p.p.m. (53). Noteworthy also is the fact that all three signals upfield of 4.0 p.p.m. in the heparin spectrum are attributable to the 2-amino-2-deoxy-D-glucose residues (27). These latter signals are absent from the p.m.r. spectrum of 1, being now replaced by those of the 2,5-anhydromannose residue which appear downfield of 4.0 p.p.m.; the inclusion of MH-6 and 6' in this region means that carbon-6 is O-sulfated.

* This spectrum was kindly recorded by Dr. G. Gatti.

Fig.4. 220 MHz p.m.r. spectrum of disaccharide 1

Fig.5. 270 MHz p.m.r. spectrum(using convolution difference technique) of disaccharide 1


DOH
ℛ denotes spinning side-band
IH-1
ℛ
MH-1
IH-5
ℛ
ℛ
IH-4
IH-3
MH-2
5
4.5
4
δ P.P.M.


DOH
$J_{3,4}=3.5$
$J_{3,2}=5.0$
$J_{1,2}=2.3$
$J_{5,4}=2.3$
$J_{4,5}=2.3$
5.0
4.5
4.0
δ P.P.M.

In conclusion, the findings of this study and those of others referred to, point to structure 2 as being the major repeating biose unit of type B heparin; that it is present in at least two-thirds of the heparin macromolecular structure, and, according to the relative intensities of the major signals (those of 1) and the minor signals in Fig.2, possibly 85%. Of the polymeric material remaining, a few possible structures will be put forward.

2.1-2. β-D-Glucuronic acid residues in type B heparin

Evidence that β-glucopyranosiduronic acid is a minor residue of the type B heparin and occurs as a non-reducing residue among the minor deamination product is abstracted from spectrum Fig.2. The ^{13}C chemical shifts for β-D-glucuronic acid (54), and calculated shifts to allow for a glycoside structure are given in Table 1, and these are compared with data for shaded peaks in spectrum Fig.2. This shows that there is a fair agreement between the calculated and the observed values. One also was able to abstract the coupling constant for the minor (U-1) peak at 103.8 p.p.m. (Fig.3). The $^{1}J_{C-H}$ valve was approx. 160 Hz, which is consistent with a β-D-glucopyranosyluronic acid structure; the β-anomer of the free acid shows a $^{1}J_{C-1-H-1}$ of 161.6 Hz(54). The 2,5-anhydro-D-mannose moiety to which the β-glucopyranosyluronic acid is attached is possibly non-sulfated; i.e., the minor peak at 84.0 p.p.m. corresponds exactly to M-5 of a non-sulfated 2,5-anhydro-mannose residue (see section 2.1-4 for chemical shifts of such a residue). It will be shown later that all other M-signals (except M-6 signal non-O-sulfated) remain the same, unaltered by a de-O-sulfation of C-6. Unfortunately it was hard to see the M-6 signal in Fig.2.

TABLE 1

Observed and Calculated ^{13}C Chemical Shifts of Minor Signals in Type-B Heparin

Carbon	β-D-glucuronic acid Obs.[a]	Correction increment[b]	Calc.	Obs.
U-1	97.0	+7	104.7	103.8
U-2	75.1	-1	74.1	74.6
U-3	76.7	-	76.7	75.9
U-4	72.9	-	72.9	73.4
U-5	77.1	-	77.1	77.0

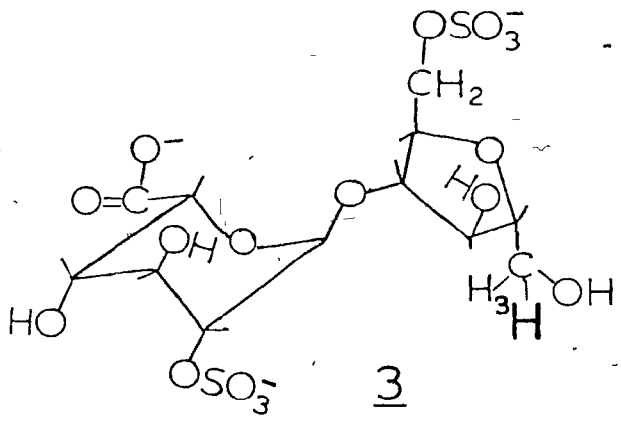
(a) values altered by -0.4 p.p.m. from those of ref.(54) to correspond to difference in reference (CH_3OH) value.

(b) Approx. change in chemical shift expected relative to the corresponding ^{13}C of the model compound; on formation of glycosidic bond at anomeric (+7 p.p.m.); due to bond introduced at adjacent position (-1 p.p.m.) (63-65).

This evidence of the existence of residues of both β-glucopyranosyluronic acid and non-sulfated 2,5-anhydro-mannose in minor amount in no way rules out the possibility that an α-L-idopyranosyluronic acid is linked to the latter moiety. In fact, paper chromatography of radioactively-labelled 1 and the minor constituents shows the presence of mono-O-sulfated disaccharides, as well as disulfated disaccharides (i.e. structure 1). This is referred to in the next section.

2.1-3. Reduction of disaccharide 1 with sodium borohydride-t

Disaccharide 1 was modified for use in collaborative studies (with Dr. E. Delvin) on its biological properties (55). That is, the aldehyde was reduced with highly radioactive sodium boro[3H]hydride-t. The ^{13}C.m.r. spectrum of its reduced form (3) was well resolved, twelve carbons being clearly accounted for. M-1, originally at 91.0 p.p.m., now reappeared at 62.6 p.p.m. as a primary carbon. Other shift differences relative to the spectrum of 1 were minor, the signals for the remaining 2,5-anhydro-D-mannitol carbons being displaced downfield by approx. 1 p.p.m.



3

When the labelled substrate (3) was analyzed by paper chromatography and radioactive counting, it was found to contain minor radioactive impurities. One of these appears to be mono-O-sulfated disaccharide. This result could mean that during the deaminative and/or reduction reactions and workup, de-O-sulfation had taken place. Alternatively, the minor component of unmodified type B heparin can be characterized as being less sulfated. Since an O-sulfate is an ester of a strong acid, and the de-O-sulfation reaction is an equilibrium reaction, hydrolysis can occur under both alkaline and acid conditions; deamination uses the latter condition, reduction, the former. The primary M-6 sulfate group is the most susceptible to de-O-sulfation.

The preparation appeared, nevertheless, to be generally suitable for the proposed assays of sulfatase and iduronidase enzyme activity (55), although results on its biological activity have not as yet been reported by Dr. E. Delvin.

2.1-4. Additional n.m.r. spectral characteristics

P.m.r.

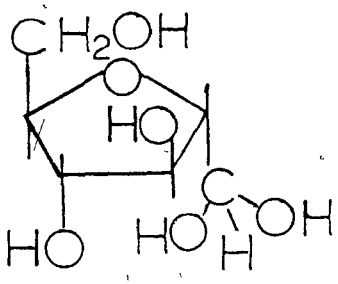
Attempts had been made to estimate the composition of various heparins by p.m.r. spectroscopy at 100 MHz (34) but it was impossible to assign most of the protons or to determine their coupling constants. At a higher frequency (i.e., 220 MHz) the spectra were analysed more thoroughly (27). Aside from assignments for the protons of the hexosamine residue and IH-1 and IH-5, tentative assignments were proposed for IH-4 and IH-2 of the L-idosyluronic acid residue. Since position-4 of the uronic acid is involved in glycosidic bonding and position-2 is O-sulfated, deshielding of the protons by 0.3 - 0.4 p.p.m. is expected relative to idopyranosyluronic acid. Therefore, the peaks at about 4.2 and 4.1 p.p.m. of the heparin p.m.r. spectrum probably

account for IH-2 and IH-4. The chemical shift of IH-3 is, tentatively, around 4.0 p.p.m.*

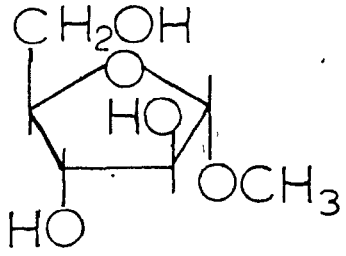
To determine the chemical shifts and coupling constants of disaccharide 1, proton shift data for related model compounds and for glycosidation and O-sulfation influences on individual protons and adjacent protons must be considered. By measuring the magnitude of proton coupling constants of the uronic acid moiety of 1, one should be able to describe its conformation and to say something about the conformation of the α-L-idopyranosyluronic acid residue in the polymer itself. Also, chemical shift data for both residues of 1 will help in elucidating the p.m.r. of higher molecular weight species of (type A) heparin.

P.m.r. data for 2,5-anhydro-D-mannose (4) are given in Table 2, and for methyl α-D-idopyranosiduronic acid in Table 3.

Since the spectrum of 4 was not first order, a complete analysis of the coupling constants was not attempted. However the proton assignments proposed are close to those for methyl α-D-arbinofuranoside (5) based on its 220 MHz spectrum. This comparison should be reasonably valid because both compounds have an analogous furanose ring (compare 4 and 5).



4



5

* A very recent study at 270 MHz has now provided a highly detailed analysis of beef lung (type B) heparin (56)

TABLE 2

Expected Chemical shifts of 2,5-anhydro-D-Mannose Moiety of 1

Proton	Obs. δ values[a] 2,5-anhydro-mannose (4)	Correction Increment[c]	δCalc for 1	Obs. coupling[b]
H-1	5.28	0	5.28	5.5 (d)
H-2	3.96	0	3.96	5.5 (t)
H-3	4.37	0	4.37	(q)
H-4	4.1	0.3-0.4	4.4-4.5	(t)
H-5	4.23	0.2	4.43	(d)
H-6	3.95	0.5	4.45	(b s)

(a) Recorded in D_2O at 100 M Hz (30°C); internal reference, sodium acetate, (at 2.1 p.p.m.)

(b) Multiplicity of signals; (b s) -broad signal due to virtual coupling (d) -doublet, (t) -triplet, (q) - quartet.

(c) Approx. change in chemical shift expected due to O-sulfation or glycosidic bonding. (27,57).

TABLE 3

Expected Proton Chemical Shifts for α-L-idopyranosiduronic Acid Moiety of 1

Proton	Methyl α-D-idopyranosiduronic acid sodium salt (53) δ value	Correction increment	δ Cal for 1	Obs. coupling
IH-1	4.73	0.27	5.0	doublet
IH-2	3.65	0.58-0.71	4.23-4.37	triplet
IH-3	3.85	0.27	4.12	triplet
IH-4	4.0	0	4.0	quartet
IH-5	4.43	can vary by ∓.3 p.p.m. (pH dependent)		doublet

Also listed in Tables 2 and 3 are approximate changes expected in observed proton chemical shifts relative to the corresponding protons of the related model compounds. These "correction increments" are based on the following (57); on introduction of an O-sulfate group geminal to an axial proton (0.6 p.p.m.), geminal to an equatorial proton (0.58-0.71 p.p.m.), vicinal to an equatorial proton (0.27 p.p.m.), vicinal to an axial proton (0.18 p.p.m.), on CH_2OH group (0.50 p.p.m.), vicinal to a CH_2OH group (0.2 p.p.m.). Introduction of a glycosidic bond deshields the geminal proton by perhaps 0.3-0.4 p.p.m. (27).

Turning to disaccharide 1, position-4 of the 2,5-anhydro-D-mannose moiety is involved in glycoside bonding (28,30); position-6 is sulfated, and position-2 of the α-L-idopyranosyluronic acid is sulfated. Finally, the proton chemical shifts calculated for the residues of 1 are listed in Tables 2 and 3. These data have been employed as an aid in the analysis of spectra of 1 (Fig.4).

Fig. 4 shows the 220 MHz p.m.r. spectrum of 1 in D_2O at 20°C. The peak assignments are tentative. IH-4 and IH-3 are expected to resonate in the 4.15 to 4.25 p.p.m. range (Table 3). Besides these, and the MH-1, IH-1, IH-5 and MH-2 signals that have already been assigned above, all other signals are found between 4.3 - 4.55 p.p.m. Either MH-5 and one MH-6 or both MH-6's may account for the two proton signals downfield at 4.43 - 4.55 p.p.m. (Table 2).

At 270 MHz and 90°C, and by using the convolution difference technique to sharpen the signals (Fig.5), the resulting superior resolution more clearly shows doublets for IH-1 (2.3 Hz) and IH-5 (2.3 Hz). Other spacings

measured from the IH-3 and IH-4 signals are $J_{4,5}$ = 2.3, $J_{3,4}$ = 3.5 Hz and $J_{3,2}$ = 5 Hz.

The convolution difference technique may have distorted the spectrum somewhat. That is, the vicinal J values for MH-1 and MH-2 are $J_{1,2} = J_{2,3}$ = 4.5 Hz, whereas at 220 MHz these constants were found to be 5.5 Hz. Perhaps, however, the higher temperature at which the 270 MHz spectrum was run (90°C) accounts for this difference. The hydrated aldehyde is favored in H_2O, but at elevated temperature shifting of this equilibrum towards the dehydrated species might become significant.

Vicinal coupling constants for methyl α-D-idopyranosiduronic acid have been shown to increase with higher temp. (53). At 85°C, the vicinal J's were $J_{1,2}$=4.5 Hz, $J_{4,5}$ = 5.0 Hz, $J_{2,3} = J_{3,4}$ = 6.0 Hz. This is an increase of 1.5, 2.0 and 0.5 p.p.m., respectively, from values at 15°C. It was postulated (53) that the α-D anomer interconverts between the two chair conformations $^1C_4$(D) and ^1C(D) with C_1(D) becoming less preponderant at elevated temperatures. The spectrum would therefore represent a time averaging. Another possible contributor to the conformation of methyl α-D-idopyranosiduronic acid is a skew form.

It is not known how the sulfate substituent at position-2 affects the conformation of the uronic acid, nor is known how the moiety to which it is attached (in 1, 2,5,-anhydromannose 6-sulfate) may contribute. Also, hydrogen-bonding and attractive or repulsive (anionic) forces may come into play. Hence, structure 1 may by no means be a true conformational model for biose repeating unit 2 of heparin. But if the smaller J values for the α-L iduronic acid moiety of 1 in Fig.5 are an indication that the uronic acid is represented more by the ^1C(L) conformation than its non-O-sulfated methyl glycoside, then it can be suggested that with residues of 2-deoxy-2- sulfamino-α-D-glucopyranose

6-sulfate on either side, the α-L-iduronic acid residue in 2 is more truly "locked" in the 1C(L) conformation.

^{13}C.m.r.

In the previous section, the assignments of the chemical shifts of the uronic acid moiety of 1 were based on the spectra of model compounds and heparin. I-3, I-4 and I-5, producing the 3-carbon ^{13}C signal at 70.9 p.p.m., are unequivocally assigned whereas I-2 (O-sulfated) is not.

In the following section (2.1-5) a thorough discussion of I-2 and the anhydromannose carbons can be found. I-2 is shown to resonate at 76.4 p.p.m., and the signal slightly downfield of it is attributed to M-2.

The fact that the anomeric carbon of iduronic acid in 1 is more shielded, and, indeed, that most of its other ^{13}C nuclei (with the exception of sulfated I-2) also resonate upfield relative to those of, e.g., methyl-β-D-glucopyranosiduronic acid (54), is consistent with the conformation 1C(L) of 1, which envisages axial C-O bonds at C-1, C-2, C-3 and C-4. Most of the ^{13}C nuclei of a sugar are more strongly shielded when C-O is axial than when equatorial (58,59). Also, Perlin et al (59) correlated satisfactorily the shielding of ^{13}C nuclei with the increase in destabilizing interactions at ^{13}C. Shielding effects are caused, therefore, by a vicinal cis arrangement of O-groups, a neighbouring axial substituent and a 1,3-syn-diaxial arrangement of C-H and C-O bonds. The conformation of the uronic acid of 1 as 1C(L) can be seen to have many of these destabilizing interactions.

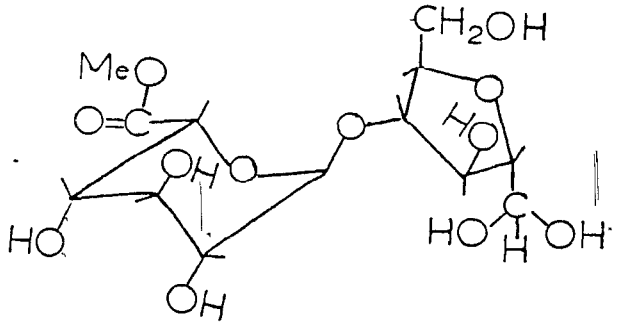
It has been found that the magnitudes of the two and three bond ^{13}C-^{1}H coupling constants in sugars depend very distinctly on the geometrical arrangement of the ^{13}C-O and C-^{1}H bonds (60-62). Accordingly, from established data, carbon I-2 of the α-L-idopyranosiduronic acid should couple with H-3 and H-4 to produce a doublet of triplets ($^{1}J_{C-H}$ being 151 Hz). If the uronic acid were in the alternative chair form, I-2 would show long range coupling only with H-3 producing a doublet of doublets. If the uronic acid were inter-

converting between one chair and the other, one would observe broading of the coupled ^{13}C-^{1}H spectrum. In the ^{1}H-coupled spectrum of 1, each half of the I-2 signal is suggestive of a triplet. The ^{2}J and ^{3}J coupling constants of I-3, 4, and 5 cannot be measured because of their coincident shifts. The lines of these carbons are remarkably sharp considering the number of protons that are near enough to be engaged in coupling (Fig.3).

These ^{13}C data, while not clear cut evidence that the α-L-idopyranosiduronic acid moiety in 1 is in a 1C(L) conformation, are consistent with this likelihood.

2.1-5. Isolation of a non-O-sulfated disaccharide methyl ester (6), and ^{13}C assignments of its 2,5-anhydro-D-mannose moiety.

Treatment of heparin with methanolic-HCl yields a methyl ester of the polymer from which the N-sulfate and most O-sulfate groups have been removed (25). Deaminative degradation of this material with HNO_2, produced a neutral disaccharide methyl ester (6), a modification of 1, which was isolated after passing the reaction mixture through an anonic exchange column.



