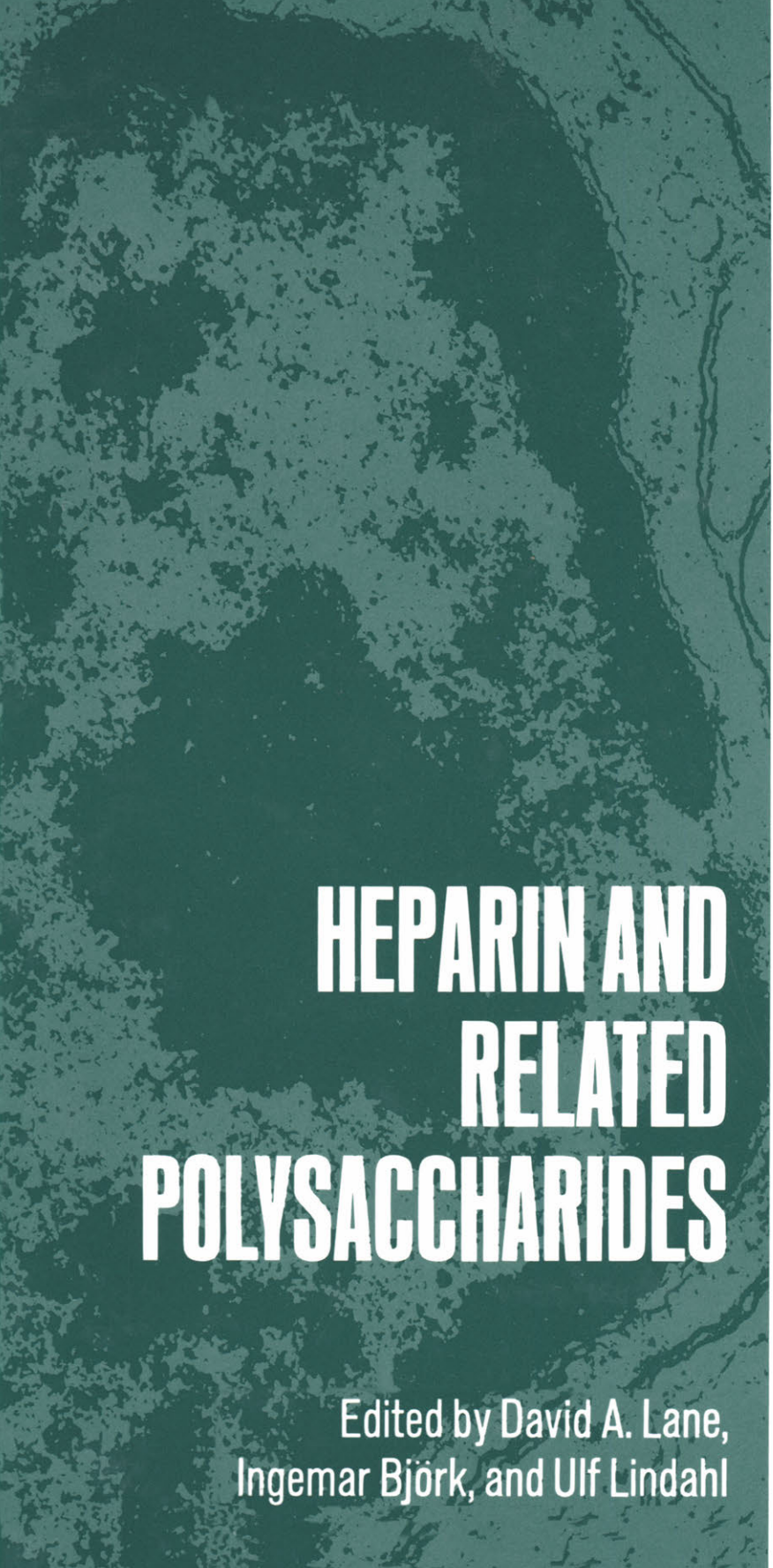

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 313

HEPARIN AND RELATED POLYSACCHARIDES

Edited by David A. Lane, Ingemar Björk, and Ulf Lindahl

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PREFACE

This volume is a record of a meeting entitled "Heparin and Related Polysaccharides" that was held at the Biomedical Center, Uppsala, Sweden between 1-6 September 1991. The meeting was hosted by U. Lindahl, L. Kjellén and I. Björk, who were helped in their preparations by a scientific advisory panel that included U. Abildgaard, B. Casu, E. Holmer and D. Lane. Altogether, 230 participants from 18 countries attended the meeting, and most were present to be included in a photograph, which is to be found at the end of this volume.

