

REVIEW ARTICLE

Heparinome routes toward novel drugs

Ulf Lindahl* and Lena Kjellén

Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Abstract

Heparin, a sulfated glycosaminoglycan composed of glucosamine and hexuronic acids in alternating sequence, binds a myriad of proteins collectively termed the “heparinome,” mainly through electrostatic interactions. Heparin prevents blood coagulation and is clinically applied to prevent thrombosis. The more sparsely sulfated polysaccharide heparan sulfate binds heparinome proteins more selectively, thereby regulating development, homeostasis, and disease. Biosynthesis of heparan sulfate yields products of variable, but controlled average composition. Dysregulation of heparinome interactions occurs in diseases, including cancer, inflammatory conditions, and amyloid diseases. Established structure–function relations provide a basis for drug development. The discovery of a specific pentasaccharide sequence in heparin, containing a rare glucosamine 3-O-sulfate group essential for anticoagulant activity, facilitated the development of various low-molecular-weight heparins with favorable pharmacokinetic properties. The 3-O-sulfate group has since been implicated in diverse aspects of heparan sulfate function. Heparin derivatives lacking anticoagulant activity can be administered at higher doses. Non-anticoagulant effects of heparin are increasingly exploited in the clinic, for instance, to curb the “cytokine storm” in severely ill COVID-19 patients. Other applications, such as in cancer and inflammatory conditions, are suggested by various incidental beneficial effects observed during heparin treatment for thromboembolic disease. Such effects are likely due to displacement of regulatory proteins from heparan sulfate proteoglycans at epithelial surfaces, or alternatively to inhibition of heparanase, a heparan sulfate-degrading enzyme. Exploitation in such cases would involve identification of key protein mediators followed by generation of minimally sulfated oligosaccharide drug candidates (or mimetics) with selective binding properties.

***Corresponding author:**Ulf Lindahl
(ulf.lindahl@imbim.uu.se)

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1. Introduction

The sulfated polysaccharide heparin is best known for its anticoagulation activity and is still a drug of choice for postoperative thromboprophylaxis. It is commercially isolated from animal sources, primarily (but not exclusively) pig intestinal mucosa. In the body, heparin is found in mast-cell granules where it interacts with histamine, proteases, and inflammatory mediators.¹ Contrary to heparin, the structurally related glycosaminoglycan, heparan sulfate (HS), occurs in virtually all mammalian cells.² Both heparin and HS form parts of proteoglycans (PGs), where the polysaccharide chains are covalently bound to core proteins. Heparin is attached to the serglycin core protein,

which also occurs in many other cell types, including hematopoietic and endothelial cells. However, in these cells, serglycin is substituted with chondroitin sulfate, another member of the glycosaminoglycan family. HS is attached to a variety of core proteins that are either secreted into the extracellular domain or associated with the cell surface. The pathophysiological feature common to heparin and HS is their ability to bind and thereby influence the functions of a large number of proteins, collectively denoted the “heparinome” or “heparin interactome.”^{3,4} Due mainly to the spatial arrangement of basic amino acid units, these proteins interact with negatively charged sulfate residues in HS chains, with variable selectivity. Highly sulfated heparin can bind a broad spectrum of heparinome proteins. The members of this group, with widely differing properties, include enzymes/inhibitors, growth factors, morphogens and their receptors, matrix proteins, and others, along with proteins of established pathophysiological roles such as chemokines, selectins, and viral coat proteins.⁵⁻¹⁰ This review discusses possibilities for further drug generation based on heparinome information, including accidental findings of unexpected beneficial effects of heparin treatment on diseases not primarily due to thromboembolism.

2. Structure and biosynthesis

Figure 1 shows the prevalent notion of heparin structure in the early 1960s, a homogeneous polymer of alternating 2-*O*-sulfated *D*-glucuronic acid (GlcA2S) and *N*,6-*O*-disulfated *D*-glucosamine (GlcNS6S) residues joined by α 1–4 bonds.