6

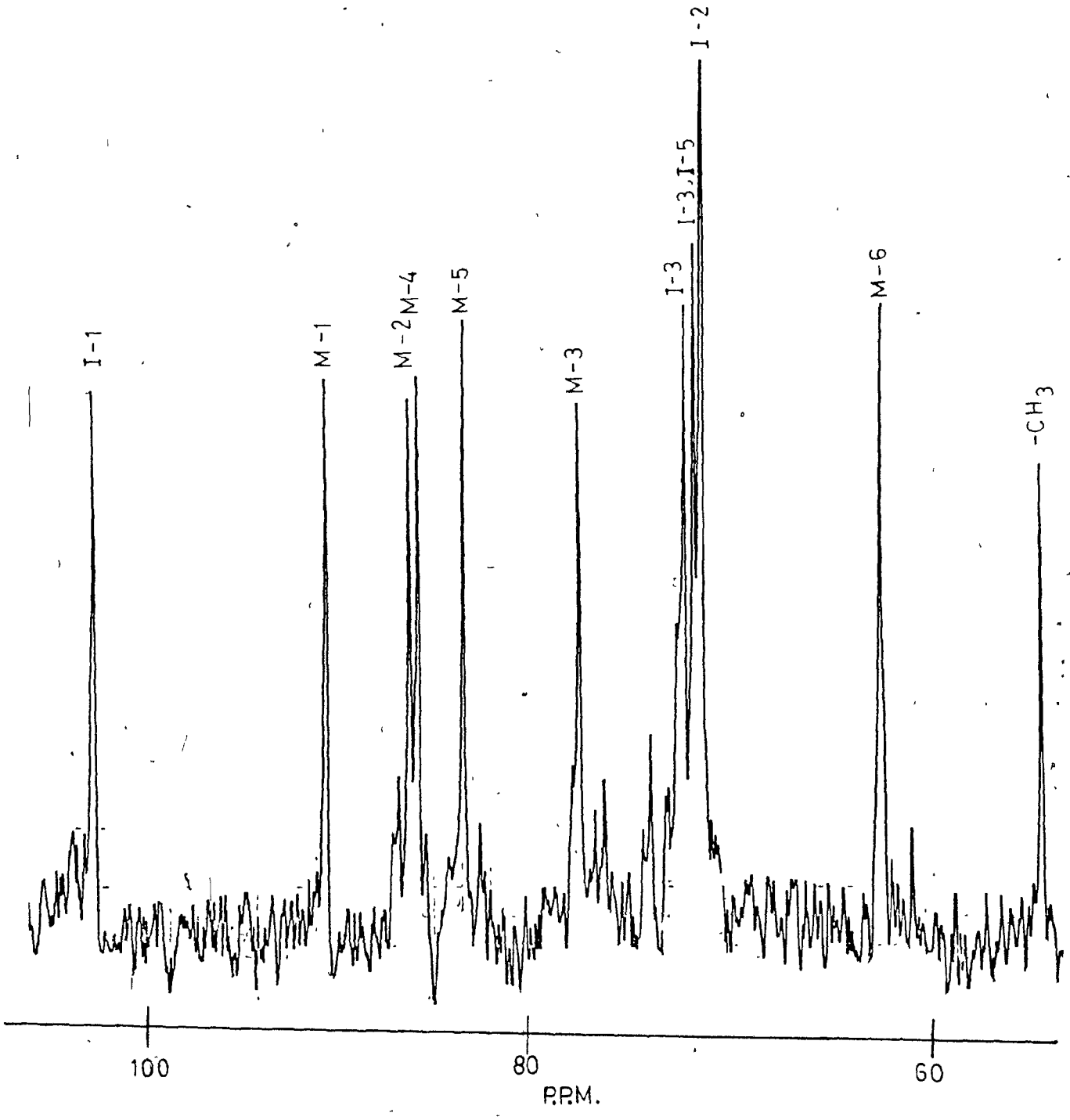
Comparing this neutral compound's ^{13}C.m.r. spectrum (Fig.6) with the spectrum of disaccharide 1, affords evidence generally confirming structure 6, and useful ^{13}C information about both compounds. There is an apparent deshielding of I-1 of 6, as compared with that of 1, by 1.7 p.p.m. This is attributable to the fact that an O-sulfate group adjacent to a ^{13}C changes the chemical shift by about -1 p.p.m. (63-65). The O-sulfate in question here is at position-2 of the uronic acid moiety of 1. Originally at 76.4 p.p.m. (compound 2), I-2 has shifted upfield by 4.7 p.p.m. to 71.7 p.p.m. I-3, now adjacent to a non-O-sulfated I-2, should also be deshielded by about 1. p.p.m. Accordingly, the ^{13}C peak of 6 at 72.6 can be assigned to this nucleus, I-3, since for 2, I-3 appears at 70.9 p.p.m. I-4 and I-5 can be attributed to either of the two remaining uronic acid signals at 71.8 and 72.1 p.p.m.

The chemical shift values of the α-L-idopyranosyluronate moiety of 6 are closely similar to shift values of methyl α-D-idopyranosiduronic acid (Na salt) (Table 3) (53).

In comparison to other data, non-sulfated I-2 of 6 is not upfield quite as much as would be expected (-4.7 p.p.m. vs. -7.0 p.p.m. (28,64)) and I-1 and I-3 have been influenced to a slightly greater extent by de-O-sulfation. One can regard these results as evidence that the α-L-idopyranosiduronic acid moiety of 6 does not have exactly the same conformation as the α-L-idopyranosiduronic acid-2-sulfate moiety of 1. Hence, an introduction of an O-sulfate group at I-2 may influence the conformation of the uronic acid.

The assignment of ^{13}C chemical shifts for the 2,5-anhydro-D-mannose moiety of 6 is more tentative. M-6 (originally sulfated in 1 and resonating at 69.4 p.p.m.) is non-sulfated and appears 6.6 p.p.m. upfield, resonating at 62.8 p.p.m. One assigns M-5 (now adjacent to a non-sulfated M-6) by observing which ^{13}C signal has moved downfield. On this basis, the ^{13}C peak at

Fig.6. Partial ^{13}C-n.m.r. spectrum of non-O-sulfated disaccharide methyl ester(6)



84.0 p.p.m. (Fig.6) can be assigned to M-5, since M-5 resonates at 81.7 p.p.m. All other ^{13}C shift values of the 2,5-anhydro-D-mannose moiety remain unchanged as compared to 1.

The remaining ^{13}C shift assignments of the 2,5-anhydro-D-mannose residue of 6 were made by comparing their observed values (Fig.6) with those of 2,5-anhydro-D-mannose (4) and 2,5-anhydro-D-mannitol (36). (The chemical shifts of M-2 and M-5 of 4 are downfield of those of M-3 and M-4 by about 7 p.p.m.). Due to glycosidic bonding the chemical shift of M-4 in 6 would increase by 7 p.p.m. Therefore, M-4 can be assigned to one of the signals at 86.4 p.p.m. and 86.8 p.p.m. (Fig.6). M-2 would then be the assignment for the second of this pair. M-3, upfield from the other signal, should not be largely affected by glycoside bond formation, and its chemical shift value is therefore at 78.0 p.p.m. for 6.

2.2 A-type Heparin

2.2-1 Deaminative degradation of heparin (A-type)

Present chemical methods of analysis do not appear to provide an adequate quantitative basis for comparing heparins (i.e. A-type vs. B-type). The current study is part of a series undertaken to see if n.m.r. spectroscopy might be useful for this purpose.

The 100 MHz p.m.r. spectra of A-type and B-type heparins (34) indicate that each spectrum is derived from the same kinds of protons, but relative intensities of the signals vary noticeably for the two group of heparins. The most striking feature distinguishing between the two polymers is the presence

or virtual absence of the signal attributed to protons of the acetamido N-acetyl group. (at 2.1 p.p.m.). Also, noticeable are differences in proton intensities in the regions of 4.3 p.p.m. and 3.7 p.p.m.; for type B heparin, the former region is stronger in intensity, for type A, the latter region is more intense. This spectral feature indicates that there is more O-sulfation present in type B heparin and therefore a stronger buildup of proton signals at lower field.

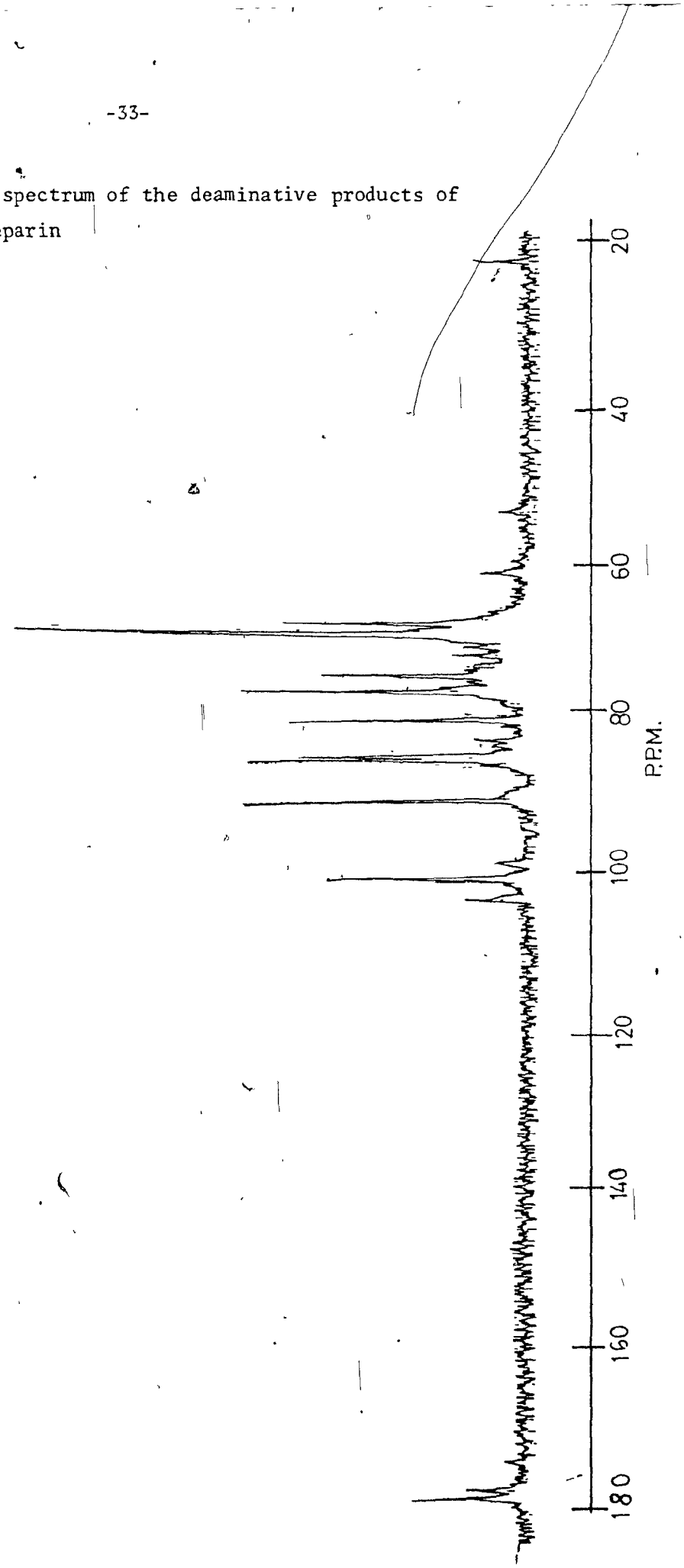
The ^{13}C spectra of A- and B types also differ noticeably in the relative intensities of their minor signals. However, it is difficult to extract more detailed information by using the heparins in their polymeric state. To elucidate fine structure the heparins have been subjected to deaminative degradation as described above for heparin of the B-type. The n.m.r. data accumulated from fragmentation products of this heparin were now found to be of help in solving the structural differences and similarities in type A heparin.

As expected, on deaminative degradation type A heparin produced disaccharide 1 in major proportion, but aside from the twelve ^{13}C signals associated with structure 1, the spectrum of the mixture showed numerous other signals, of minor intensity. (Fig.7). There are 14 or more distinct peaks, varying in intensity, including the methyl and carbonyl ^{13}C of the N-acetyl group at 24.6 p.p.m. and at 176.8 p.p.m., respectively.

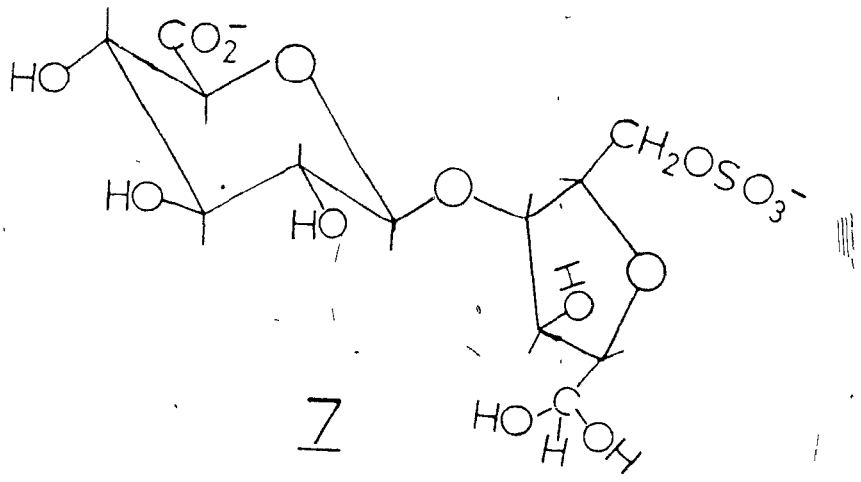
The minor peaks at 103.8 p.p.m. and at 99.1 p.p.m. are attributable to G-1 of β-D-glucosiduronic acid residues, and A-1 of the 2-deoxy-2-acetamido-α-D-glucopyranose residue, respectively. The magnitude of these signals indicates that there is more of the former residue than the latter in A-type heparin.

Molecular weight fractionation of the mixture on Sephadex G-25 was then

Fig.7. ^{13}C-n.m.r. spectrum of the deaminative products of A-type heparin



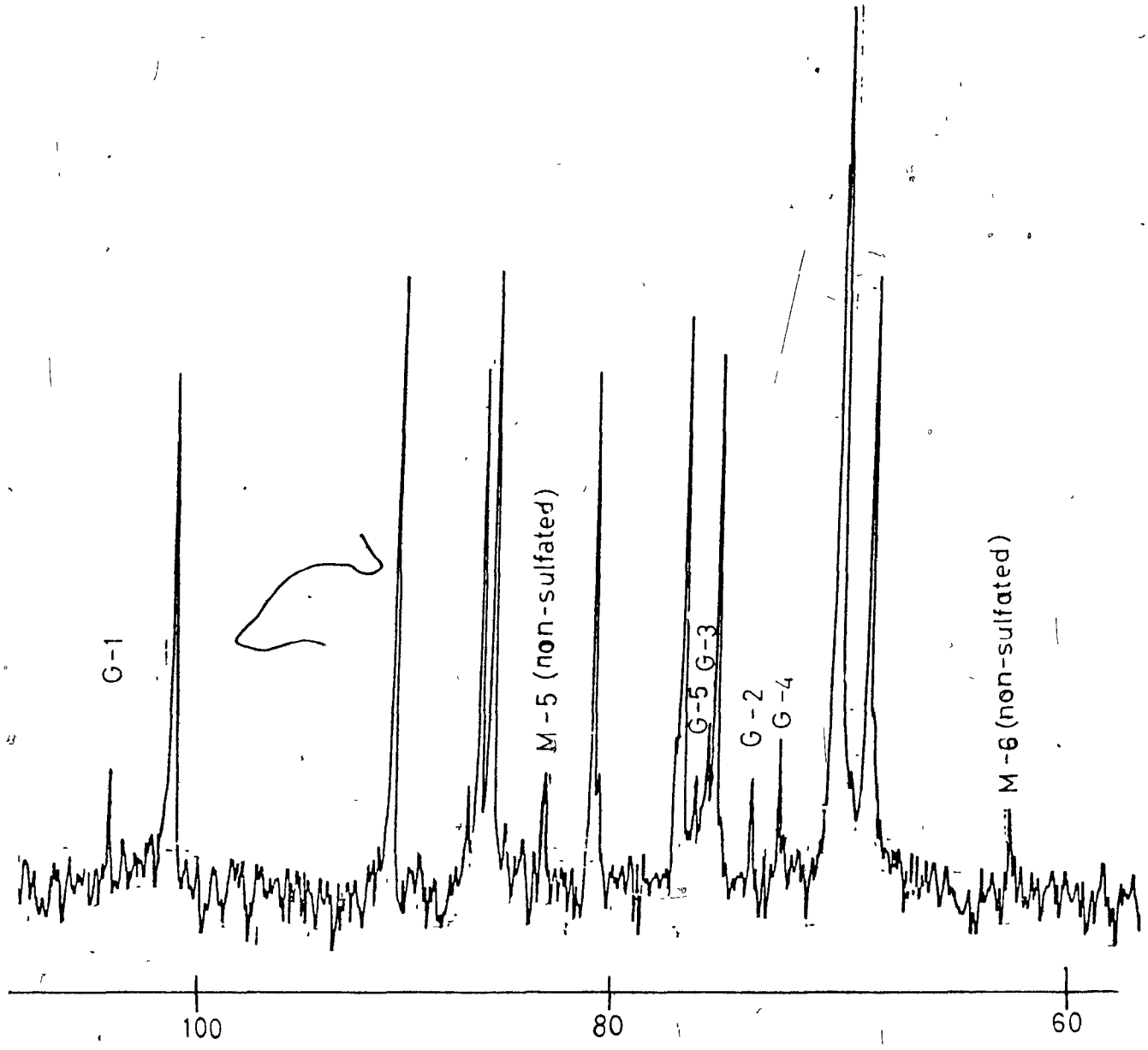
performed. Fig. 8 shows the spectrum of the lowest molecular weight material collected. The minor signals due to the 2-acetamido-2-deoxyhexose, as well as a few other signals, have gone. What remains are the twelve distinguishable ^{13}C resonances of 1, and seven minor signals. The latter compare closely in chemical shift with the minor signals of B-type heparin (Fig. 2). Focusing specifically on Fig. 8, one can clearly account in substantial detail for disaccharide structure 7: G-3, G-5, G-2 and M-6 (non-sulfated) are now clearly distinguishable at 76.6 p.p.m., 77.2 p.p.m., 74.6 p.p.m. and 69.4 p.p.m., respectively. These values are in very good agreement with those calculated for residues of β-D-glucopyranosiduronic acid (Table 1) and 2,5-anhydro-mannose (section 2.1-5)



7

P.m.r. spectra of the above samples were not very informative. Although there were minor signals throughout, their assignments were not straight-

Fig.8. Partial ^{13}C-n.m.r. spectrum of the disaccharides(1 and 7) from the deaminative products of A-type heparin



forward. This illustrates the sometimes superior capability of ^{1}H-decoupled ^{13}C.m.r. spectra in distinguishing minor components in samples, and in furnishing an additional criterion of purity.

The larger molecular weight components of the deaminative degradation products are discussed in the next section.

2.2-2 Examination of higher M.W. components: Proposed structures for minor tetrasaccharide sequences in A-type heparin.

^{13}C.m.r. spectroscopy is an illuminating adjunct to ^{1}H.m.r. for studies in oligosaccharide structure elucidation. Its value derives in large measure from the wide range of chemical shifts involved (20 fold that of protons) which favours a much better separation of nuclear signals than usually found in ^{1}H.m.r. spectra. Thus, with the use of the proton-decoupled F.T. ^{13}C.m.r. spectrum of oligosaccharides, one should be able to count the number of different carbons present and deduce the size of the molecules. With extensive reference data available for both ^{13}C and ^{1}H chemical shift values of the possible saccharides present, structural assignments for larger deaminative fragments that have been isolated, are proposed below.