The selection of presentations for inclusion in the Symposia of the meeting was made on the basis of the known high quality of the work of the individuals or groups involved. This, we believe, is reflected in the contents of the enclosed articles, which collectively give a comprehensive overview of the present state of knowledge of heparin and related compounds.

Some of the areas covered are evolving or controversial and the views expressed in each article should be regarded as those of the author(s). The authors have taken various amounts of care to define all their abbreviations and some familiarity with the different forms of nomenclature used in the fields of polysaccharide chemistry and of the coagulation proteinases and their inhibitors will assist the reader.

The meeting in Uppsala was made possible by financial contributions from the following sponsors: Alfa Wassermann S.p.a., Carmeda AB, The City Council of Uppsala, Crinos Industria Farmacobiologica S.p.A., Diosynth bv, AB Draco, Erik Jorpes Memorial Fund of the Swedish Society of Medicine, Italfarmaco S.p.A., Kabi Pharmacia Cardiovascular, Lilly Research Laboratories, Mediolanum Farmaceutici S.r.l., The Nobel Committee for Chemistry of the Royal Swedish Academy of Sciences, Novo Nordisk A/S, Opocrin S.p.A., Rhône-Poulenc Rorer S.A., Sanofi Recherche, The Swedish Medical

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November 1991

U. Lindahl
I. Björk
D.A. Lane

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HEPARIN - AN INTRODUCTION

Lennart Rodén, Sandya Ananth, Patrick Campbell, Tracy Curenton, Göran Ekborg, Stephen Manzella, Dennis Pillion, and Elias Meezan

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INTRODUCTION

Seventy-five years after its discovery (1,2), heparin remains an important tool in medicine. Among its established uses are the prevention of postoperative thrombosis, the treatment of acute venous thrombosis, and the prevention of clot formation in the heart-lung machine (3). Little was known about the structure of heparin when the first clinical trials began in the mid-1930s, and it is only during the 1980s that the detailed structural basis of heparin action has been elucidated through the characterization of a specific, antithrombin-binding pentasaccharide segment in the polysaccharide molecule. In the following, we shall retrace some of the steps that have led to this goal.

THE YEAR 1935

The year 1935 was a remarkable time in the history of heparin. By then, it was apparent that heparin was not a phospholipid, as had initially been assumed on the basis of the original mode of isolation. Analysis by the naphthoresorcinol reaction in Howell's laboratory at Johns Hopkins University had shown that heparin contained carbohydrate, more specifically a uronic acid, and this conclusion was confirmed by Jorpes at the Karolinska Institute, who published a landmark paper on the chemistry of heparin in 1935 (4). In addition to the colorimetric test, which was not always reliable, Jorpes used decarboxylation in strong acid for the qualitative and quantitative analysis of uronic acid in heparin. (It is of interest to note that, in 1936, two other pioneers in heparin research, Charles and Scott, in Toronto, still did not believe that heparin contained uronic acid.) Other basic features of the composition of heparin were also established by Jorpes in 1935. The newly developed Elson-Morgan procedure helped provide evidence for the presence of hexosamine in heparin, and, in accordance with the belief at the time that all hexosamine residues in complex carbohydrate molecules were N-acetylated, the presence of acetyl groups was also verified experimentally. A major finding in Jorpes' work was the high content of sulfate groups in heparin, determined by analysis of the ash, and this observation led Jorpes to conclude that heparin was the most highly charged polyanion in Nature.