The isolated yield of oligosaccharides in the deaminative degradation product of A-type heparin was approx. 15% of the total material collected. These fractions were rich in acetamido groups, as observed by ^{1}H.m.r. spectroscopy. In A-type heparins, the acetamidodeoxyhexose (measured from the proton integral of the acetyl group (34)) is from 15 to 25% of the total hexosamine content. Thus, the oligosaccharides collected (above) are highly representative of the N-acetyl containing protons of these polymers.

Two fractions of molecular weight higher than disaccharide were obtained; the first, fraction I, accounting for about one-third of the N-acetyl

rich material. The 2nd fraction (i.e., fraction II), the major component, will be discussed initially.

Fraction II - tetrasaccharide 8

The partial ^{13}C spectrum of compound 8 (Fig.9) shows about twenty-three carbon signals, seven of which overlap in varying degrees. Signals for the carbonyl ^{13}C are not seen because of the low sample concentration, but allowing one carbonyl for the N-acetyl group and two carbonyls for two uronic acid residues gives a total of twenty-six carbons: i.e., 8, a tetrasaccharide composed of two uronic acids, one acetamidodeoxyhexose and one anhydromannose residue.

The 2,5-anhydro-D-mannose 6-sulfate signals can be easily accounted for by reference to data above and are marked as M-# in Fig 9. (It appears that M-2 is overlapped by two other carbon signals at 77.6 p.p.m.).

There are three anomeric carbon signals downfield, at 103.6, 101.1 and 98.5 p.p.m. That at 101.1 coincides with I-1 of the α-L-idopyranosiduronic acid moiety of 1. Because one may account for three of the four carbon signals at 70.7 p.p.m. as being associated with this uronic acid I-3, I-4, and I-5 are at 70.6 p.p.m., whereas sulfated I-2 resonates downfield at 76.3 p.p.m. Therefore, this corresponds to a residue situated at the non-reducing end of the tetrasaccharide.

The anomeric carbon signal at 103.6 is very close to the G-1 signal of the β-glucopyranosiduronic acid moiety of 6 (Table 1). If this type of uronic acid residue is present in 8, and linked through G-4, there should be predictable effects on the chemical shift of this and other carbons. By reference to Table 1 for β-glucuronic acid shifts, and using a downfield displacement correction of approx. +9 p.p.m., one finds a calculated chemical shift for G-4 of 81.9 p.p.m. The signals closest to this value are ones at 78.9 and

77.6 p.p.m. Nevertheless, it has been reported (54) that G-4 of the 4-O-linked β-D-glucuronic acid moiety in chondroitin A resonates at 77.7 p.p.m.* Because of this similarity in observed shifts for G-4 and because chondroitin A also is made up of β-glucuronic acid and acetamidodeoxyhexose residues, it should be very appropriate to compare the other G-signals of chondroitin A with various signals observed in Fig.9.

For chondroitin A*: G-1 = 104.6 p.p.m.
G-2 = 73.4 "
G-3 = 74.7 "
G-5 = 77.7 "

For signals in Fig.9 : G-1 = 103.6 p.p.m.
G-2 = 73.0 "
G-3 = 74.8 "
G-5 = 77.6 "

These latter signals are now designated accordingly in the spectrum of Fig.9.

What are left to be resolved are the six carbons of the 2-acetamido-2-deoxy-α-D-glucopranose unit. Table 4 gives the calculated and observed ^{13}C chemical shift values for this residue (4-O-linked).

The values correspond very well. This strongly indicates that the hexosamine residue is non-sulfated, and is attached to the β-D-glucopyranosyluronic acid residue through an α-linkage at A-1, and to the α-L-idopyranosyluronic acid 2-sulfate residue through A-4 as in formula 8.

* Values abstracted from this paper (54) are adjusted by -0.4 p.p.m. to correct for difference in reference (CH_3OH) value.

TABLE 4

Calculated and Observed ^{13}C chemical shift values for the acetamidodeoxyglucose moiety in Tetrasaccharide 8.

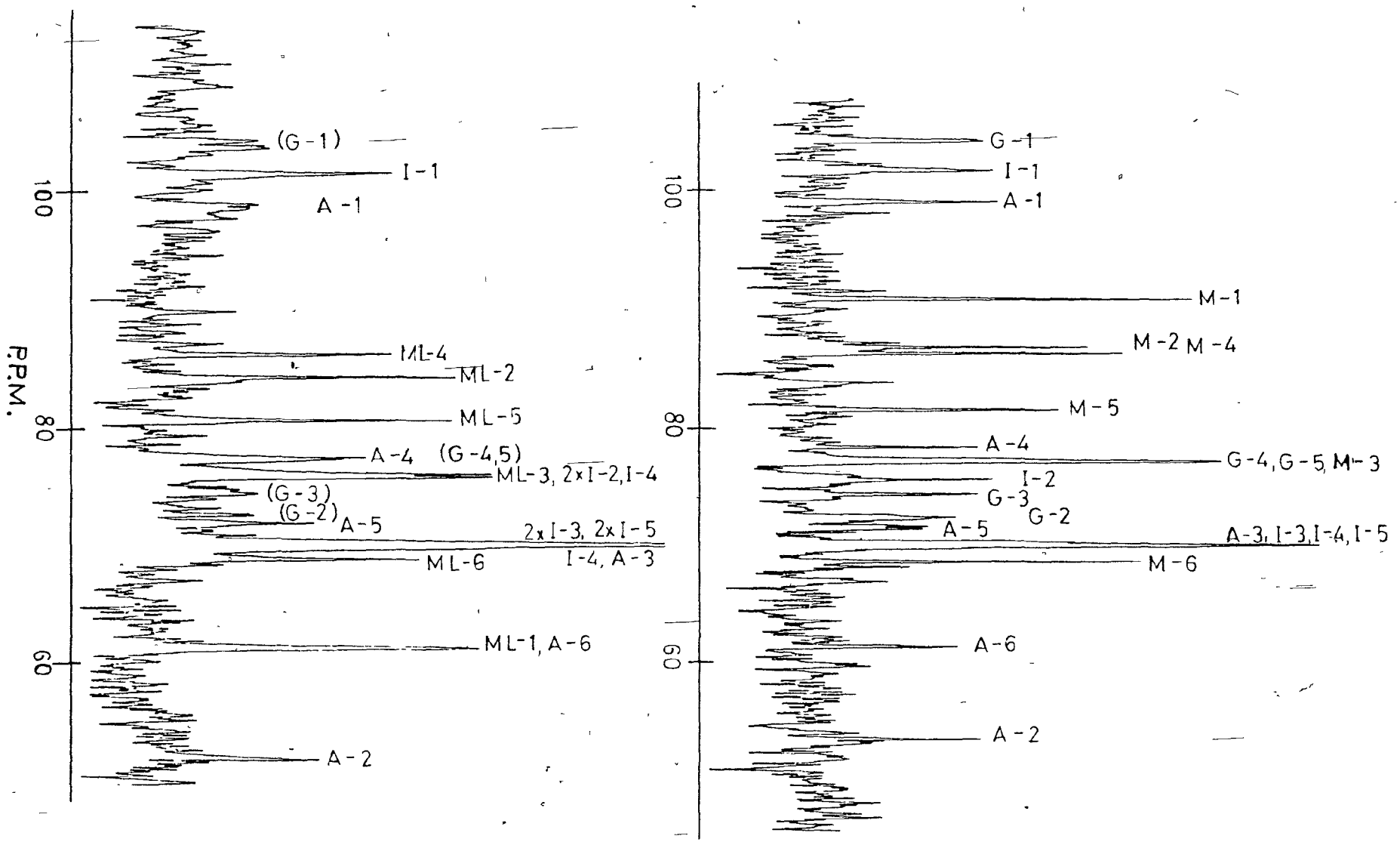
Carbon	2-Acetamido-2-deoxy-α-D glucopyranose[a]	Calc. Increment[b]	for 8 Calc.	Obs.
A-1	91.7	+ 7	98.7	98.5
A-2	54.9	- 1	53.9	55.0
A-3	71.6	- 1	70.6	70.6
A-4	71.0	+ 9	79.0	78.9
A-5	72.4	- 1	71.4	71.9
A-6	61.5	0	61.5	62.4
$-CH_3$	23.3	0	23.3	24.6
CO	175.7	0	175.6	-

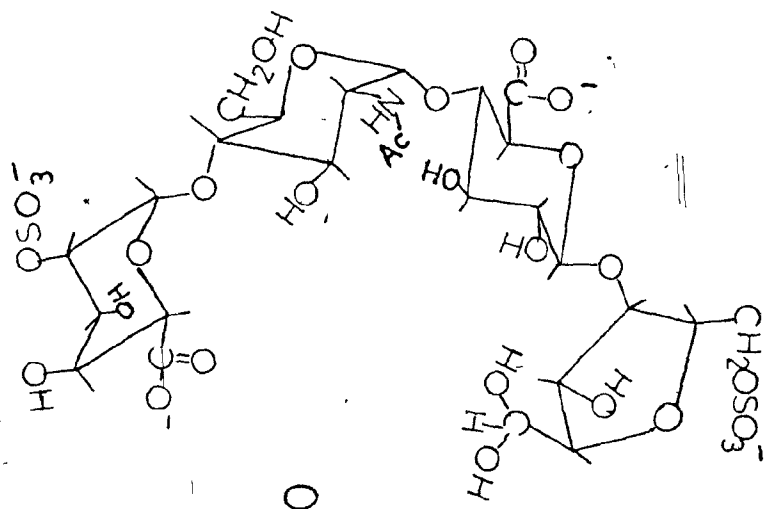
(a) values of ref. 58 - adjusted by -0.4 p.p.m. due to difference in reference (CH_3OH) value.

(b) expected chemical shift changes due to glycosidic bonding (63-65).

Fig.9. Partial ^{13}C-n.m.r. spectrum of fraction II: tetrasaccharide 8

Fig.10. Partial ^{13}C-n.m.r. spectrum of fraction I (after reduction): tetrasaccharide 9


R.P.M.
100
80
60
(G-1)
I-1
A-1
ML-4
ML-2
ML-5
A-4
(G-4,5)
ML-3, 2xI-2, I-4
(G-3)
(G-2)
A-5
2xI-3, 2xI-5
ML-6
I-4, A-3
ML-1, A-6
A-2
G-1
I-1
A-1
M-1
M-2
M-4
M-5
A-4
G-4, G-5, M-3
I-2
G-3
G-2
A-5
A-3, I-3, I-4, I-5
M-6
A-6
A-2



10

The p.m.r. spectrum of fraction II Fig. 11(A) produces some complementary evidence for structure 8. Values for the proton signal (AH) for the 2-acetamido-2-deoxy-α-D-glucose unit compare well with those exhibited (27) in a 220 MHz spectrum of B-type heparin for the N-sulfated 6-O-sulfate residue, allowance being made for small shielding differences. AH-6 (non-sulfated) and AH-5 of 8 would resonate in the 3.85-4.0 p.p.m. region, AH-3 around 3.85 p.p.m., AH-4 at 3.7 and AH-2 at 3.5 p.p.m. as will be seen below. These calculated values are, in fact, close to those indicated in Fig.11(B) for the acetamidodeoxyhexose residue of tetrasaccharide 9. There is a broad peak attributable to AH-1 at 5.55 p.p.m., this signal also being present in the 220 MHz spectrum of A-type heparin.

Other signal assignments in Fig.11(A) are suggested by reference to the spectra of 1 (i.e. its residues of α-L-idopyranosiduronic acid-2-sulfate and 2,5-anhydromannose 6-sulfate), and to methyl β-D-glucopyranosiduronic acid. The GH-1 chemical shift of the latter model compound is

consistent with the observed value of about 4.85 p.p.m. for the corresponding proton of 8. To accommodate the integral of 3 protons for the group of peaks at 4.24 p.p.m. (probably including IH-4 and IH-3) GH-5 is tentatively assigned to this region.

The integral ratio for AH-1 and IH-1 is approximately 0.8 to 1.0 which suggests that another type of uronic acid, namely β-D-glucuronic acid, must be present.

Fraction I - tetrasaccharide 9

The 220 MHz spectrum of fraction I was poorly resolved in the anomeric region of 5.0 - 5.5 p.p.m. To confirm that the doublet at 5.25 p.p.m. in the p.m.r. spectrum of fraction I is due to MH-1 and also, hopefully, to clarify this part of the spectrum, fraction I was reduced with sodium borohydride. The resulting spectrum is shown in Fig. 11(B)

Since removal of the MH-1 signal from the region of the anomeric protons was effected, the anomeric proton, IH-1, can be clearly seen.

The insert, Fig.11(C), is a partial 90 MHz F.T. spectrum of fraction I-reduced. It shows AH-1 and IH-1 with an integral ratio of close to 1:2. This suggests there are two iduronic acid residues present per hexosamine. IH-5 in Fig.11(B) also has an integral of about 2 protons. Since, there is observed to be one 2,5-anhydro-D-mannitol residue per hexosamine residue, it appears that another type of tetrasaccharide is present in the heparin deaminated material. But in contrast to the fraction II tetrasaccharide, this molecule contains only small amounts of β-glucuronic acid residues.

In agreement with this, signals attributable to glucuronic acid in both the ^{13}C and ^{1}H n.m.r. spectra are small. There is little evidence of GH-1 around 4.85, and in the region of 4.0 p.p.m. there is no evidence of GH-3 and GH-4. MLH-2 of the 2,5-anhydro-D-mannitol is less deshielded than

the corresponding proton of the aldehyde and resonates further upfield at 3.9 p.p.m. All signal assignments proposed in Fig.11(B) correspond well to the integrals measured.

Two distinct features in the partial ^{13}C spectrum of this reduced material (Fig.10) that add to the p.m.r. evidence are, first: the signal for I-1 at 101.1 p.p.m. of the iduronic acid is twice as intense as that of the ^{13}C of the N-acetyl hexosamine. (though signal A-1 at 98.5 p.p.m. is rather indistinct, the signal of A-2 at 53.5 p.p.m. of this latter residue is clearly due to one carbon); secondly, the signal intensities at 76.3 and 77.6 p.p.m. are reversed as compared with those of fraction II (Fig.9). That is, there is evidence of a buildup of iduronic acid signals: 2xI-2 (sulfated), I-4 (linkage) and ML-2, at 77.6 p.p.m. There is evidence in Fig.10 that a low proportion of residues of β-D-glucuronic acid is present in this fraction. The minor signals attributed to such residues are designated by the use of brackets. Signal G-1 could coincide with I-1 of a non-sulfated iduronic acid unit, as shown in section 2.1-5, but the other minor signals also are consistent with the β-gluco structure.

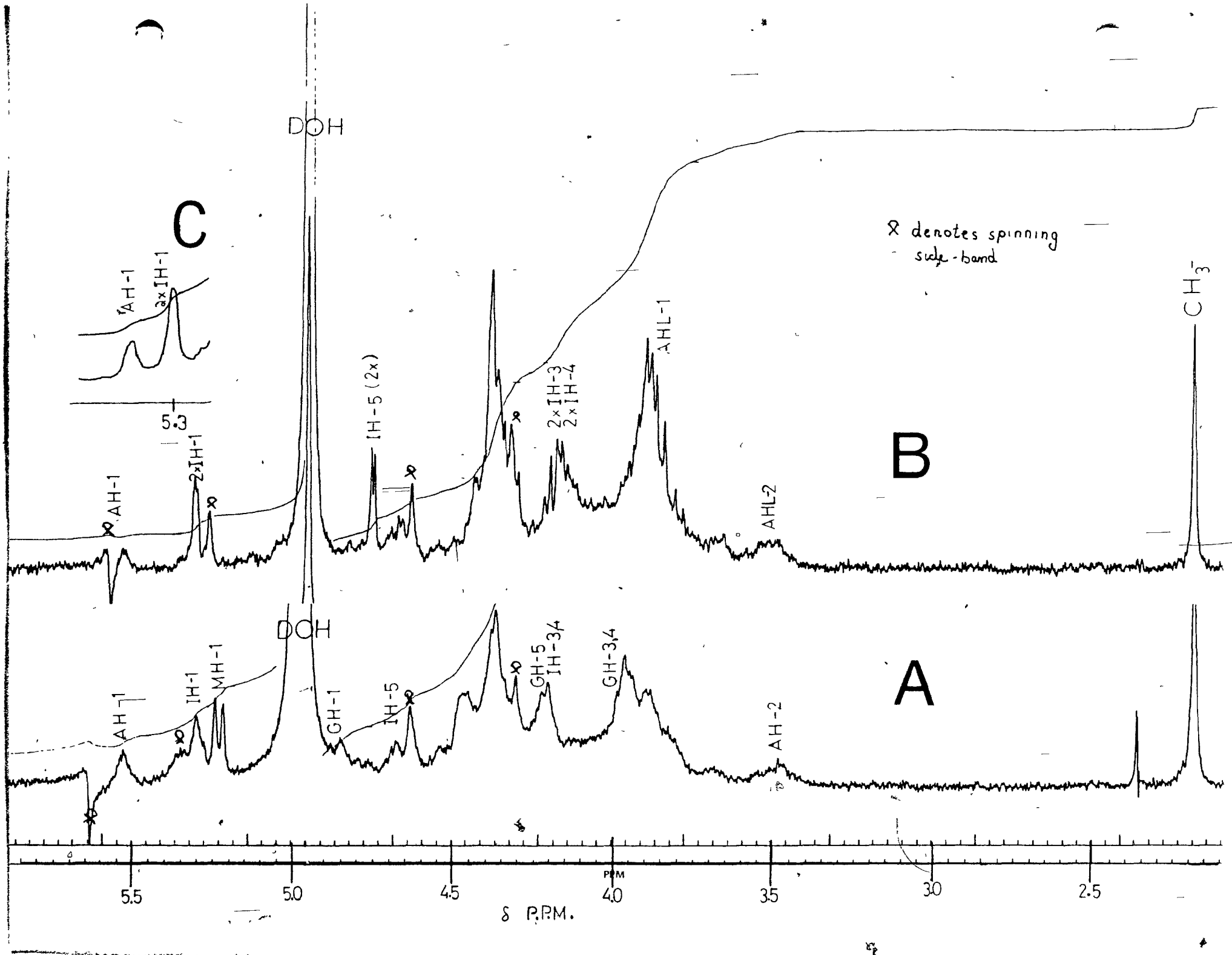
The assignments of ^{13}C signals (ML) for the 2,5-anhydro-D-mannitol residues were based on the ^{13}C spectrum of the reduced disaccharide (3). C-6 of this unit is sulfated and resonates at 69.7 p.p.m. It can be seen that the N-acetyl-glucosamine residue is not 6-O-sulfated by comparing Fig.10 with Fig.9 (tetrasaccharide 8)

It is interesting to note variations in the ^{13}C chemical shifts of the acetamido methyl group and A-2, the carbon to which this group is attached. The methyl signal of 9 (not shown in Fig.10) is at 21.2 p.p.m., and A-2 resonates at 53.5 p.p.m. By contrast, the corresponding values for 8 (Fig.9) are 24.6 and 55.0 p.p.m., i.e., much closer to the values for

Fig.11. A. 220 MHz p.m.r. spectrum of fraction II: tetrasaccharide 10

B. 220 MHz p.m.r. spectrum of fraction I (after reduction): tetrasaccharide 9

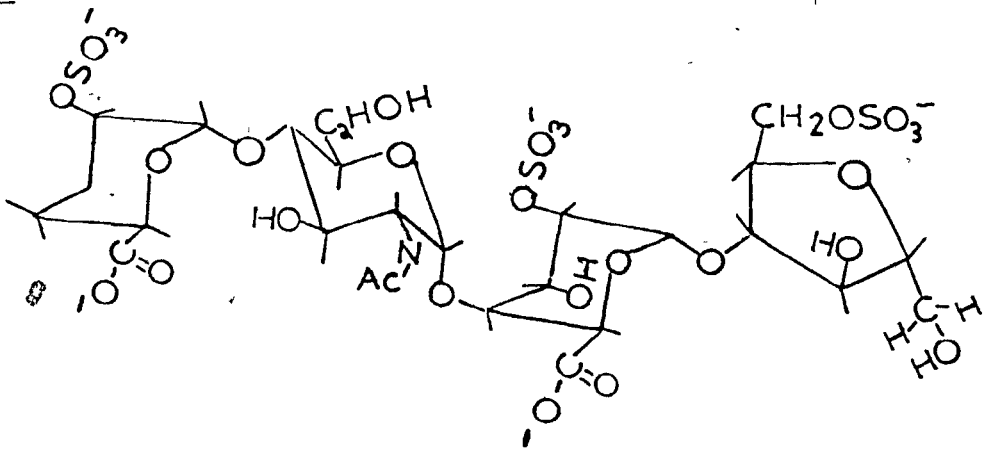
C. Partial F.T. 90 MHz p.m.r. spectrum of fraction I (after reduction): tetrasaccharide 9


C
AH-1
a×IH-1
5.3
DOH
IH-5 (2x)
2×IH-3
2×IH-4
AHL-1
CH3
AH-1
2×IH-1
AHL-2
B
denotes spinning side-band
DOH
AH-1
IH-1
MH-1
GH-1
IH-5
GH-5
IH-3,4
GH-3,4
AH-2
A
5.5
5.0
4.5
4.0
3.5
3.0
2.5
PPM
δ P.P.M.