In 1935, there were two major centers in heparin research in the world, Stockholm and Toronto. Under the direction of Charles Best, a team in Toronto had developed methods for the purification of heparin, and large-scale preparations were being undertaken at the Connaught Laboratories, affiliated with the University of Toronto. In Stockholm, Jorpes was building on the experience gained by Charles and Scott of the Toronto team, and large-scale preparations were being made possible through the participation of the Vitrum Company in this endeavor. (The difficulties in obtaining useful amounts of heparin on a laboratory scale were made abundantly clear to the senior author when, early in his career, he was given 80 kg of pig intestinal slime by Jorpes and eventually succeeded in isolating 112 mg of heparin with an anticoagulant activity of 56 units per mg (L. Rodén, unpublished results). Despite the considerable effort, this accomplishment did not a thesis make.)

The obvious goal of the researchers in Stockholm and Toronto was to develop heparin into a therapeutically useful drug. As a young surgeon in Stockholm, Clarence Crafoord had observed only too many times how the best efforts at the operating table had subsequently been thwarted by the development of postoperative venous thromboses resulting in fatal lung emboli. His zeal in wanting to eliminate this seemingly unnecessary complication helped Jorpes focus his efforts on the rapid development of heparin preparations fit for use in humans. After such preparations had been tested on medical students working in the Department of Medical Chemistry at the Karolinska Institute, Crafoord began clinical trials in August of 1935. A few months earlier, the team in Toronto had embarked on similar studies, and the efforts of the two groups soon proved the value of heparin as a clinically useful drug (5-8). Crafoord was the first to publish the results of this work (5) and provided convincing evidence that heparin was effective in the prevention of postoperative thrombosis. Early ambulation has subsequently lessened the incidence of postoperative thrombosis in general, but certain groups of patients are still at high risk, and the development of appropriate antithrombotic prophylaxis remains an area of active investigation even today.

Yet another line of research, relevant to heparin, came to fruition in 1935. Charles A. Lindbergh - the pilot of Spirit of St. Louis - had been working for several years in the laboratory of Nobel Laureate Alexis Carrel at the Rockefeller Institute on the construction of a mechanical heart pump, and in June of 1935 it was announced that this endeavor had succeeded. The Lindbergh apparatus allowed investigators to keep organs from experimental animals alive for more than one month by perfusing them with nutrient fluids of appropriate composition under strictly sterile conditions. Although perfusion of organs had been carried out earlier by many investigators, this was the first time that such experiments could be extended beyond a period of only a few hours. Whole blood was not used in Lindbergh's and Carrel's experiments in 1935, but in their book, "The culture of organs," published in 1938 (9), they reported that they had obtained heparin from the Connaught Laboratories in Toronto and had thus been able to compare the performance of whole blood and other nutrient fluids in their systems.

In the cardiovascular literature, the Lindbergh apparatus is described as one of the forerunners of the heart-lung machines, and its successful use in Carrel's laboratory and by other investigators undoubtedly stimulated the development of the technology necessary for application in humans. When heart-lung machines were first used for open-heart surgery in the early 1950s, Crafoord was again one of the leaders and reported the second successful operation of this kind in 1954.

THE PIECES OF THE PUZZLE

Monosaccharide Composition of Heparin

Five different monosaccharides have been identified as components of the heparin molecule: D-glucosamine, D-glucuronic acid, L-iduronic acid, D-galactose, and D-xylose. The definitive identification of the monosaccharide components of heparin began in 1936 and was not completed until 1964. In 1936, Jorpes and Bergström (10) (who was then a medical student like McLean) isolated the hexosamine component of heparin and showed that it was glucosamine. Jorpes' initial belief that heparin was an oversulfated chondroitin sulfate thus proved incorrect, and it was concluded, instead, that heparin was akin to mucoitin sulfuric acid, a glucosamine-containing polysaccharide isolated from gastric mucosa. (Mucoitin sulfuric acid has subsequently receded into oblivion.)