2-acetamido-2-deoxy -α-D-glucose: 23.3 and 55.3 p.p.m. (58). These large differences in shift between 8 and 9 are most directly attributable to the presence of different uronic acid units attached at either side of the N-acetyl hexosamine. Perhaps, for example, the carboxyl group of the interior iduronic acid of 9 is in closer proximity to the N-acetyl group than is the carboxyl of the glucuronic acid residue in 8. Repulsive forces between the O-sulfate groups might help to ensure a conformation in which the COO^- and $COCH_3$ of 9 are brought close together.

Hence these ^{13}C nuclei may serve as an analytical probe to determine the spatial surroundings of the N-acetyl group and indirectly, to deduce the type of uronic acid attached to the hexosamine. This could be generally useful in the field of mucopolysaccharides where N-acetyl groups are sometimes present in large proportions.

It is noteworthy that tetrasaccharide 9 emerged from the Sephadex column before 8. Although 9 contains an extra O-sulfate group, this relatively small difference in molecular weight would not alone be expected to account for such a good separation. Since, however, the gel support is slightly anionic, the additional effect of higher electrostatic repulsion might make for this overall difference in the chromatographic behavior of 8 and 9.



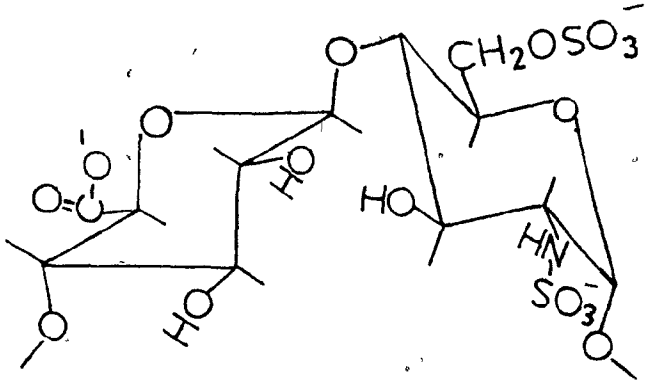
9

2.3 Summary of data on the structure of heparins

The results of the present investigation confirm that the biose repeating unit represented by 2 constitutes as much as 85% of B-type heparin, and suggest that it makes up for approximately 60 - 70% of A-type heparin.

While the interpretation of the spectra of the minor degradation fragments from heparin are to some degree tentative, certain general conclusions about these fragments can be reached. It is especially helpful to survey the literature in evaluating the evidence for structures 7, 8 and 9.

Evidence has been obtained that β-glucuronic acid is present in both types of heparin from the fact that disaccharide 7 was isolated from each by gel permeation chromatography, along with the major disaccharide (1). That this uronic acid need not be linked to residues of N-acetyl hexosamine, is shown by the fact that in disaccharide 7, the β-glucuronic acid is attached to a 2,5-anhydro-D-mannose residue. Both ^{13}C chemical shifts and $^{1}J_{C-H}$ of G-1 are characteristic of a β-linked glucopyranosyluronic acid residue. The distribution of the biose units from which 7 is derived, i.e., 4-O-(β-D-glucopyranosyluronic acid)-2-deoxy-2-sulfamino-D glucopyranose -6-sulfate (10) is not known. They might be randomly arranged, or could be confined to limited regions of the molecular chain.

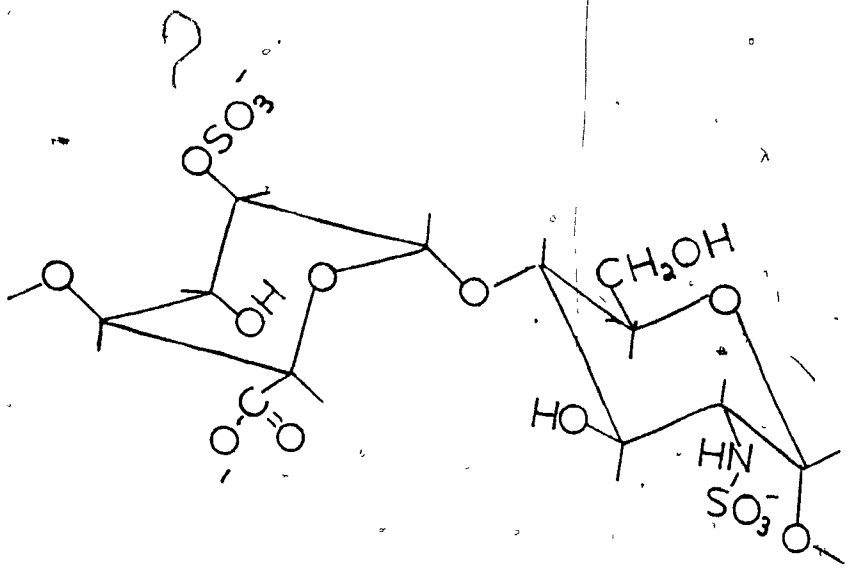


10

There are, presumably, small amounts of non-O-sulfated hexosamine residues in addition to the hexosamine of biose repeating units 2. This is borne out by the identification of non-sulfated 2,5-anhydro-D-mannose residues in the ^{13}C.m.r. spectra of the disaccharide material (i.e., Fig.2 and Fig.8).

Work done by J.A. Cifonelli et al(67) showed the formation of two mono-O-sulfated disaccharides along with the major disulfated disaccharide (1) on deaminative degradation of beef or hog mucosal heparin. Paper chromatography of the acid hydrolyzate of these mono-O-sulfates showed that glucuronic acid was present and also a smaller amount of iduronic acid. A later paper by M. Hook et. al (26) confirms these findings. These results are evidence for some of structure 10 in heparin, and also for one in which α-L-iduronic acid is attached to a non-O-sulfated deoxy-sulfaminohexose residue (11); the former disaccharide sequence being sulfated at M-6 of the 2,5-anhydro-mannose moiety, the latter being sulfated at I-2 of the uronic acid moiety. Confirmatory reports (19,23)show that L-iduronic acid residues in heparin are sulfated, in contrast to the D-glucuronic acid, which is not. Thus, the repeating disaccharide residues containing L-iduronic acid (2) are trisulfated, and those containing D-glucuronic acid (10) are mostly disulfated (O and N),although there is presumably a small content corresponding to 2 but non-O-sulfated at M-6, i.e., 11.

It has been reported (19) that there is an average of 2.5 sulfate groups for each disaccharide unit of A-type heparin. Since repeating unit 2 contains three sulfate groups, this average is lowered by the contributions from such structures as represented by 10 and 11, and by non-O-sulfated acetamidodeoxyhexose residues.



11

The heparin-protein linkage region (21) contains two non-sulfated glucuronic acid residues and one non-sulfated N-acetylglucosamine, as well as a neutral trisaccharide which is linked to serine. This region is common to each heparin chain but it would account for only a small contribution to the lowering of the sulfate content from the value of 3.0 per disaccharide in 2. The present study was not able to account for the linkage region, either because the analysis was insensitive to such a small component, or because of the way this commercial heparin was prepared.

Cifonelli et al (67) studied the distribution of 2-acetamido-2-deoxy-D-glucose residues in mammalian heparin. Their results showed that the N-acetylglucosamine residues are distributed approximately equally between the linkage region and the interior of the polymer. Preparative

chromatography of the cleavage products formed after deaminative degradation of both beef and hog mucosal heparin indicated that most of the N-acetyl-glucosamine residues occur singly in tetrasaccharide fragments that originate from random arrangements in the interior of the polymeric chain. However, small amounts of degradation fragments containing two, or possibly three, N-acetyl hexosamine residues were also found. T. Helting and U. Lindall (33) isolated a tetrasaccharide in similar fashion and proposed its structure to be 12.

uronosyl—N-acetylglucosamine—uronosyl
|
2,5-anhydromannose

12

This structure is basically of the same kind as tetrasaccharides 8 and 9 isolated in the current study. No sulfate groups are indicated for 12 because the material was subject to de-O-sulfation before isolation.

The sulfate contents of these N-acetyl-containing tetrasaccharides described in the literature vary. Cifonelli et al (67) found only 0.5 sulfate groups per uronic acid present, and the location of this substituent in the structure was not determined. Hook et al (26) found approximately one sulfate group per uronic acid, and in other oligosaccharide fragments (i.e., hexa-to octa-), the sulfate content per uronic acid was marginally higher. Again, they did not obtain evidence as to which residues the sulfate groups were attached. The

molar ratio of their di-, tetra-, hexa-, and octa- saccharides was 1.0:0.3:0.1:0.08, At such a low molar concentration, the higher molecular weight fragments would have been difficult to examine by n.m.r. spectroscopy in the present study, unless much larger quantities of heparin had been used.

As just indicated, there is evidence in the literature of tetrasaccharide structures akin to 8 and 9. These contain from perhaps one, to as high as three sulfate groups per tetrasaccharide. What about the nature and location of the uronic acids present in these fragments?

When Helting et al (33) treated their tetrasaccharide (12) with a β-glucuronidase enzyme, only 28% of the uronic acids (which would have been located at the terminal, non-reducing position of the molecule) was released. No further glucuronic acid was released when an enzyme preparation shown to contain α-glucuronidase activity was introduced. It seemed possible, therefore, that 12 was a mixture of tetrasaccharides and that L-iduronic acid residues were located at the non-reducing ends of at least two-thirds of the molecules in the mixture, and β-glucuronic acid, in the remainder. The isolation of tetra- and hexasaccharide fractions with molar ratios of iduronic acid/total uronic acid exceeding 1:2 and 1:3, respectively (26), shows that the iduronic acid may occupy more than one position in those fragments; i.e., iduronic acid residues can be linked to both C-1 and C-4 of N-acetylglucosamine in some of the oligosaccharide structures.

In the light of the foregoing results on tetrasaccharide structures, tetrasaccharides 8 and 9 are very good candidates to account for the minor N-acetyl-containing components existing in heparin.

The occurence, as in 9, of iduronic acid residues on either side of of an N-acetylglucosamine residue suggests that in biosynthesis (26), C-5

epimerization of a uronic acid (i.e., gluco→ido) does not require N-sulfation of the adjacent amino sugar. Conversely, N-sulfation of two alternate glucosamine residues does not necessarily result in epimerization of the intervening uronic acid, as shown by the isolation of glucuronic acid-containing disaccharide 7. These conclusions conform to recent results obtained with ^{14}C-labelled microsomal heparin (68). The importance of O-sulfation of chain units in the biosynthesis of iduronic acid (i.e., on C-5 epimerization of glucuronic acid) is not known.

It seems from the earlier and current data on deaminative degradation that N-acetylhexosamine units are an integral part of the heparin biopolymer and not due to contamination by other mucopolysaccharides (i.e., heparan sulfate).

Similarities in the detailed molecular structure of heparin and heparan sulfate (69) are of particular interest in relation to the biosynthesis of these glycosaminoglycans from a 2-acetamido-2-deoxy-D-glucose containing "precursor substance" (70). The results from the chemical studies are in accord with the suggestion of a biosynthetic mechanism (70,71) whereby N-acetyl groups are replaced by N-sulfate groups. However it appears, from the evidence presented, that the formation of L-iduronic acid in heparin requires an initial O-sulfation of the glucuronic acid residues in the polymer.

Structural differences between heparin and heparan sulfate are undoubtedly fundamental in determining their differences in biological roles as anticoagulant agents and in other roles. Further studies in this area, as well as in the biosynthetic area, should lead to advances in understanding biochemical deficiencies and in elucidating the functioning of these biopolymers.

CHAPTER 3

SYNTHETIC STUDIES RELATED TO HEPARIN

3.1 Introductory remarks

An important component of studies on heparin is the examination of low molecular weight model compounds. 2-Amino-2-deoxy-D-glucose and D-glucuronic acid have long been known as constituents of heparin. Thus much work has been done in synthesizing heparin saccharides related to heparin as well as other biopolymers that contain the above sugar units. A few of these saccharides are illustrated in Fig. 1 (Introduction). The presence of L-iduronic acid residues in heparin has been recognized only in the last decade; hence, there have been few syntheses involving this acid although recent work by Kiss and Wyss(42) has produced derivatives of the anomeric benzyl-L-idopyranosiduronates.

The purpose of this study was to devise a simple synthetic route to L-idopyranose; to produce the sugar in reasonable amounts so as to permit the synthesis of higher saccharides, and in the form of a derivative convenient for synthetic manipulation.

Methods for synthesis of L-idose and its uronic acid are discussed in the Introduction; some give reasonable yields of product but most are lengthy and involve cumbersome separation of anomeric and configurational mixtures of derivatives.

Since D-glucurono -6,3-lactone (13) is an abundant compound, attempts were made during this study to invert the configuration at C-5 and thus produce an L-idurono-6,3-lactone derivative (Fig.12 and Fig. 13). Although this was not achieved, a successful, novel route has been devised for the synthesis of 2,3,4,6-tetra-O-benzyl-L-idopyranose (37). This compound is stable and is suitable for glycoside and other (i.e., uronic acid) synthesis. Fig. 14 illustrates

the scheme for the synthesis of compound 37; tritium and deuterium can be incorporated at C-5 by the use of $NaBT_4$ and $NaBD_4$ during the reduction. The former labelled compound could be used for bio-assays, the latter for conformational studies.

3.2 Attempted synthesis of L-idofuranurono-6,3-lactone derivatives

Scheme A

Fig. 12 describes the attempted synthesis of 1,2-O-isopropylidene-L-idofuranurono-6,3-lactone (16) via configurational inversion at C-5 by base-catalysed enolization.

The 1,2-O-isopropylidene derivative of 13, i.e., 14, synthesized by the acetone-sulfuric acid method (72), was treated with dilute sodium deuteroxide, thus permitting incorporation of deuterium at C-5 via an enol (15)-keto (14 and 16) equilibrium. N.m.r. spectroscopy could readily detect such an equilibrium.

As the pH of the solution of 14 was slowly raised to a value of 8.0 it became clear that enolization of the lactone structure was not sufficiently rapid to compete successfully with ring opening, and at pH 9.0 the sugar existed entirely in the open chain form (17) and decomposition started. Since carboxylate ions are not enolizable, the experiment was terminated.

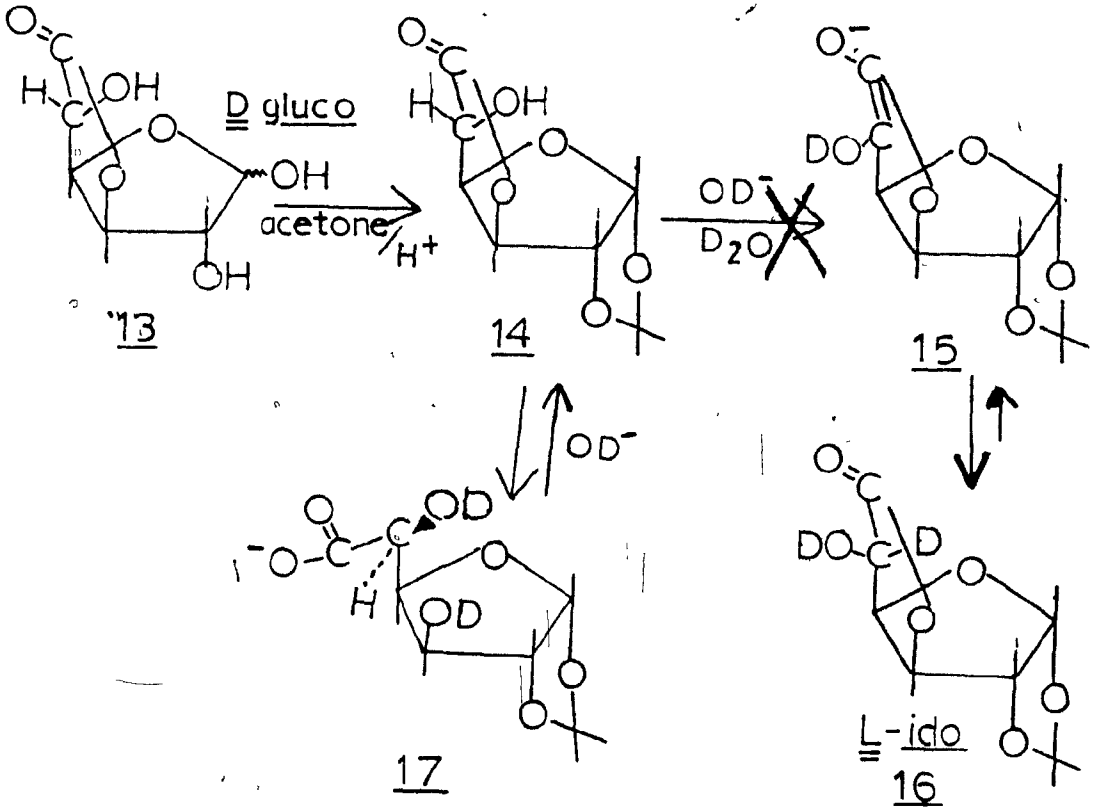
Alternatively, since it is possible to enolize an ester of a carboxylic acid, the methyl ester of 14, methyl 1,2-O-isopropylidene-α-D-glucofuranuronate (18) was prepared from the lactone (14) by adding sodium methoxide dissolved in deuterated methanol (CH_3OD). The methyl ester formed readily, but no enolization was detected even at pD of ≈10.

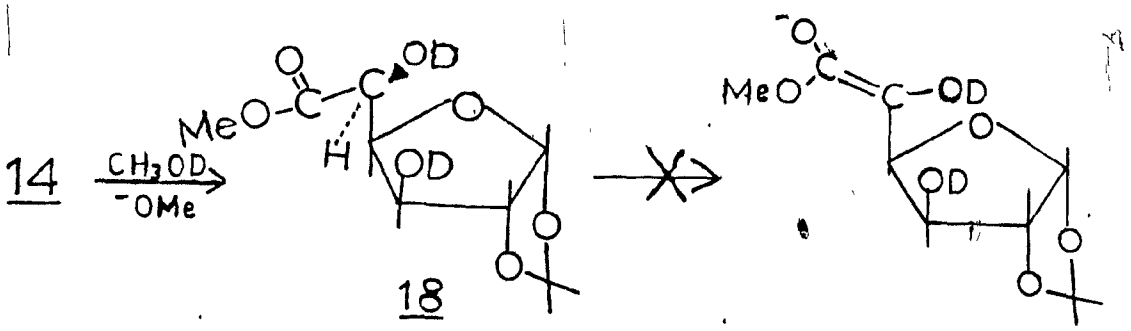
Scheme B

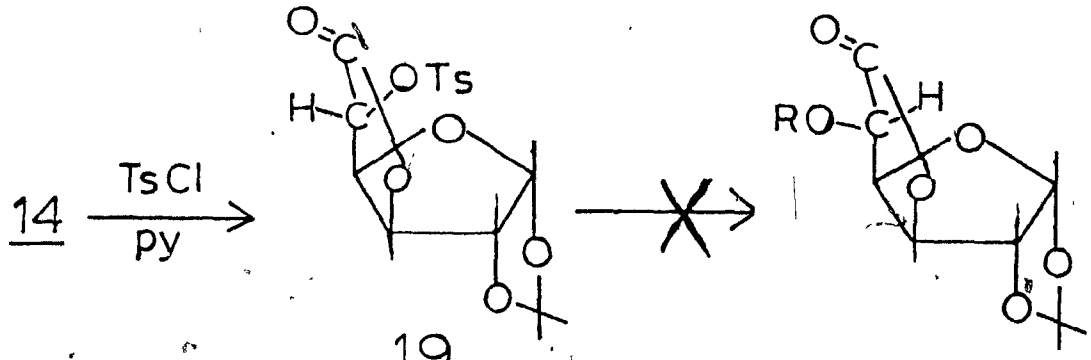
It appeared that a convenient approach to iduronic acid was to

Fig.12. Scheme A: Attempted synthesis of L-iduronic acid via enolization

Fig.13. Scheme B: Attempted synthesis of L-iduronic acid via inversion at C-5


D gluco
acetone
H+
OD-
D2O
13
14
15
17
16
L-ido


14
CH3OD
-OMe
18


14
TsCl
py
19

invert C-5 of glucuronolactone by Walden inversion of a sulfonic ester at C-5 with a strong nucleophile. Several syntheses based on this principle have been published (45,46,47) all of them involving the displacement-inversion of a 5-O-sulfonyl group of a 1,2-O-isopropylidene-α-D-glucofuranose derivative by means of a strong nucleophile: potassium acetate in acetic anhydride (44), sodium benzoate in N,N-dimethyl formamide (73), or acetate ion-exchange resin in acetic anhydride (47).

Fig. 13 illustrates the synthetic route that was attempted. It involved the tosylation of 14 to produce 5-O-tosyl-1,2-O-isopropylidene- -D-glucofuranurono-6,3-lactone (19). Unfortunately no displacement of this tosyl group occurred without decomposition, using either potassium acetate in acetic anhydride, or acetic anionic-exchange resin in acetic anhydride as nucleophile.

It seems likely that displacement with inversion of the endo group in the 5-membered ring of 19 can not be effected, due to steric restrictions, although de-O-tosylation might be accomplished with an exocyclic epimer. In this context, it seems possible that an ester of 19 would be suitable for such a method of synthesis, but this was not tested.

3.3 Synthesis of 2,3,4,6-tetra-O-benzyl-L-idopyranose

3.3-1 Introduction

Synthesis of aldohexoses are usually performed by either lengthening an aldopentose sugar chain by one carbon to produce the higher, six carbon sugar, or by manipulating a hexose through inversion of the configuration at a designated carbon to produce its epimer.

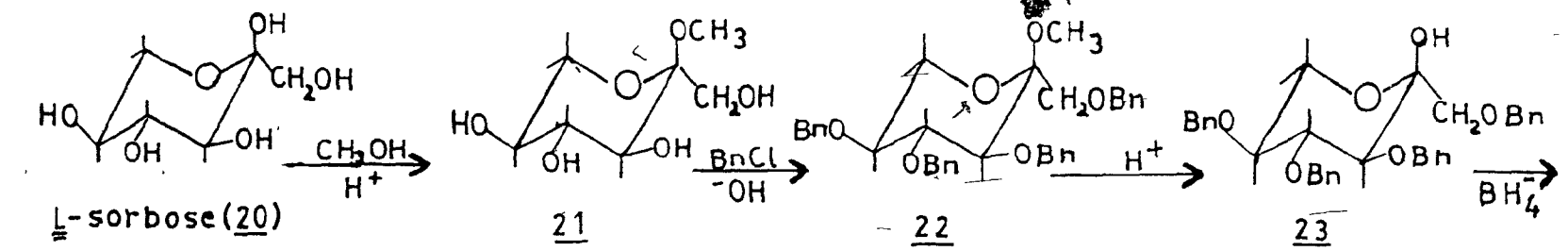
Another feasible method of synthesizing an aldohexose is by starting from its corresponding ketohexose. The opposite route was

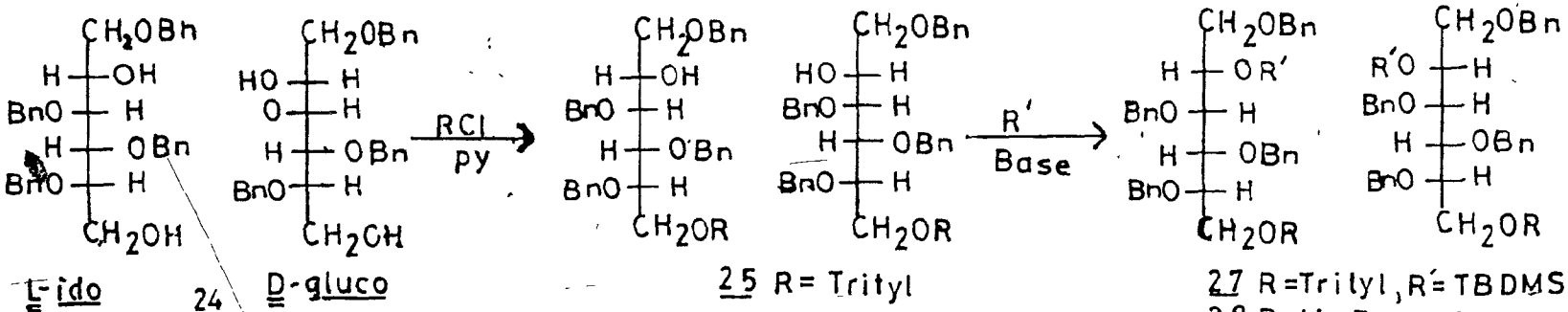
studied by Rabinsohn and Fletcher (74). They described a scheme by which an aldose was converted into a ketose in a sequence of reactions which involved the reduction of C-1 in a partially benzylated aldose, and subsequent oxidation of C-4 or C-5. In the hexopyranose series, they transformed 2,3,4,6-tetra-O-benzyl-D-glucose into 1,3,4,5-tetra-O-benzyl-L-sorbopyranose.

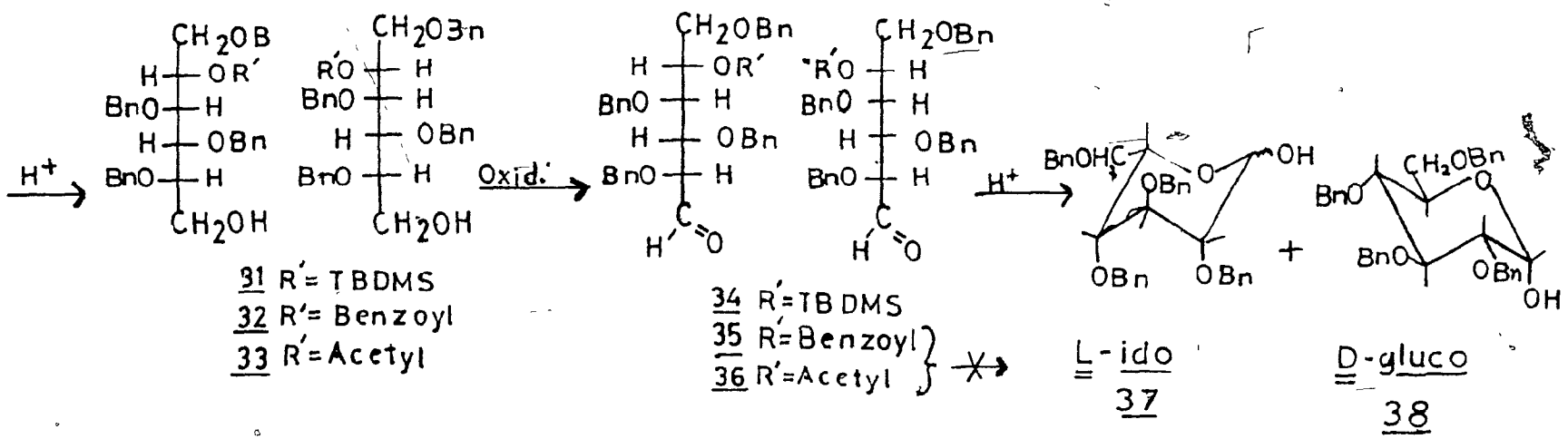
An inverted sequence of reaction steps has been utilized here to produce 2,3,4,6-tetra-O-benzyl-L-idopyranose (37), as depicted in Fig. 14. This has involved the reduction of 1,3,4,5-tetra-O-benzyl-L-sorbopyranose (23) to give an alditol mixture of partially benzylated epimers (i.e., 24, gluco and ido). The secondary hydroxyl group, at C-2 of these alditols, was then masked by a protecting group and the remaining, primary, hydroxyl (i.e., as in 31) oxidized to an aldehyde. Subsequent removal of the masking group would give compounds 37 and 38 (both together if separation of diastereomers could not be effected en route).

There were two additional requirements. First, the primary alcohol group had to be selectively blocked with a labile protecting group, and the secondary one with another type of blocking group. It was necessary that the latter be stable under conditions that would subsequently be used to remove the substituent on the primary alcohol, and then be easily removed after the oxidation step, without affecting the O-benzyl groups or the aldehyde. Secondly, a procedure was needed to separate the mixture of diastereomers, i.e., to recover the L-ido isomer. As illustrated in Fig. 14, both diastereomers were carried together throughout the scheme, because it was only at the last step that one was able to separate them with ease.

Fig.14. Scheme C: Synthesis of 2,3,4,6-tetra-O-benzyl-L-idopyranose(37)


L-sorbose (20)
21
22
23
BnCl
-OH
H+
BH4-


L-ido
24
D-gluco
RCl
py
R'
Base
25 R= Trityl
26 R= Me-Trityl
27 R=Trityl, R'=TBDMS
28 R=Me-Trityl, R'=TBDMS
29 R=Trityl, R'=Benzoyl
30 R= " , R'=Acetyl


H+
Oxid.
31 R'= TBDMS
32 R'= Benzoyl
33 R'=Acetyl
34 R'=TBDMS
35 R'=Benzoyl
36 R'=Acetyl
L-ido
37
D-gluco
38

Several protecting groups were tested during the synthesis; for simplicity, these groups are referred to by letter in Fig. 14. Both ether and ester groups were tried at C-2; the former type of group was found to be more satisfactory. The terms aldoses and alditols are used in the Discussion and refer to the mixture of ido- and gluco-aldose derivatives and iditol and glucitol derivatives, respectively.

The overall yield of 2,3,4,6-tetra-O-benzyl-L-idopyranose (from compounds 21 through to 37 (via 6-O-methoxy trityl derivative)) was 18% (in 90% purity).

3.3-2 Discussion

(A) Synthesis of 6-O-trityl-1,3,4,5-tetra-O-benzyl alditols (25)

When L-sorbopyranose (20) was treated by conventional methods (75,76) for glycosidation of aldoses (i.e., methanol-containing HCl or a cationic resin, under reflux), decomposition of the starting material occurred and only a small amount of methyl-L-sorbopyranoside was formed. Instead, conditions described by Arragon and Bertrand (77) were tested, i.e., the use of low reaction temperatures, a low concentration of acid catalyst, and a large excess of dry methanol. Actone proved to be a highly satisfactory crystallizing solvent for purification of the methyl-L-sorboside (21). Most of the latter was present as its α-anomer, because the large benzoxy methyl group prefers an equatorial orientation and the anomeric effect favours the axial methoxyl group.

Benzylation of 21 was carried out according to the procedure of Glaudemans and Fletcher (78) using benzyl chloride and powdered potassium hydroxide. Methyl 1,3,4,5-tetra-O-benzyl-α-L-sorboside (22) was produced as a yellow oil in over 90% yield. The yellow colour de-

veloped during the initial minutes of the reaction, when the starting material (21) was in contact with the hot suspension of potassium hydroxide.

An attempt to hydrolyse compound 22 with hot acetic acid-2 N sulfuric acid (78) was unsuccessful; under these conditions, 22 decomposed within twenty min. It was found also that exposure of 22 to light caused decomposition. However, under reflux in the dark in 0.1 N HCl-dioxane (1:3), hydrolysis of 22 proceeded in under 3.5 h. The product, 1,3,4,5-tetra-O-benzyl-L-sorbose (23), isolated by column chromatography, remained as an oil although partial crystallization occurred at low temperature. The α-anomer of 23 was preponderant in solution as indicated by its optical rotation. Also the ^{13}C.m.r. spectrum of 23 showed only 10 carbon signals (4 methylene ^{13}C of the benzyl groups and 6 ring carbons) along with the numerous phenyl ring carbons signals.

The partially benzylated ketose (23) was reduced either with lithium aluminum hydride in tetrahydrofuran or with $NaBH_4$ in methanol. Yields by both procedures were the same, but the latter method of reduction was used most often because workup of the products was simpler.* The ^{13}C.m.r. spectrum of the product showed that both diastereomeric alditols were formed in substantial proportion, but it was unclear as to whether the reduction was partially selective in producing more of one isomer than the other; the ^{13}C.m.r. spectrum of their O-trityl derivatives (below) was more helpful.

* A by-product (5% yield) having an r.f. value on t.l.c. greater than that of the alditol derivatives was isolated during purification of the products by column chromatography, although not identified. Perhaps under basic conditions of the reduction, the free ketone is subject to decomposition and/or condensation.

Mixture 24 did not separate satisfactorily on a column, and neither they nor their di-O-acetyl derivatives crystallized. Thus there was no success at this point of the synthesis in separating the diastereomers.

Since the primary alcohol had to be selectively protected by a group which could be readily removed later on, the O-triphenylmethyl (trityl) ether derivatives of 21 were synthesized, using close to an equimolar amount of trityl chloride. The 6-O-trityl-1,3,4,5-tetra-O-benzyl alditols formed failed to crystallize. Although O-trityl derivatives are known to crystallize easily, O-benzyl ether derivatives frequently do not. The ^{13}C.m.r. spectrum of the O-trityl derivatives now indicated that the reduction step had produced approx. a 2:1 mixture of the alditols; it will become evident from the isolation of the idose derivative that this epimer was the more abundant one.

(B) Use of TBDMS as the secondary hydroxyl protecting group

Initially, silyl ether substituents were used essentially for producing volatile derivatives of sugars for gas-chromatoghrphic analysis (79). Trimethyl silyl ethers are too susceptible to solvolysis in protic media (either acid or base) to be broadly useful in organic synthesis. But the t-butyldimethylsilyloxy group (TBDMS), which is about 10^4 times more stable than trimethylsilyloxy (80), seemed promising as a hydroxyl protecting group. The advantages of using TBDMS as a protecting group have been cited by Corey (81), and subsequent papers have appeared (82-84) which involve the use of TBDMS in carbohydrate chemistry. Furthermore Olgivie et al (84) had noted a large difference in the rate of acid hydrolysis between a primary O-trityl group and a TBDMS group on a secondary hydroxyl of ribonucleosides, the trityl group being removed selec-

tively and rapidly.

Mono-O-silylation of compounds 25 required forcing conditions (excess catalyst and reagent); nevertheless, about 10% of starting material remained. The products, 6-O-trityl-1,3,4,5-tetra-O-benzyl-2-O-TBDMS-alditols (27), after column chromatography were amorphous, although they gave satisfactory p.m.r. spectra.

Selective hydrolysis of the trityl group of 27 proved to be difficult, and the TBDMS group more labile than expected. The use of cold 30% HBr in acetic acid resulted in very little selective hydrolysis and, moreover, t.l.c. showed that a substantial amount of O-debenzylation had occurred. Thus, milder hydrolysis conditions were needed.

Accordingly, compounds 24 were dissolved in glacial acetic acid and, while heated on a steam bath with stirring, the solution was diluted with 5 volumes of preheated 70% aqueous acetic acid. Caution was taken not to allow the compound to oil out. The addition required no longer than 10 — 15 min, at which time most of the trityl ether had been hydrolysed, (t.l.c. evidence), although if the reaction was prolonged substantial hydrolysis of the TBDMS ether occurred. Even with such precautions, about 10-15% of the product was de-O-silylated.

Other treatments, using acidic silica gel or gentle MeOH-HCl hydrolysis, were non-selective. It appears that the difference in rates of hydrolysis of the O-trityl and O-TBDMS group is not as great in an open chain structure, such as 27, as on a furanose ring, where the TBDMS group is on a more sterically hindered secondary hydroxyl group.

Difficulty was experienced in separating the 1,3,4,5-tetra-O-benzyl-2-O-TBDMS-alditols (31) on a column from the relatively large amount of trityl alcohol, but the latter was removed, together with

some unhydrolysed starting material (27) by high vacuum distillation. Further purification was effected by column chromatography.