The basis of the successful identification of glucosamine by Jorpes and Bergström was the resistance of this sugar to treatment with strong acid at high temperature, which made it possible to subject heparin to acid hydrolysis and to isolate the sugar in good yield in crystalline form from the hydrolysate. In contrast, uronic acids are not as stable to acid treatment, and the conditions required for their release from a polymer are such that free uronic acids are rapidly destroyed. Today, it would have been relatively easy to show the presence of free glucuronic acid in a heparin hydrolysate, since we now have access to more sensitive analytical methods. At the time, however, it was not possible to detect the small quantities present, and it was only through indirect methodology that Wolfrom and Rice (11) succeeded in obtaining information about the identity of the uronic acid component of heparin. In 1946, these authors reported results of experiments in which bromine had been included in the reaction mixture during acid hydrolysis (bromine-sulfuric acid at about 3 °C for one week), thus effecting immediate oxidation of the released uronic acid to the more stable dicarboxylic acid. Having isolated and identified the latter as glucaric acid (saccharic acid), Wolfrom and Rice concluded that the uronic acid of heparin was D-glucuronic acid. It should be noted, however, that glucaric acid may also be derived from L-guluronic acid, but this possibility was not mentioned in the paper by Wolfrom and Rice and was perhaps considered too remote. Subsequently, more stringent proof for the D-gluco configuration was obtained, as has been described by Brimacombe and Webber (ref. 12 and refs. cited therein).

The third monosaccharide component of heparin was discovered by Cifonelli and Dorfman in 1962 (13). Facing the same difficulty as Wolfrom and Rice, they chose another approach and first removed the N-sulfate groups (see below) by mild acid hydrolysis; the free amino groups were then acetylated to yield a structure in which the glucosaminidic linkages were more susceptible to hydrolytic cleavage than in the starting material. After acid hydrolysis of the modified polysaccharide and repeated N-acetylation and hydrolysis, paper chromatography showed the presence of iduronic acid and iduronolactone in the hydrolysate. Further characterization of the iduronolactone was carried out by measurement of its optical rotation (indicative of the L-configuration) and reduction to idonolactone, which was clearly separated from gluconolactone, gulonolactone, and mannonolactone by paper chromatography. Initially, the work of Cifonelli and Dorfman was met with skepticism, but their important discovery was eventually confirmed by several investigators (14-17), including Perlin and his collaborators (15,17), whose

NMR studies showed that L-iduronic acid was the major uronic acid component of heparin.

The discovery of the two remaining monosaccharides, galactose and xylose, was incidental and occurred in 1964 in the course of a project aimed in a different direction (18,19). Although it had been known since Mörner's investigations in 1889 (20) that chondroitin sulfate was associated with protein, the prevalent notion during the first several decades of this century was that this association was effected by strong ionic interactions. It was only in the 1950s that convincing evidence for a covalent linkage between the protein and the polysaccharide emerged, mainly through the investigations of Schubert and Mathews and their collaborators. Based on amino acid analyses of chondroitin sulfate that had been subjected to extensive proteolysis, Helen Muir (21) concluded in 1958 that the polysaccharide was attached to serine residues in the core protein of the proteoglycan. The same conclusion was subsequently reached by Meyer and his collaborators on the basis of characterization of the products formed on alkaline cleavage of the protein-polysaccharide bond. As a consequence of the investigations of the chondroitin sulfate proteoglycan and its carbohydrate-protein linkage, the possibility naturally had to be explored that other polysaccharides, including heparin, were also covalently bound to protein. To test this hypothesis, Lindahl et al. (22) subjected partially processed heparin preparations to amino acid analysis and found that some such preparations contained essentially only serine. Mild acid hydrolysis yielded two tell-tale fragments, xylosylserine and galactosylxylosylserine, and it was therefore concluded that not only was native heparin covalently bound to peptide or protein, but the carbohydrate-protein linkage region contained two sugars that had not previously been recognized among the monosaccharide components of heparin (18,19).