Oxidation of the primary alcohol to an aldehyde was first attempted with chromium trioxide-pyridine complex (85,86). The use of a 12:1 molar ratio of oxidant to substrate succeeded in oxidizing only 50% of alditols, 31, to the 2,3,4,6-tetra-O-benzyl-5-O-TBDMS-aldoses (34) (measured by the intensity of the aldehydic protons in the p.m.r. spectrum).

However, the pyridinium chlorochromate reagent (87) proved to be a more effective and convenient method of oxidation. Using only 1.5 molar equivalents of the oxidizing reagent produced the required aldehydes (34) in a yield of 74%.

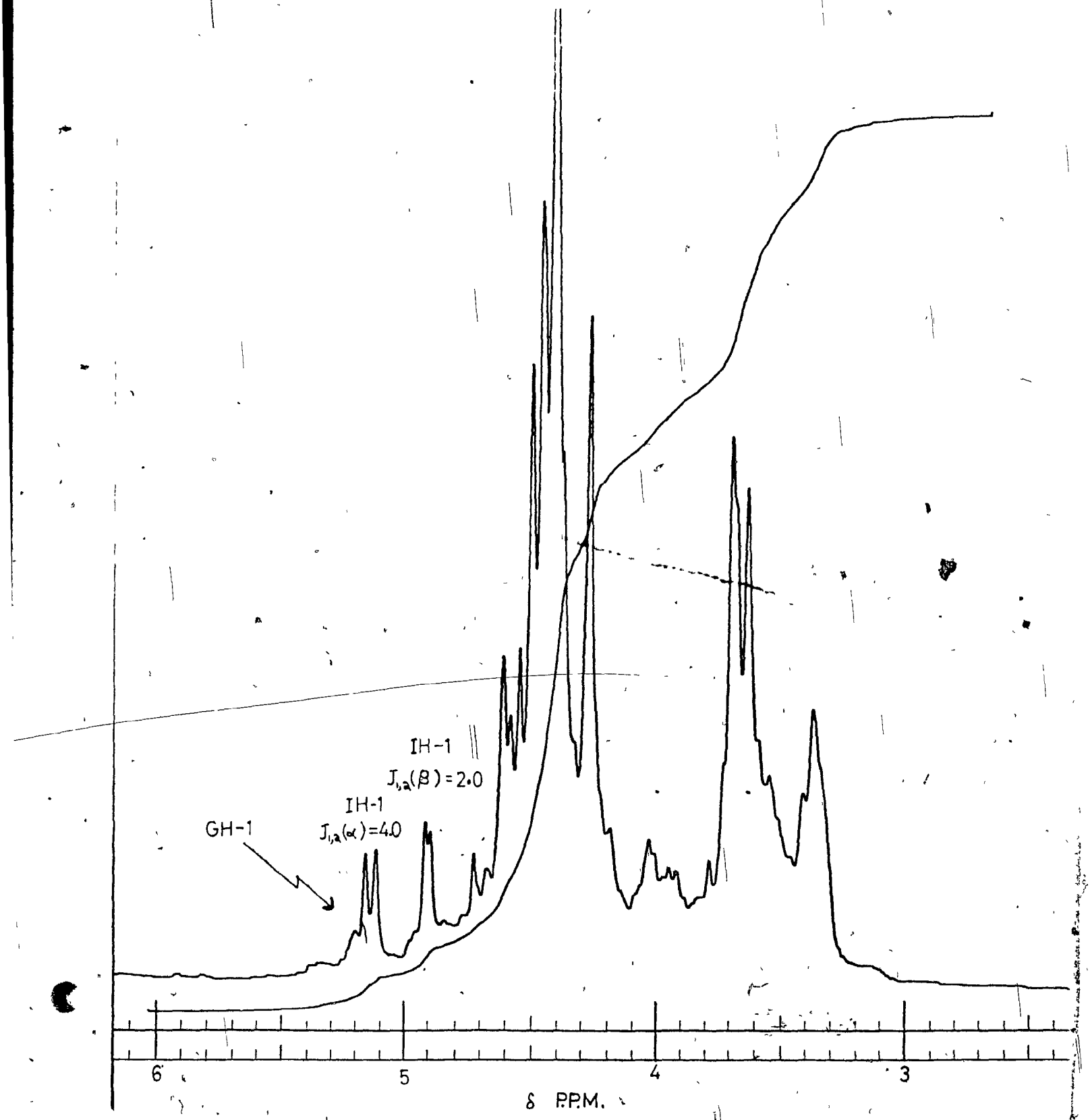
These products were isolated by column chromatography (since they were faster moving components than the unreacted alcohol (31), the latter was readily collected and re-oxidized). Their I.R. spectrum showed a strong adsorption band for aliphatic aldehydes, at 1730 cm^{-1}, and there was no hydroxyl adsorption band. According to the p.m.r. spectrum, a small proportion of benzaldehyde was present (weak signal at 9.86 p.p.m.). The p.m.r. spectrum of the purified material showed that the acyclic aldoses (34) were not present in equal proportion. That is, one of the aldehydic proton signals (at δ=9.7) was twice as intense as that of its diastereomer (δ=9.62). As shown below, the ido compound accounts for the major signal.

De-O-silylation of 34 was tried with tetrabutyl ammonium fluoride in T H F but this appeared to cause extensive decomposition. It proved more satisfactory to use the same conditions as for de-O-tritylation except that the hydrolysis required approx. 3 hr. as compared to 10-15 mins.

for the O-trityl protecting group. On cooling the reaction mixture, a substantial amount of 2,3,4,6-tetra-O-benzyl-α-D-glucose (38) crystallized out. By increasing the water content of the aqueous acetic acid solvent to 20%, larger amounts of crystalline 38 were obtained. Finally, the solution was concentrated and the residual sirup dissolved in methanol, producing more tetra-O-benzyl-D-glucose.

The material remaining consisted of about 90% of 2,3,4,6-tetra-O-benzyl-L-idose (37) and 10% of 2,3,4,6-tetra-O-benzyl-D-glucose (38). This composition was indicated by the p.m.r. spectrum (Fig. 15): the two L-ido anomers are seen to be present in nearly equal proportion, from the relative intensities of their H-1 signals (at 4.92 and 5.15 p.p.m.). A minor, partially obscured signal at 5.2 p.p.m. is attributable to H-1 of tetra-O-benzyl-α-D-glucose (by comparison with authentic material) showing that the gluco isomer can account for very little of the product. Column chromatography of this mixture was not successful in removing the D-glucose derivative. The p.m.r. spectrum showed that both anomers of tetra-O-benzyl-L-idopyranose (18) were present in equal amounts (Fig. 15). From the amount of crystalline tetra-O-benzyl-D-glucose recovered and from what was left of this compound in solution, the yield of tetra-O-benzyl-L-idose (34) produced in the synthetic route was estimated to be twice that of its D- gluco counterpart (38).

Fig.15, Partial 100 MHz p.m.r. spectrum of 2,3,4,6-tetra-O-benzyl-L-idopyranose (37)



The L-idose configuration of 37 was confirmed in the following way. The compound was reduced with borohydride in methanol, the O-benzyl groups were then hydrogenolyzed catalytically with palladium black, and the product was peracetylated. This gave L-iditol hexaacetate, which was identified by a mixed melting point and optical rotation.

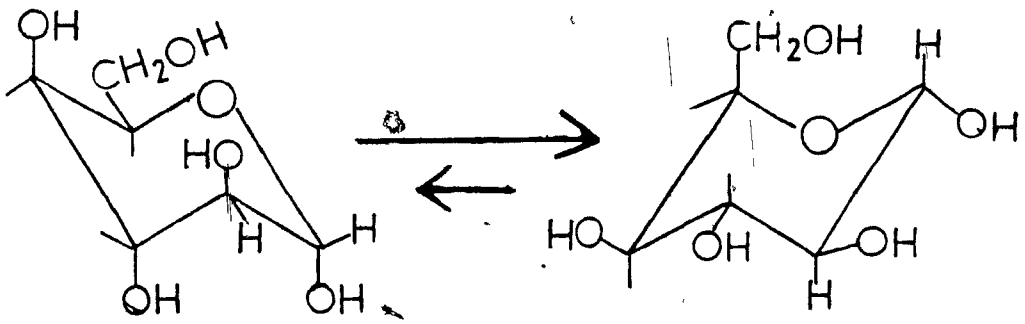
(C) N.m.r. characteristics of 2,3,4,6-tetra-O-benzyl-L-idopyranose (37) and its derivatives

A partial p.m.r. spectrum of product 37 is shown in Fig.15. Aside from the small H-1 signal of 2,3,4,6-tetra-O-benzyl-α-D-glucopyranose, there are two anomeric H-1 signals of 2,3,4,6-tetra-O-benzyl-α- and β-L-idopyranose resonating at 4.92 p.p.m. ($J_{1,2}$ = 2.0Hz) and at 5.15 p.p.m. ($J_{1,2}$ = 4.0 Hz) and of approx. equal intensity. The assignment of each anomeric signal is tentatively made on the following basis.

To help with these assignments, the 1-O-acetyl derivatives of both 2,3,4,6-tetra-O-benzyl-D-glucopyranose and of sirupy 37 were made. Since the H-1 signal of 1-O-acetyl-2,3,4,6-tetra-O-benzyl-α-D-glucose was now clearly resolved (at 6.36 p.p.m.) its low intensity confirmed that only a small amount of the gluco isomer was present in the mixture. The two anomeric protons of 1-O-acetyl-2,3,4,6-tetra-O-benzyl-α,β-L-idopyranose, resonated at 6.2 p.p.m. ($J_{1,2}$=4.0 Hz) and at 6.1 p.p.m. ($J_{1,2}$=2.5 Hz), the relative intensities of their signals being 4:1. Because the α anomer is expected to predominate over the β due to the anomeric effect, the major H-1 signal ($J_{1,2}$=4.0 Hz) is associated with the α configuration. A comparison of the $J_{1,2}$ values for 2,3,4,6-tetra-O-benzyl-L-idose (Fig. 15) with those of the 1-O-acetyl derivatives suggests, therefore, that one can assign the down-

field anomeric proton, at 5.15 p.p.m., to the α-anomer and the upfield one to the β-anomer.

The conformation of L-idopyranose and its derivatives in solution are of much interest in conformational analysis and stereochemistry. Angyal and Pickles (88) have stated that D-idose at equilibrium in D_2O exists in both furanose and pyranose forms. The β-pyranose ($J_{1,2}$=1.5 Hz) is in the C1(D) conformation, whereas the α-pyranose (its H-1 proton resonating upfield from that of the β-anomer, $J_{1,2}$=5.6 Hz) exists as interconverting 1C(D) (2/3) and C1 (D) (1/3) conformations (39). Both the higher field chemical shift and the larger $J_{1,2}$ values of the α-anomer are explained by this equilibrium. Methyl-α-D-idopyranoside, with a $J_{1,2}$ value of 4.0 Hz (88) is likely to be represented by a higher proportion of C1(D) owing to the larger anomeric effect of the methoxyl group.



small $J_{1,2}$
C1(D)

large $J_{1,2}$
1C(D)

39

The anomers of tetra-O-benzyl-L-idose (38) show a marked difference in their H-1 chemical shift and $J_{1,2}$ values over L-idopyranose. Hence, the H-1 signal of the α anomer is to the low field of that of the β anomer, and $J_{1,2}$ (4.0 Hz) is moderately lower. It may be said, then, that tetra-O-benzyl-α-L-idopyranose is preponderantly in the 1C(L) conformation. This suggestion can be supported by the fact that the conformation of α-D-idopyranose pentaacetate exists wholly in the C1(D) chair conformation (89).

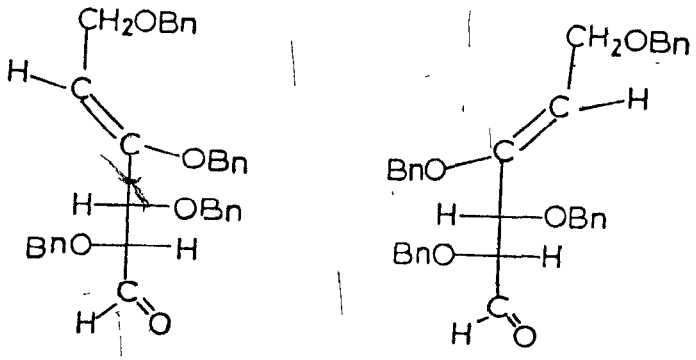
(D) Unsuccessful use of ester substituents as secondary hydroxyl protecting groups

A logical step in improving the yields of tetra-O-benzyl aldoses is to optimize the yield at the de-O-tritylation step by use of a more acid-stable protecting group on the secondary position (R' in Fig. 14). Since esters are relatively stable in the aqueous acetic acid employed for the trityl ether hydrolysis, the 6-O-trityl-2-O-benzoyl-1,3,4,5-tetra-O-benzyl alditols (29) and 6-O-trityl-2-O-acetyl-1,3,4,5-tetra-O-benzyl alditols (30) were synthesized. De-O-tritylation was then performed as before, and was accompanied by very little hydrolysis of the ester group. Yields were 15-20% higher than those involving the use of TBDMS as the protecting group.

In the subsequent oxidation step, yields were the same as before, although, in contrast to the greater abundance of ido aldehyde in the oxidation products, the proportions of ido and gluco derivatives in these experiments were, unaccountably, about equal. Again, benzaldehyde showed up as an impurity of the oxidation step.

Catalytic deacylation, with sodium methoxide, of the ester group of the aldehydes (5-O-benzoyl-2,3,4,6-tetra-O-benzyl aldoses (35)

and 5-O-acetyl-2,3,4,6-tetra-O-benzyl aldoses (36)) failed to produce the desired tetra-O-benzyl aldoses (37 and 38). Complete de-esterification required longer reaction times and greater amounts of sodium methoxide than normally required for catalytic transesterification, and the end products could not be fully identified. Their r.f. values were approximately the same as those for the tetra-O-benzyl aldoses, but their p.m.r. spectra were substantially different. Two signals at 9.15 p.p.m. were likely due to aldehydic protons, and this was supported by I.R. and ^{13}C.m.r. spectra. However, there was a broad doublet at ≈6 p.p.m., suggestive of a proton from an alkene group. I.R. bands for an alkene group such as C=CH- also were observed. From these features and additional one from the n.m.r. spectra it is suggested that elimination products such as those illustrated below are formed from 35 and 36 by the alkaline conditions.



Acid hydrolysis of the O-acetyl derivatives (36) using a cationic exchange resin in methanol did not afford methyl tetra-O-benzyl aldopyranoside, and HCl-methanol produced only small amounts of glycosides, and other products that have not been identified.

(E) Proposed methods for improving yields of tetra-O-benzyl-L-idopyranose

(i) As noted above, moderate and variable yields of 1,3,4,5-tetra-O-benzyl-2-O-TBDMS alditols (31) resulted because of partial hydrolysis of the TBDMS group during de-O-tritylation. A preliminary investigation was undertaken of the use of a methoxytrityl (p-anisyl diphenyl methyl) ether as a replacement for the conventional O-trityl group on the primary alcohol (e.g. 14, 26, 28, 31). The overall increase in yield of 31 was about 10%.

(ii) Another possibility is to use the TBDMS substituent for masking both the primary and secondary hydroxyl groups. Selective hydrolysis of the TBDMS at the primary hydroxyl might occur to produce 31 in a similar fashion. Hence, Oglivie et al (90) have demonstrated that the stability of secondary TBDMS ether is twice that of a primary TBDMS ether in ribose nucleosides.

(iii) Hydrolysis of methyl 1,3,4,5-tetra-O-benzyl-α-L-sorboside (22) to give the ketose (23) was moderately difficult, its yields variable, and some decomposition occurred. To avoid such difficulties a benzylated allyl glycoside might be used (91). Alkaline rearrangement would then afford the benzylated 1-propenyl glycoside, which is notably labile to acid.

(iv) A recent communication by Hanessian and Lavallee (92) illustrates the t-butyldiphenylsilyl ether as being a very useful secondary hydroxyl protecting group in that this ether has much greater stability to acid and to conditions of hydrogenolysis, than related silyl and trityl ethers. Therefore preferential removal of a trityl or other silyl group (in this synthesis at the primary alcohol) might

be effected in the presence of a TBDPSi ether, and treatment with fluoride ion, or stronger acid conditions should cause cleavage of the latter.

(F) Initial investigation of glycoside synthesis of 37

To investigate glycoside synthesis with 2,3,4,6-tetra-O-benzyl-L-idopyranose (37), a recent route (93,94), which employs the conversion of some tetra-O-benzyl aldopyranoses into glycosyl through the successive intermediacy of a 1-triflate and a glycosyl bromide, was examined. However, preliminary results of this reaction were inconclusive. Only small amounts of methyl glycosides of 37 were produced and the rest of the reaction products could not be identified. Chromatographic and n.m.r. evidence on the debenzylated material suggest that other types of glycosides (i.e., disaccharides) had been produced during the glycoside synthesis. Nevertheless, when the reaction products were treated with methanolic-HCl, the resulting material (after debenzylation) was a mixture of methyl glycosides consisting of about 80% of methyl-α-L-idopyranoside.

CHAPTER 4

EXPERIMENTAL

4.1 Analytical methods

General methods

Evaporations were carried out under reduced pressure at a bath temperature below 60°. All samples were stored in a freezer.

Melting points (m.p.) were determined on a Fisher-Johns (hot plate) apparatus and were uncorrected.

Infrared spectra were measured as a film on silver chloride discs using a Unicam SP 200 model spectrophotometer.

Optical rotation measurements were made with a Perkin-Elmer 141 model polarimeter using the indicated solvent, at room temperature.

Proton magnetic resonance spectra were recorded with a Varian HA 100 spectrometer, using tetramethylsilane (TMS) as an internal standard and lock signal. An external tetramethyltin (TMT) capillary was used to provide a lock signal for samples dissolved in deuterium oxide.

Fourier transform ^{1}H.m.r. spectra were recorded using a Bruker W-90 spectrometer. F.T. spectra were measured using a pulse width of 2.5 μsec (70°) and a sweep width of 900 Hz.

Carbon-13 magnetic resonance spectra were recorded at 22.63 MHz using a Bruker WH-90 spectrometer. Proton-decoupled F.T. spectra were measured using a repetition time of 0.6 sec, pulse width of 18 μsec (70°), and usually a sweep width of 4000 Hz. For ^{1}H-coupled spectra the repetition time was 1.2 sec, decouple time 0.5 sec and pulse width 24 μsec (90°). Chemical shifts (p.p.m.) are given with respect to internal tetramethylsilane, using methanol as an internal reference; at 35°C, the chemical shift of methanol in deuterium oxide relative to that of internal tetramethylsilane

was 50.35 ± 0.1 p.p.m. The heparin samples were examined as solutions in D_2O. All other samples were examined in $CDCl_3$.