Other Components

Substituents of three kinds are found on the sugar residues of heparin: acetyl, sulfate, and phosphate groups. The first analyses of the purified polysaccharide in Jorpes' laboratory indicated that, on a molar basis, the acetyl content approximately equalled that of hexosamine. At the time, this finding was almost a foregone conclusion, since in all hexosamine-containing complex carbohydrates examined previously, the amino groups of the hexosamine components had always been found to be N-acetylated. When Jorpes' landmark paper of 1935 (4) was published, however, it had become clear that the N-acetyl content of some heparin preparations was much lower than expected. The explanation of these results was not immediately obvious, but with his dogged perseverance, Jorpes did not let go of the problem and eventually arrived at the solution in 1950 (23) in the course of studies of the second class of substituents, the sulfate groups.

Although the presence of sulfate in heparin preparations was known to Howell, it was only through Jorpes' meticulous analytical work that it became clear that the sulfate was part of the polysaccharide molecule and that the sulfate content was much higher than in any other sulfated macromolecule known at the time. We now know that the sulfate groups may be found in five different locations in mammalian heparin, i.e., linked to the amino groups of the glucosamine moiety (N-sulfate) and as ester sulfate (O-sulfate) on C-6 and C-3 of glucosamine and on C-2 of iduronic and glucuronic acid. The assignment to these five positions was not easy and spanned over half a century, following Jorpes' discovery of the sulfate groups in 1935 (on average one position every 10 years!). The N-sulfate groups, which are

unique to heparin and the closely related heparan sulfate, were discovered by Jorpes, Boström, and Mutt in 1950 (23) and the last assignment (to C-2 of some of the glucuronic acid residues) was made in 1985 by Bienkowski and Conrad (24). (See ref. 2 and refs. cited therein for details on other structural studies concerning the sulfate groups.)

Since heparin was originally thought to be a phospholipid and the earliest preparations were undoubtedly contaminated with such substances, it was not surprising that Howell's first analyses showed the presence of phosphorus. As the isolation procedures improved and heparin became a complex carbohydrate, the phosphorus disappeared. Recently, however, following the discovery by Oegema and his collaborators (25) that a phosphate group may be present at C-2 of the xylose residue in chondroitin sulfate, similar analyses by Fransson et al. (26) have shown that the same is true for heparan sulfate from bovine lung, and Rosenfeld and Danishefsky (27) have shown the presence of phosphate in the same location in heparin. The function of the phosphate group is not known, nor has the reaction by which it is introduced into the polysaccharide molecules been demonstrated.

Bigger Pieces of the Puzzle

The heteropolysaccharides found in Nature are often (but not always) composed of small repeating subunits, containing two or more monosaccharides. The determination of their detailed structure, including the positions and anomeric configurations of the glycosidic linkages, therefore may be facilitated by depolymerization and isolation of small fragments that are more amenable to analysis than the intact macromolecules. Acid hydrolysis has for many years been a mainstay in the degradation of polysaccharides by chemical methodology and is, of course, generally applicable to the study of all polysaccharides. For example, acid hydrolysis of hyaluronan produces the disaccharide, hyalobiuronic acid, which is composed of glucuronic acid and glucosamine, linked by a ß1,3 linkage. Our knowledge of heparin structure is, likewise, based in part on the characterization of hydrolytic fragments generated from native or chemically modified heparin, and the results of this work have indicated that heparin is composed of alternating glucosamine and uronic acid residues. Interestingly, the hydrolysis pattern of heparin is different from that of hyaluronan, partly because most of the glucosamine amino groups in heparin carry an N-sulfate group, while those in hyaluronan are N-acetylated. The rapid removal of the N-sulfate groups upon acid hydrolysis and the ensuing protonation of the amino groups render the glucosaminidic linkage highly resistant to hydrolysis, and the heparin molecule, therefore, breaks preferentially at the uronidic linkages. As a consequence, the predominant disaccharide fragments generated from heparin are glucosaminyl-iduronic acid and glucosaminyl-glucuronic acid. This is in contrast to the hydrolysis of hyaluronan and other N-acetylated glycosaminoglycans, which proceeds by preferential cleavage of the N-acetylhexosaminidic linkages. The extensive studies of the disaccharides from native and chemically modified heparin are described in detail elsewhere (2,12,28,29).

By virtue of the presence of the unique N-sulfate groups in heparin, the polysaccharide may also be degraded by a chemical method which specifically targets these groups, i.e., deaminative cleavage by nitrous acid. Well known in organic chemistry as a reagent that converts a primary amine to the corresponding alcohol and free nitrogen, nitrous acid has also been found to react with unsubstituted as well as N-sulfated glucosamine amino groups. If the glucosamine is polymer-bound, as in heparin, the treatment with nitrous acid also results in cleavage of the adjacent glucosaminidic linkage and in the

conversion of the glucosamine to anhydromannose. Deaminative cleavage by nitrous acid, as used by the Birmingham group in England in the 1950s and subsequently in the laboratories of Cifonelli, Lindahl, and Conrad, has been the single most useful method for the generation of heparin disaccharides and larger fragments and has facilitated greatly the structural studies as well as metabolically oriented investigations (30-32). Recently, its application to the production of low-molecular-weight heparin has also opened up a new field in the therapeutic use of heparin (33). It should be noted that an obvious but important difference between acid hydrolysis and deaminative cleavage is that the latter procedure does not affect the O-sulfate groups and that the fragments obtained therefore represent the original structure of the polymer, albeit with anhydromannose at the reducing terminus instead of glucosamine.

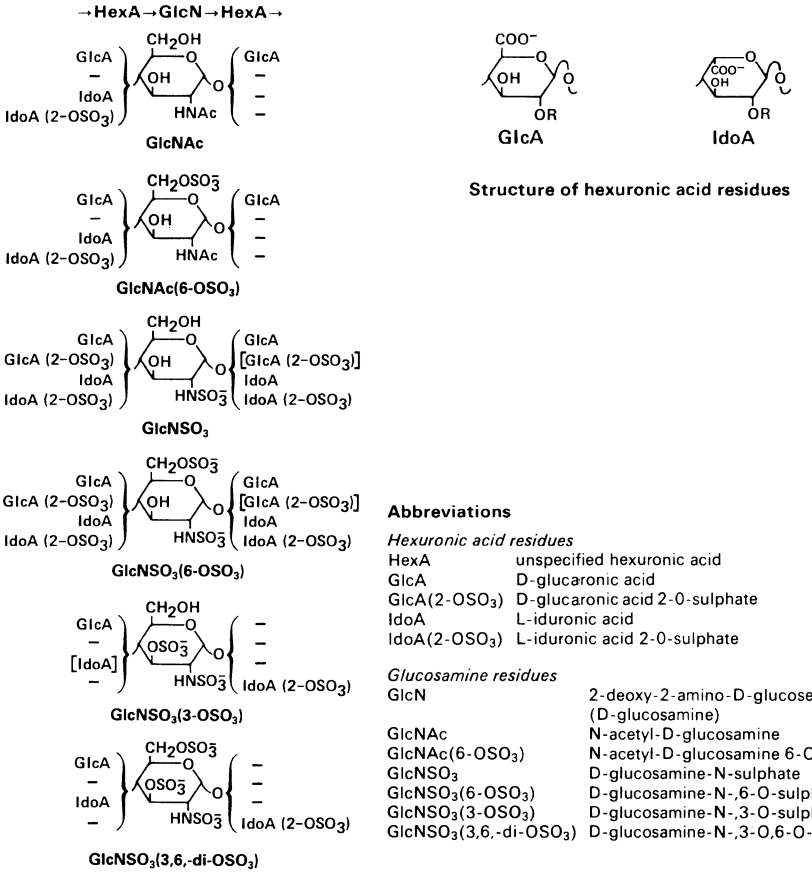
In addition to chemical methods of degradation, enzymatic depolymerization has also played an important role in the elucidation of the structures of mammalian polysaccharides. Testicular hyaluronidase cleaves the endo-hexosaminidic linkages of hyaluronan and the chondroitin sulfates to yield tetrasaccharides as the major products, and leech hyaluronidase specifically cleaves the endoglucuronidic linkages of hyaluronan, yielding "reverse" oligosaccharides with glucuronic acid at the reducing terminus. The study of heparin structure has, likewise, benefited from the use of degradative enzymes of mammalian as well as of bacterial origin. A host of exoenzymes are present in mammalian tissues, which, in concerted action, are capable of degrading the polymer to its constituents (34). In addition, an endoglycosidase has also been found that specifically attacks endoglucuronidic linkages in the heparin molecule and whose action results in the formation of relatively large heparin fragments with a reducing terminal glucuronic acid residue (34).