The 220 MHz p.m.r. spectra were recorded at the Canadian 220 MHz n.m.r. Centre, Sheridan Park, Ontario.

The 270 MHz p.m.r. spectrum was recorded by Dr. G. Gatti (Milan, Italy).

Chromatographic methods

Thin layer chromatography (tlc) was carried out with MN silica gel (G/U.V.) as adsorbent. Visualization was effected, unless otherwise indicated, by spraying with sulfuric acid (50%,v/v), and heating the sprayed plate at 120°C in an oven. Preparative t.l.c. plates were visualized by U.V. irradiation using a Miner shortwave lamp.

Column chromatographic separations were carried out on columns packed with MN silica gel (grain size 0.8mm) or with fine MN silica gel (grain size 0.063 - 0.200 mm). The following solvent systems were used:

A.	chloroform:ether	9:1 v/v
B.	benzene:ether	3:2 v/v
C.	chloroform:ether	9.5:0.5 v/v
D.	benzene:ether	9.6:0.4 v/v
E.	methylene chloride	

Molecular weight gel filtration was carried out with a Sephadex G-15 column (2.8cm x 103cm). The column was washed with distilled H_2O, with the eluant being collected in 10 ml fractions at a rate of 30-35 ml/h. Fractions were examined by weight and by n.m.r. (presence of acetyl groups), and pooled where appropriate.

4.2 Structural analysis of the heparins

A. Deaminative degradation of B-type heparin

To a cooled solution of 0.63 g of B-type heparin (sodium salt, Upjohn, beef lung, lot #093CE, assay-140 units/mg) in 6 ml of H_2O, sodium nitrite (0.3 g) and M HCl (2.4 ml) were added, producing a vigorous effervescence (95). The solution was stored at 20°C for 24 h, its pH maintained at 3.0-3.5, then the pH was adjusted to 6.0, and the reaction mixture was concentrated to a syrupy residue. A ^{13}C.m.r. spectrum was recorded (see Discussion).

The sample was dissolved in 3 ml of H_2O and applied to a Sephadex G-15 column. Most of the product was recovered by concentrating those fractions of the eluate comprising 380-450 ml; yield, 0.35 g. Very little material was contained in fractions emerging ahead of the major fraction.

^{13}C.m.r. and 220 MHz p.m.r. spectra were recorded for the materials collected. Discussion of these spectra can be found in section 2.1.

Radioactive-labelled substrate 3 was prepared by dissolving the material (containing mostly disaccharide, 1) collected above (0.15 g) in 2 ml of H_2O, and adding a freshly prepared solution of sodium borotritide (0.1 ml) (100 mCi/ml) after 2 h. An additional amount of sodium borohydride (0.015 g in 1 ml of H_2O) was added to the reaction to ensure complete reduction of the aldehyde. After 18 h at 20°C Amberlite IR-120 resin was introduced to remove sodium ions, the filtrate was concentrated and methanol was used to remove borate. (Care was taken to thoroughly wash and safely dispose of all radioactive residues.) The syrupy product (0.11g) obtained was found

by paper chromatography (descending, Whatman 3 mm paper, using a developing solvent of (4:1 (v/v)) 95% aqueous ethanol: M ammonium acetate) and by radioactive monitoring for tritium on the paper, to contain at least two components. By radioactive counting and sulfate analysis*, it was found that major amounts of di-O-sulfated disaccharide (1) and a smaller amount of mono-O-sulfated material (also radioactively labelled) were present.

B. Deaminative degradation of A-type heparin

To a cooled solution of 1 g of A-type heparin (sodium salt, Upjohn, hog mucosal, lot #1415B, assay-152 units/mg) in 10 ml of H_2O was added sodium nitrite (0.5 g) and M HCl (4 ml). The reaction conditions and work up were identical to those above for the B-type heparin. A ^{13}C.m.r. spectrum of the pure product was recorded, and is discussed in section 2.1.

The product was dissolved in 3 ml of H_2O and applied to a sephadex G-15 column. A major fraction was recovered by concentrating those eluates (devoid of N-acetyl protons, according to F.T. p.m.r. spectroscopy) comprising 380-440 ml; yield, 0.55 g. ^{13}C.m.r. spectroscopy showed this material to consist of disaccharides. Two minor products that were formed in the deamination reaction emerged from the Sephadex column ahead of the disaccharides: fraction I was recovered from eluate volume 270-320 ml (43 mg), fraction II from eluate volume 320-380 (70 mg). These fractions gave spectra which indicated

* The sulfate analysis was performed by Dr. E. Delvin, Montreal.

they were N-acetyl containing tetrasaccharides.

Fraction I was subjected to reduction by adding sodium borohydride (15 mg) in water (0.5 ml) to a cooled solution of this material. After 18 h, the reaction mixture was worked up by treatment with Amberlite IR-120 resin, and with methanol to remove borate (yield, 30 mg). A detailed n.m.r. spectral study of these fragments is found in section 2.2.

C. Non-O-sulfated disaccharide methyl ester **6**

A sample*, which was obtained after treatment of heparin with methanolic-HCl (25) (which yields a totally de-N-sulfated, partially de-O-sulfated methyl ester of the polymer), was deaminatively degraded to smaller units in which 6 was present. The degradation was effected by dissolving the polymer (0.8 g) in H_2O (8 ml) and adding sodium nitrite (0.4 g) and M HCl (3.2 ml). The reaction conditions and work-up were the same as before.

The deamination product was dissolved in 3 ml of H_2O and applied to Sephadex A-25 (HCO_3^- form) column (2 cm x 15 cm) and the column was washed thoroughly with distilled water. The only material to emerge from the anionic exchange matrix was a neutral disaccharide methyl ester that was characterized (section 2.1-5) as 6. No other material was eluted from the column. Yield, 0.15 g.

A ^{13}C.m.r. spectrum was obtained. (After a few weeks, the compound decomposed probably due to instability of the 2,5-anhydro-D-

* Donated by Dr. G.R. Sanderson.

mannose residue).

4.3 Synthetic experiments

1. 1,2-O-Isopropylidene-α-D-glucofuranurono-6,3-lactone(14)

Anhydrous D-glucurono-6,3-lactone(13) (20g) was stirred with dry acetone (1 l.) containing sulfuric acid (conc., 8 ml). After 4 h, when all of the sugar had dissolved, an excess of anhydrous sodium carbonate was added, the suspension was filtered, and the filtrate was evaporated to a light yellow sirup. 1,2-O-Isopropylidene-α-D-glucofuranurono-6,3-lactone(14) (16 g) crystallized from ether-petroleum ether as colourless needles, m.p. 119-120°, $[\alpha]_D$ +68° (c 2.0, water). (Lit. (59), m.p. 120°, $[\alpha]_D$+70° (c 1.0, water).

2. Attempted enolization of the lactone (14)

Compound 14 (0.25 g) was dissolved in 1 ml of D_2O in a ^{13}C.m.r. sample tube. The spectrum was recorded, and then the pH of the solution was raised at intervals of 0.5 by the addition of 0.1 N NaOD, a spectrum being taken after each pH rise. There was no evidence of an appreciable enol-keto equilibrium (14 ⇄ 15 ⇄ 16) i.e., there was no decrease in the intensity of the C-5 signal (α carbon), which would be expected if deuterium exchange had occured. (Deuterated carbons (e.g., C-5 of 14 and 16) give much less intense signals than protonated carbons.) As the pH of the solution was raised to 8.0, an equilibrium between the lactone (14) and its acyclic form (17) was established, and at a pH of 9.0, the compound was totally in its open ring form (17).

C.m.r. data for 14: 23.5 p.p.m., 24.0,(isopropyl); 67.5 (C-5); 75.6 (C-3); 78.8 (C-4); 80.0 (C-2); 103.5 (C-1); 110.0 (isopropyl); 172.0 (C=O).

C.m.r. data for 17: 23.7 p.p.m., 24.2 (isopropyl); 68.0 (C-5); 172.0 (C-3); 80.0 (C-4); 82.5 (C-2); 102.8 (C-1); 176.3 (C=O).

3. Attempted enolization of methyl 1,2,-O-isopropylidene-D-gluco-furanuronate (18)

Compound 14 (0.25 g) was dissolved in 1 ml of CH_3OD in a ^{13}C.m.r. sample tube. The pH of the solution was raised to 8.5 by addition of sodium methoxide at which time the methyl ester (18) was in equilibrium with the lactone (14) (i.e., 18 ⇄ 19). At higher pH there was no indication of ionization nor of deuterium exchange.

Partial c.m.r. data for 18: 54.0 p.p.m. ($CO\underline{C}H_3$); 67.8 (C-5); 71.0 (C-3); 80.1 (C-4); 82.0 (C-2); 102.6 (C-1); 173.1 (CO).

4. 5-O-Tosyl-1,2-O-isopropylidene-α-D-glucofuranurono-6,3-lactone (19)

A solution of p-toluenesulfonyl chloride (10 g) in chloroform (20 ml) was added dropwise with stirring to a cold (0°) solution of 14 (10.7 g) in pyridine (50 ml). Cooling was continued for 1 h after the addition was complete, then the reaction mixture was allowed to stand at room temperature for 6 h, after which it was poured with stirring into 100 ml of ice water. Chloroform (50 ml) was added, and the organic layer was washed with ice water (2x), cold 10% sulfuric acid (2x), cold sodium bicarbonate (2x) and then ice water. The chloroform solution was dried, and concentrated, giving crystals of 19 (9.2 g); m.p. 185-187°, $[\alpha]_D$ = 50.8 [c 2.5, chloroform]. ^{17}C.m.r. showed the presence of a tosyl group at C-5; I.R. showed no -OH absorption band.

P.m.r. data: (solvent CD_3COCD_3) δ4.8-5.0, m(H-2,3,4); 5.6, d(H-5); 6.0, d(H-1); isopropylidene: 1.3, 1.45(s,6H); tosyl: 2.5, s(CH_3-); 7.5, d, 7.9, d, (4H).

5. Attempted configurational inversion at C-5 of 19 with sodium benzoate

Compound 19 (2 g) was dissolved in dry N,N-dimethylformamide (75 ml), sodium benzoate (6.5 g) was added, and the suspension was heated under reflux for 6 h (the reaction mixture turned dark). Water (100 ml) was then added to dissolve the sodium benzoate, and the solution was extracted with 60 ml (2x) of chloroform. The organic layer was washed 6 times with water, dried, and evaporated to dryness. A brown residue was recovered which appeared to be composed mainly of p-toluenesulfonic acid.

6. Attempted configurational inversion at C-5 of 19 with acetate ion resin and acetic anhydride

Acetic anhydride (30 ml) was added to a mixture of 19 (1 g) and Dowex 1-X8(OAc^-) (15 g). With stirring, the reaction was heated under reflux for 4 h (the mixture became dark), then methanol (10 ml) was introduced to destroy excess acetic anhydride. The reaction mixture was cooled, the resin filtered off, the filtrate was extracted with chloroform, and the extract was concentrated. No product was identified from the dark oily residue obtained.

7. Methyl α-L-sorbopyranoside (21)

The procedure was similar to that of Arragon et al. (77) Dry L-sorbose (20) (80 g) was added to a 5% HCl-methanol solution (2.7 l, 5°) (23.6 ml of acetyl chloride in 3 l of dry methanol) with stirring. After 3 days silver carbonate was added to neutrality, the solution was filtered, treated with Norite, and then concentrated. The sirupy residue was exhaustively extracted with hot acetone (approx. 2.5 l) (on a steam bath). On cooling, 21 crystallized out. Yield, 60 g (70%). M.p. 119-120°, $[\alpha]_D$=-86.5 (c 1.0, water). Lit.(77):

m.p. 118.5°; $[\alpha]_D$=-90.2°(c 1.0, water)

8. Methyl 1,3,4,5-tetra-O-benzyl-α-L-sorbopyranoside (22)

The procedure was similar to that of Glaudemans and Fletcher (78). Methyl α-L-sorboside (21) (17.5 g) was suspended in dry dioxane (100 ml) together with powdered potassium hydroxide (100 g), the mixture being constantly stirred and gently boiled under reflux (caution was taken to stir vigorously to prevent charring). Benzyl chloride (125 ml) was added dropwise over a period of 20 min, and 1 h later the dioxane was allowed to distill off. The residue was cooled, sufficient water was added to dissolve the crystalline material, this being followed by an extraction with ether. The organic layer was washed with water, treated with Norite, and concentrated. Benzyl alcohol, benzyl chloride and dibenzyl ether were removed by distillation in a high vacuum (0.02 mm, 140°), leaving 22 as a light yellow oil. Yield, 46 g (92%). $[\alpha]_D$=-14.2° (c 3.1, chloroform).

C.m.r. data: 48.3 p.p.m. (methoxy); 60.9 (C-6); 68.9; 73.0; 73.3, 75.4(2x); 78.3; 79.2; 82.4; 100.4 (C-2); 127-129(20x); 137.5; 138.4; 138.6; 138.9.

P.m.r. data: δ3.2 (methoxyl); 3.3-4.1 (7 ring protons); 4.4-4.95 (8 methylene); 7.1-7.4 (20 phenyl).

9. 1,3,4,5-Tetra-O-benzyl-α-L-sorbopyranose (23)

Hydrolysis of 22 following the procedure by Glaudemans and Fletcher (78) (for the hydrolysis of 2,3,4,6-tetra-O-benzyl glucopyranose) did not produce a satisfactory product, as extensive decomposition occured.

Therefore, the following milder method was used: 18.5 g of 22 in dioxane (125 ml) was heated under reflux with stirring and

while both the reaction flask and condenser were covered with aluminum foil to exclude light, 0.5N HCl (pre-heated to 90°) was added dropwise to the solution until the compound just began to oil out of solution (approx. 32 ml of 0.5N HCl). The reaction was continued until t.l.c. (solvent A) showed that the hydrolysis of 22 had occured (approx. 3-4 h), 100 ml of ice water was then added, whereupon much product oiled out. A chloroform extract (200 ml) of the reaction mixture was washed with water, saturated sodium bicarbonate, 5% HCl, and water, then dried over $MgSO_4$, filtered, and concentrated. A yellow oil (23) remained; yield, 15.3 g (83%). When stored in the cold, the product partially crystallized, m.p. 47-50°, $[\alpha]_D$=-11.3° (c 1.5, chloroform). Lit. (74): m.p. 48-51°, $[\alpha]_D$=-12.9° (c 3.3, chloroform). Attempts at recrystallization from a variety of solvents were unsuccessful.

C.m.r. data: 60.1 p.p.m. (C-6); 71.2; 72.2; 72.9; 74.5; 74.8; 77.7; 78.0; 81.9; 96.6 (C-1); 126.6-127.6(20x); 136.7; 137.1; 137.5; 137.9.

P.m.r. data: δ3.3-4.1(7x); 4.4-4.9 (methylene 8x); 7.0-7.4(20x).

10. 1,3,4,5-Tetra-O-benzyl alditols(24)

(a) Product 23 (14 g) was dissolved in dry methanol (100 ml) and the solution was cooled to 5°. Sodium borohydride (3 g) was added slowly to the stirred solution over a period of 0.5 h, the temperature being maintained at <10°, then the reaction mixture was kept at r.t. for 3 h at which time excess Amberlite IR-120 resin (H^+, approx. 50 g) was added. The resin was filtered off and rinsed twice with methanol, the washings and solution were combined, concentrated, and the residue was treated with successive amounts of

methanol to remove borate. The yellow sirup remaining was chromatographed on silica gel (750 g) using solvent A. Small amounts of 22 and 23 and of an unidentified product were isolated before product 24 was collected; yield, 12.8 g (91%); the amorphous, homogeneous material could not be induced to crystallize; $[\alpha]_D$=+10.3° (c 2, chloroform). (A small amount of product was used to prepare the diacetate derivative using sodium acetate-acetic anhydride but this did not afford crystalline material).

C.m.r. data: 61.0 p.p.m., 61.1 (C-1's); 68.6; 69.8; 70.4-73.8(12x); 76.7-78.8, (6x, methylene); 127.0-127.6 (40x); 137.3 (8x).

P.m.r. data: δ2.68 (2x, hydroxyls); 3.3-4.1 (8x); 4.35-4.7 (8x, methylene); 7.18-7.3 (20x).

(b) To a cooled suspension of lithium aluminum hydride (1.0 g) in dry tetrahydrofuran (200 ml) a solution of 22 (9 g) in dry tetrahydrofuran (100 ml) was added dropwise. After the mixture had been stirred at 10° for 1.5 h, ethyl acetate was added to decompose excess lithium aluminum hydride, followed by ice and dilute hydrochloric acid. Methylene chloride was used to extract the crude product and the extract, after being washed with water, was dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a column of silica gel (solvent A) to give 8.1 g (89%) of 24 as sirup. An unidentified product was also isolated from the reaction mixture, as well as small amounts of unreduced 23. $[\alpha]_D$=+10.0° (c 2.0, chloroform).

11. 6-O-Trityl 1,3,4,5-tetra-O-benzyl alditols(25)

Compound 24 (10 g, 18 mmoles) and chlorotriphenylmethane (5.5 g) were dissolved in dry pyridine (60 ml), the solution was stored at r.t. for 2 days, then poured into ice-cold water with stirring.

The clear supernatant solution was decanted off, a white sirup which had deposited was dissolved in methylene chloride and washed successively with cold 5% hydrochloric acid, saturated bicarbonate solution, and water. Removal of the methylene chloride yielded a sticky sirup, which was chromatographed on silica gel (700 g) using solvent C. Product 25 was obtained in a yield of 89% (12.9 g). It was found that partial de-O-tritylation of 25 can occur on silica gel support so that the chromatographic step might, advisedly, be omitted. $[\alpha]_D$=+9.12° (c 1.5, chloroform).

C.m.r. data: 62.3 p.p.m., 62.5(2x C-1's); 69.2; 70.0; 70.6(2x); 72.1-72.5 (4x); 73.5; 73.9 (3x); 126.2-128.0 (35x); 137.5 (4x); 143.2 (3x).

From evidence of ^{13}C signal intensities it seems that one diastereomer is present in larger amount.

P.m.r. data: δ2.55, 2.84, (hydroxy); 3.1-4,1 (8x); 4.1-4.7 (8x); 7-7.5 (35x).

12. 6-O-Methoxy-trityl 1,3,4,5-tetra-O-benzyl alditols (26)

The 1-O-methoxy-trityl derivative (26) was produced in the same fashion as compound 25 by using p-anisylchlorodiphenylmethane. Yields of 26 were the same as those of 25. $[\alpha]_D$=+9.04° (c 1.0, chloroform). Its c.m.r. and p.m.r. spectra were closely analogous to those of compound 25.