Bacterial endoglycosidases of three types are known, which all cleave glucosaminidic linkages in heparin by an eliminase reaction that yields a Δ4,5-unsaturated uronic acid residue at the nonreducing terminus of the pertinent fragment (34). The bacterial enzymes may, in a sense, be regarded as tools that are complementary to deaminative cleavage, since the glucosamine moiety participating in the reaction remains intact, while the uronic acid residue at the cleavage point undergoes a structural change. When needed, the unsaturated uronic acid may be removed by a short treatment with mercuric acetate at room temperature (35).

In summary, the several procedures described above - acid hydrolysis, deaminative cleavage, and degradation with mammalian and bacterial enzymes - have allowed heparin researchers to degrade the polysaccharide to fragments of various sizes - disaccharides and larger - which are amenable to structural analysis. Aided by a wide range of additional methods - e.g., methylation analysis, periodate oxidation, and NMR spectroscopy - they have succeeded in establishing the basic structural features of heparin, including its monosaccharide composition, the positions and configurations of the glycosidic linkages, and the locations of the sulfate groups (Fig. 1). This work has eventually led to a deeper insight into the molecular mechanisms of action of heparin as an anticoagulant, and this aspect of heparin biology will be discussed briefly in the following.

THE STRUCTURAL BASIS OF THE ANTICOAGULANT ACTION OF HEPARIN

In 1935, Jorpes (4) pointed out that heparin is the most highly charged polyanion in Nature, and this is probably still true. It was then reasonable



Abbreviations

Hexuronic acid residues

HexA	unspecified hexuronic acid
GlcA	D-glucaronic acid
GlcA(2-OSO_3)	D-glucaronic acid 2-0-sulphate
IdoA	L-iduronic acid
IdoA(2-OSO_3)	L-iduronic acid 2-0-sulphate

Glucosamine residues

GlcN	2-deoxy-2-amino-D-glucose (D-glucosamine)
GlcNAc	N-acetyl-D-glucosamine
GlcNAc(6-OSO_3)	N-acetyl-D-glucosamine 6-O-sulphate
$GlcNSO_3$	D-glucosamine-N-sulphate
$GlcNSO_3$(6-OSO_3)	D-glucosamine-N-,6-O-sulphate
$GlcNSO_3$(3-OSO_3)	D-glucosamine-N-,3-O-sulphate
$GlcNSO_3$(3,6,-di-OSO_3)	D-glucosamine-N-,3-O,6-O-sulphate

Fig. 1. Scheme of disaccharide sequences identified in heparin and heparan sulphate. The six variously substituted glucosamine residues in the middle (GlcN), are combined with the hexuronic acid units (HexA) at C4 and C1 to give 17 (possibly 18) different →HexA→GlcN→ sequences, and 10 (possibly 12) →GlcN→HexA→ sequences, respectively. The structures of the hexuronic acid residues are indicated in the upper right hand corner. Gaps indicated by (-) denote combinations that have not been found, either because they do not exist (e.g.→ GlcNAc→IdoA→), or because they occur very infrequently. Probable sequences, still to be uneqivocally demonstrated, are indicated by [] around the hexuronic acid concerned. Reproduced with permission of the author (U. Lindahl) and the publisher from "Heparin", D.A. Lane and U. Lindahl, eds., Edward Arnold, London (1989).