13. 6-O-Trityl-1,3,4,5-tetra-O-benzyl-2-O-TBDMS alditols (27)

A solution of compound 26 (10 g, 12.7 mmoles), dimethyl-tert-butylsilyl chloride (2.3 g, 15.2 mmoles), and imidazole (2.2 g, 31.7 mmoles) in dry dimethylformamide (40 ml), was heated at 35° for 24 h, poured into ice water, and the mixture was stirred for 2 h and left

overnight in the cold. The supernatant solution was decanted off, and the sirupy product was dissolved in methylene chloride. After being washed successively with cold 5% hydrochloric acid, saturated bicarbonate solution and water, the solution was dried and concentrated. The yellow residue was chromatographed on silica gel (700 g) using solvent C, affording sirupy 27 in a yield of 9.8 g (86%). $[\alpha]_D$=+5.7° (c 2.5, chloroform).

P.m.r. data: δ0.05 (6x, TBDMS), 0.82 (9x, TBDMS); 3.1-4.1 (8x); 4.1-4.8 (8x); 7.0-7.4 (15x).

14. 6-O-Methoxy-trityl-1,3,4,5-tetra-O-benzyl-2-O-TBDMS alditols (28)

Compound 28 was synthesized in the same fashion as compound 27; the yield was marginally higher, $[\alpha]_D$=+4.2° (c 1.5, chloroform).

15. 6-O-Trityl-2-O-benzoyl-1,3,4,5-tetra-O-benzyl alditols (29)

To a cooled pyridine (20 ml) solution of 25 (5 g, 6.4 mmoles) was added dropwise 1 ml of benzoyl chloride (8.3 mmoles). The solution was left overnight at r.t., 5 ml of ice-water was added to destroy excess benzoyl chloride, and 200 ml of methylene chloride was introduced. The organic layer was washed successively with H_2O, cold 5% hydrochloric acid, saturated bicarbonate, and water, dried over $MgSO_4$ and concentrated to a sirup. The material was chromatographed on silica gel (250 g) using solvent C. Compound 29 was obtained as a sirup (yield, 5.1 g (88%)), $[\alpha]_D$=+4.1° (c 2.1, chloroform).

P.m.r. data: δ3.5-4.1 (7x); 4.25-4.65 (8x, methylene); 5.5, m (H-5); 7.0-7.4 (38x); 7.8-8.0 (2x, benzoyl).

16. 6-O-Trityl-2-O-acetyl-1,3,4,5-tetra-O-benzyl alditols (30)

Compound 25 (5 g, 6.4 mmoles) was dissolved in acetic anhydride (16 ml) and dry pyridine (40 ml), the solution was kept at

r.t. for 18 h, and poured into ice water with stirring. An oily residue that deposited out was worked up in the same fashion as for compound 29. Yield, 4.2 g (82%). $[\alpha]_D$=+4.0° (c 2.7, chloroform).

P.m.r. data: δ1.93, s(acetyl); 3.4-4.1 (7x); 4.2-4.8 (8x); 5.35 (m, H-5); 7.0-7.4 (35x).

17. Attempted selective hydrolysis of trityl group of 27

(a) Using MeOH-HCl:

Compound 27 (0.6 g) was dissolved in methanol (10 ml), 0.1 N methanolic-HCl (2 ml) was added, and the reaction followed by t.l.c. After 20 mins, it was evident that both O-silyl and the O-trityl groups were being hydroxyled, and the reaction was terminated.

(b) Using silica gel:

To a column of silica gel (25 g of a mixture of 60-200 mesh (Fischer grade) and 28-60 mesh (Fischer grade)) a solution of 27 (0.5 g) in benzene (5 ml) was applied, and 50 ml of benzene was used to develop the chromatogram. After 12 h the compounds were eluted from the column with solvent D: the products were found to represent a mixture of de-O-tritylation and, to a lesser extent, de-O-tritylation and de-O-silylation, of 27.

18. 1,3,4,5,Tetra O-benzyl-2-O-TBDMS alditol (31) (via trityl derivative)

Compound 27 (4.7 g) was quickly dissolved in twice its weight of glacial acetic acid (i.e., 9.5 ml). While the solution was heated and stirred vigorously, preheated (90°) 70% aqueous acetic acid (38 ml) was added batchwise slowly enough not to oil out the material. The total time of hydrolysis did not exceed 12 mins, at which time the reaction mixture was poured into ice water (300 ml) with stirring. A precipitate which formed was collected by decantation

and centrifugation and dissolved in methylene chloride (250 ml). This solution was washed successively with saturated sodium bicarbonate and water, and concentrated. Most of the trityl alcohol and small amounts of unhydrolysed 24 were removed from the product by vacuum distillation (140°/0.02 mm). (Yield of the trityl alcohol, 1.25 g). The remaining material was chromatographed on silica gel (300 g) using solvent D, to yield 2.4 g of 31 (65 %); $[\alpha]_D$=-6.9° (c 1.5, chloroform).

P.m.r. data: δ0.05, 0.13 (6x, TBDMS); 0.87 (9x, TBDMS); 2.35 (hydroxyl); 3.4-4.2 (8x); 4.3-4.9 (8x, methylene); 7.1-7.4 (20x, phenyl).

19. 1,3,4,5-Tetra-O-benzyl 2-O-TBDMS alditols (31) (via methoxytrityl derivative)

To a solution of 28 (2 g) glacial acetic acid (6 ml), stirred and heated at 50°, was added batchwise 70% aqueous acetic acid (preheated to 70°)(18 ml) at such a rate as to avoid precipitation of the compound. After 45 min the solution was poured over ice water, and the product recovered as in procedure 17. Yield of 31, 1.1 g(74%), $[\alpha]_D$=-6.5° (c 1.1, chloroform). The p.m.r. spectrum was indistinguishable from that of 28, produced by procedure 17.

20. 2-O-Benzoyl-1,3,4,5-tetra-O-benzyl alditols (32)

Hydrolysis of the O-trityl group of compound 29 was performed as for 27 (procedure 17), except that the reaction was allowed to proceed for 0.5 h instead of 12 min. High vacuum distillation removed most of the trityl alcohol, and the residue was chromatographed on silica gel (solvent D); yield, 77%, $[\alpha]_D$=+4.1° (c 1.6, chloroform).

P.m.r. data; δ2.25 (m, hydroxyl); 3.5-4.1 (7x); 4.2-4.6 (8x, methylene); 5.5 (m, H-5); 7.0-7.4 (23x); 7.8-8.0 (2x, benzoyl).

21. 2-O-Acetyl-1,3,4,5-tetra-O-benzyl alditols (33)

Hydrolysis of the O-trityl group of compound 30 was performed as with the O-benzoyl derivative (procedure 19). Yield, 75%, $[\alpha]_D$=+5.0° (c 1.3, chloroform).

P.m.r. data: δ1.95, s(acetyl); 3.4-4.1 (7x); 4.2-4.8(8x); 5.40, m(H-5); 7.0-7.4 (20x).

22 2,3,4,6-Tetra-O-benzyl-5-O-TBDMS aldoses (34). Oxidation with chromium trioxide-pyridine

The oxidation procedure was similar to that described by Arrick, Baker and Horton (86). The reaction was carried out in a dry box. Chromium trioxide (1.08g, 12 M equiv./M equiv of alcohol) was added to a solution of dry pyridine (1.7 ml, 2 M equiv./M equiv. of chromium trioxide) in dry methylene chloride (20 ml). The oxidant was stirred for 20 min, at which time compound 31 (0.6 g, 0.9 mmole) dissolved in 3 ml of methylene chloride, was added. A tarry deposit formed at once; the suspension was stirred for 20 min at r.t., the supernatant solution was decanted, and the tar was extracted twice with ether. The combined washings and supernatant were concentrated, and the residue was chromatographed on silica gel (50 g) using solvent E. The aldehyde (34) was collected (first compound to emerge from the column) in a yield of 0.3 g (50%). $[\alpha]_D$=-2.7° (c 0.8, chloroform).

P.m.r. data: δ0.08, 0.15 (6x, TBDMS); 0.85 (9x, TBDMS); 3.3-4.25 (6x); 4.3-4.9 (9x, methylene); 7.1-7.4 (20x, phenyl); 9.6, 9.68 (s, aldehydic protons).

23. 2,3,4,6-Tetra-O-benzyl-5-O-TBDMS aldoses (34). Use of the pyridinium chlorochromate oxidation method

The oxidation procedure was similar to that of Corey and Suggs (87). Pyridinium chlorochromate (prepared as in ref 87 and stored dry and in the dark) (1.03 g, 4.76 mmoles) and anhydrous sodium acetate (0.08 g) were suspended in dry methylene chloride (5 ml). Compound 31 (2.1 g, 3.15 mmoles), in dry methylene chloride (10 ml) was added in one portion to the stirred suspension of oxidant, followed after 3 h by 50 ml of dry ether. The suspension was decanted and the tarry deposit washed twice with ether, and the combined solutions were concentrated, giving a black residue that was chromatographed on silica gel (100 g) using solvent E. Aldehyde 34 was obtained in 74% yield (1.54 g). $[\alpha]_D$=-2.5° (c 2.0, chloroform). Preparative t.l.c. (solvent D) of the aldehyde mixture (34) succeeded in separating a fraction which contained 80% of the gluco isomer, the aldehydic proton of which resonates at 9.6 p.p.m. Column chromatography was less successful. P.m.r. spectrum was indistinguishable from that of product given by procedure 21.

C.m.r. data: -5.8 p.p.m., -5.6, -5.2, -5.1 (2x, methyls of TBDMS); 25.1 (3x, tert-butyl $\underline{CH}_3$); 70.6; 71.0; 71.6;71.9; 72.2 (3x); 72.4; 72.5; 72.6; 73.3; 73.4; 77.2; 77.7; 79.3; 79.6; 79.7, 79.9 (6x, methylene to secondary carbon); 126.7-128.3 (40x); 136.4-137.6 (8x); 198.6 (aldehyde, 2x).

24. 5-O-Benzoyl-2,3,4,6-tetra-O-benzyl aldoses (35)

The oxidation of compound 32 was similar in procedure to the previous oxidation method (i.e., 22); the yield of compound 35 was 72%. $[\alpha]_D$=+0.4° (c 1.0, chloroform).

P.m.r. data: δ3,3-4.0 (5x); 4.1-4,8 (9x); 5.5, m(H-5); 7.0-7.5 (23x); 7.8-8.0 (2x, benzoyl); 9,6, 9.65 (s, aldehydes, equal intensity).

25. 5-O-Acetyl-2,3,4,6-tetra-O-benzyl aldoses (36)

Oxidation of compound 33 by the procedure described in 22, gave 36 in 73% yield. $[\alpha]_D$=+1.6° (c 1.2, chloroform).

P.m.r. data: δ1.96, s(acetyl); 3,35-4.0 (5x); 4.05-4.8 (9x); 5.35, m(H-5); 7.0-7.4 (20x); 9.55, 9.6 (s, aldehydic proton).

26. 2,3,4,6-Tetra-O-benzyl aldoses: L-ido (37), D-gluco (38)

The use of tetrabutyl ammonium fluoride in THF (procedure of Ogilvie et al (84) to de-O-silylate compound 34 produced side products. An alternative, more satisfactory, procedure used acidic conditions. Compound 34 (3.3 g) was dissolved in 30 ml of acetic acid, water (8 ml) was added slowly to the heated and stirred solution, the apparatus was covered with aluminum foil, and the reaction allowed to proceed for 4 h. On cooling, 2,3,4,6-tetra-O-benzyl-α-D-glucose (38) crystallized out (yield, 0.80 g). Water was added, the solution was concentrated, methanol (6 ml) was used to dissolve the residue sirup, and on cooling more of 38 (0.1 g) crystallized out. The total yield of 38 was 33%. M.p. 150-152°, $[\alpha]_D$=+20.8°(c 2.4, chloroform). Lit (78): m.p. 151-152°; $[\alpha]_D$=+21.2° (c 2.19, chloroform).

It was estimated that 90% of the residual sirup (1.7 g, 63% yield) was 2,3,4,6-tetra-O-benzyl-L-idose (37). $[\alpha]_D$=-1,0° (c 2.4, chloroform).

Additional p.m.r. data(crude 37): δ3.3-4.1 (6x); 4.2-4.8 (8x); 4.92, d(β, H-1); 5.15, d(α, H-1); 7,0-7,3 (20x).

C.m.r. data (crude 37): 67.6 p.p.m.; 68.0; 68.5 (2x); 71.7-72.9 (10x); 74.6 (2x); 75.5; 77.1; 91.8 (α, C-1); 93.3 (β,C-1);

126.6-127.6 (40x); 136.6-138 (8x).

27. Catalytic methanolysis of the ester groups of compounds 35 and 36

To a solution of each compound (0.5 g) in dry methanol (10 ml) was added 1.5 ml of freshly prepared 0.5% sodium methoxide. The reaction mixture was stored at 12° for 18 h, then it was neutralized with cationic resin, the solvent was removed and the dark residue (0.32 g) was chromatographed on t.l.c. plates. The product showed evidence of extensive decomposition, as described in section 3.3-2 (D). $[\alpha]_D$(from benzoate)=+2.6°; $[\alpha]_D$(from acetate)=+1.8°.

28. Acid hydrolysis of 5-O-acetyl-2,3,4,6-tetra-O-benzyl aldose (36)

Compound 36 (0.5 g) was dissolved in 5 ml of dry methanol containing Dowex-50 resin (H^+) (1.0 g), the suspension was stirred for 2 days (t.l.c. showed no significant amount of methyl glycoside), the resin was removed, and 1 N HCl-methanol (2.5 ml) was added. After 2 days at r.t. only 30% of the material was converted into methyl glycosides, as indicated by p.m.r. spectroscopy, the rest of the material being unreacted 36 and decomposition products.

29. 1-O-Acetyl-2,3,4,6-tetra-O-benzyl-D-glucopyranose

To a cooled mixture of dry pyridine (3.5 ml) and acetic anhydride (0.3 ml) was added compound 38 (0.5 g), and after 14 h at r.t. the solution was poured dropwise over 15 g of ice. The mixture was extracted with chloroform, and the organic layer washed with water, dried, and concentrated, giving a clear oil (0.45 g, 84%). The p.m.r. spectrum showed the oil to be a mixture of α:β anomers in the ratio of 5:1. The observed chemical shifts were in close agreement with those reported by Leroux (97).

30. 1-O-Acetyl derivative of crude 37

To a cooled mixture of dry pyridine (2.5 ml) and acetic anhydride (0.2 ml) was added sirupy crude 37 (0.3 g). After 14 h at r.t., workup was performed as described in the previous section; yield, 0.25 g (83%), $[\alpha]_D$=+1.8° (c 2.4, chloroform).

P.m.r. data: δ1.9(acetyl, α anomer); 1.95(acetyl, β anomer); 3.45-4.9 (5x); 5.1-5.7 (9x); 6.1 d($J_{1,2}$=2.5 Hz, β-anomer); 6.2 d($J_{1,2}$=4.0 Hz, α-anomer); 7.0-7.4 (20x).

31. L-Iditol hexaacetate

To a cooled methanolic solution (5 ml) of the crude preparation of 37 (0.25 g) sodium borohydride (0.05 g) was added slowly. After 14 h, an excess of Dowex-50 resin (H^+) was introduced, the solution was evaporated to dryness, and methanol was added (2x) and then removed to remove the borate.

The reduced material was dissolved in 90% aqueous dioxane (12.5 ml), palladium black (0.04g) was added, and hydrogen was introduced by means of a balloon reservoir. The stirred suspension was left at r.t. for 18 h, filtered through Celite pad, the filtrate was evaporated, and the residue dried thoroughly under vacuum. To the residue was added acetic anhydride (2 ml) and anhydrous sodium acetate (0.2 g), the mixture was heated at 95° for 1.5 h and evaporated to dryness. The product was extracted into chloroform, the solvent was removed and crystallization occurred when the residue was dissolved in ethanol; yield, 0.06 g (66%), m.p. and mixed m.p. 122-124°, $[\alpha]_D$=+20.1° (c 0.3, chloroform)

32. Methyl 2,3,4,6-tetra-O-benzyl-α,β-L-idopyranoside

Methyl glycoside synthesis was attempted by the procedure of Leroux (94) by using triflic anhydride, tetrabutylammonium bromide and collidine, followed by the addition of methanol. The main product recovered after chromatographic purification was not identified, only a small proportion of methyl glycoside being present. (p.m.r. evidence). A part of the material was de-O-benzylated by hydrogenation (aqueous dioxane-palladium black), the product of which appeared to be composed of 4 or 5 compounds: 2 showed r.f. values (t.l.c., ethyl acetate:methanol=8.5:1.5) characteristic of disaccharides, whereas another 2 corresponded to methyl glycosides. The p.m.r. spectrum was complex, and appeared to contain 4 small anomeric signals.

Another portion of the product (above) was treated with 3% methanolic-HCl under reflux for 6 h. The material recovered had $[\alpha]_D$=+1.0° (c 1.0, chloroform). Its p.m.r. spectrum indicated that it was composed of approx. 70% methyl 2,3,4,6-tetra-O-benzyl-α-D-idopyranoside, 20% of the β-anomer, and 10% of methyl 2,3,4,6-tetra-O-benzyl-α-D-glycopyranoside.

P.m.r. data: δ3.3(methoxy,α-anomer, glucose); 3.38(methoxy, α-anomer ido); 3.42(methoxyl β-anomer, ido); 3.3-4.3(9 protons); 4.3-5.0 (9 protons); 7.0-7.4 (20x).

The mixture of methyl glycosides was de-O-benzylated by hydrogenolysis in aqueous dioxane containing palladium black (procedure 30). The methyl glycosides that resulted were present in the same proportion as before de-O-benzylation; $[\alpha]_D$=-30.5° (c 1.5, chloroform). The p.m.r. signals ascribed to the methyl-α-L-idopyranoside present (70%) corresponded closely to the spectrum of authentic methyl-α-D-

idopyranoside.

Partial p.m.r. data: δ3.45(methyl, α-anomer, ido); 3.55(methyl, β-anomer, ido), 4.7 d($J_{1,2}$=4.0 Hz, α-anomer, ido); 4.9, d($J_{1,2}$=1.4, β-anomer, ido).

C.m.r. data for methyl-α-L-idopyranoside: 56.4 p.p.m. (CH_3-); 60.7 (C-6); 70.7 (C-4); 71.2 (C-5); 71.5 (C-5); 72.0 (C-3); 102.0 (C-1). For methyl-β-L-idopyranoside: 57.4 (CH_3-); 62.0 (C-6); 69.2 (C-5); 70.1 (C-4); 70.4 (C-2); 76.0 (C-3); 100.9 (C-1).

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CLAIMS TO ORIGINAL RESEARCH

1. N.m.r. spectroscopy has been used to obtain detailed structural information about minor components of type A and type B heparins.

2. A comprehensive n.m.r. spectral study has been carried out on the major product formed by deaminative degradation of heparin, i.e., 4-O-(α-L-idopyranosyluronic acid 2-sulfate)-2,5-anhydro-D-mannose 6-sulfate. (1) Also, characterization of a de-O-sulfated, methyl ester analog of 1 has been effected.

3. A new synthetic route to L-idopyranose has been developed; it involves the synthesis of 2,3,4,6-tetra-O-benzyl-L-idopyranose, starting from L-sorbose.