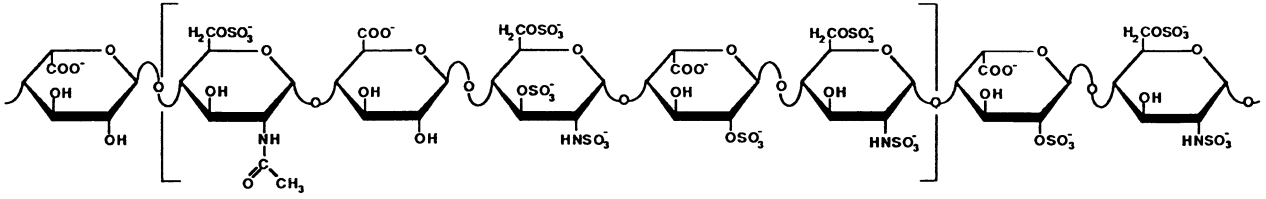


Fig. 2. Structure of a heparin octasaccharide sequence that displays most of the variously substituted monosaccharide components identified to date. The pentasaccharide sequence within brackets represents the antithrombin-binding region.

to assume that the anticoagulant activity of heparin was due, in part at least, to its high negative charge density, and this hypothesis was supported by the finding that chemical sulfation of neutral polysaccharides endows them with a measure of anticoagulant activity. A high negative charge density, however, is not the sole requirement for a high anticoagulant activity, and we now know that this property resides in a segment of specific structure in the heparin molecule. The first indication to this effect came in 1976, when Lam, Silbert, and Rosenberg (37) found that only a portion of the molecules in a standard heparin preparation interacted with antithrombin, as shown by centrifugation in a sucrose gradient. The same conclusion was reached by Höök, Björk, Hopwood, and Lindahl (38), who demonstrated that only about one third of a similar heparin preparation was bound strongly to an antithrombin-agarose column and that the anticoagulant activity was associated with this fraction of the material. Since the chemical composition, including the sulfate content, of the antithrombin-binding fraction did not differ substantially from that of the inactive material, it was apparent that charge density was not the only determining factor in the interaction between antithrombin and heparin. A search for the specific structural features responsible for the biological activity was then launched, which culminated in the discovery by Lindahl and collaborators (39,40) that a sulfate group is present at C-3 of a glucosamine unit in the antithrombin-binding segment of the molecule (Fig. 2).

Through careful dissection of the heparin molecule in Lindahl's laboratory, it is now well established that the antithrombin-binding segment of heparin is a sulfated pentasaccharide of unique structure, in which not only the 3-O-sulfate group but also other sulfate residues are essential for the biological activity (40,41). The analytical studies have been complemented, in an impressive manner, with chemical synthesis of the antithrombin-binding pentasaccharide and other relevant oligosaccharides (42), and the chemical manipulation of the pertinent structures has set the stage for detailed conformational studies of the interaction between antithrombin and heparin. A description of the results to date is beyond the scope of this presentation, and reference is made to other articles (40,43) and works cited therein.

BIOSYNTHESIS OF HEPARIN

In the earliest studies of heparin biosynthesis - where credit goes particularly to Jeremiah Silbert - the formation of a nonsulfated polysaccharide was shown to occur when the microsomal fraction of a mouse mastocytoma was incubated with UDP-glucuronic acid and UDP-N-acetylglucosamine (44-47). Although this polysaccharide had the same charge density and, presumably, the same composition as hyaluronan, it was not degraded by testicular hyaluronidase. Instead, its susceptibility to digestion with heparinase from *Flavobacterium heparinum* indicated that it was related to heparin. In the presence of 3'-phosphoadenylylsulfate (PAPS), a sulfated polysaccharide was formed, which was similar to heparin in its charge density and contained N-sulfate groups. The uronic acid composition of the polysaccharide products was not determined, and at the time there seemed to be no particular reason to do so, since it was well known that the uronic acid component of heparin was D-glucuronic acid. (Even in 1967 (45-47) it was not yet known that the predominant uronic acid is L-iduronic acid.)

Following Silbert's pioneering investigations, Lindahl and his collaborators adopted a similar experimental system, using the Furth mastocytoma rather than the Dunn-Potter tumor, and conducted a series of elegant studies that have led to our current picture of the biosynthetic process (36,48-51).