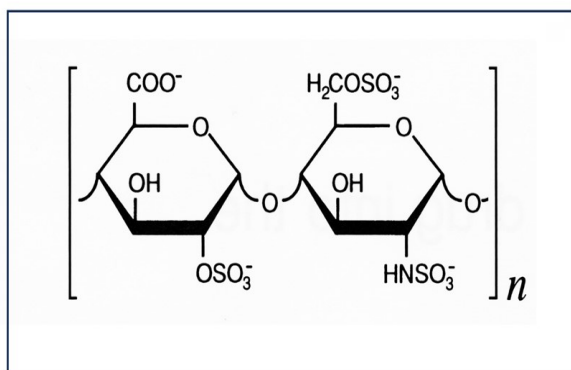


Figure 1. Heparin in the 1960s

However, subsequent studies revealed that GlcA residues are relatively rare and are β -linked rather than α -linked and, that L-iduronic acid is the predominant *O*-sulfated hexuronic acid unit (IdoA2S). Moreover, the GlcN residues are occasionally *N*-acetylated (GlcNAc) and not exclusively *N*-sulfated (Figure 2, lower structure). The partial heterogeneity of heparin can be explained by its

biosynthesis, which involves the concerted, stepwise, but incomplete action of multiple enzymes (Figure 2).

HS, initially considered a “heparin side fraction” but later recognized as a distinct glycosaminoglycan species, is generated by the same families of enzymes, acting on the same $[\text{GlcA}\beta 1\text{--}4\text{GlcNAc}\alpha 1\text{--}4]_n$ precursor polymer (Figure 2, upper structure). However, HS is less extensively modified than heparin and contains domains of variable sequence and sulfate density.^{5,11} Evidence indicating strictly regulated polymer modification was obtained through structural analysis of HS from different murine tissues (Figure 3).¹² These results demonstrated distinct average distributions of variously modified sugar residues, depending on cellular origin. Yet the lack of precise sequence conformity of any HS preparation, no matter how purified, points to an element of stochasticity. There is no indication of “template” control or any “sulfation code.” Instead, the “GAGosome” concept^{13,14} predicts complexes of biosynthesis enzymes interacting with each other and with their substrates, in potentially regulatory mode,¹⁵⁻¹⁹ such that the relative amounts of the different enzymes may determine the outcome of biosynthesis. Recent findings implicating complexes of interacting enzymes in the control of HS chain length are summarized in a recent review.¹¹ Seemingly incompatible observations need to be rationalized, such as the apparent rapidity of the overall polymer-modification process, completed in less than 30 s for a single heparin chain,²⁰ as contrasted by the apparent inability of the two major *O*-sulfotransferase reactions to operate simultaneously.^{21,22} Further studies along these lines will hopefully broaden our understanding of the role of enzyme interactions in the overall regulation of HS/heparin biosynthesis. More detailed information regarding the biosynthesis processes is found in Refs.^{21,23}

3. Heparinome interactions

Biological “activities” of heparin/HS saccharides, in the broad context of this review, are generally due to interactions with heparinome proteins. The members of this group, with widely differing properties, include enzymes/inhibitors, growth factors, morphogens and their receptors, matrix proteins, and others, along with proteins of established pathophysiological roles such as chemokines, selectins, and viral coat proteins.⁵⁻¹⁰ Notably, whereas mast-cell heparin generally has a broader interactive capacity than HS (Section 3.2), the actual number of endogenous target proteins is restricted compared to HS, with its wide distribution throughout the body. HS/protein interactions with defined roles in normal homeostasis can be exploited to entirely different ends in disease. Some distinctive features of the protein-binding saccharide domains are notable.

3.1. Interactions involving “rare” sugar units

The protein-binding saccharide domains mostly consist of fewer than 10 sugar units, generally involving a combination of the “common” residues (GlcA, IdoA, IdoA2S, GlcNAc, GlcNS, GlcNAc6S, GlcNS6S) (Figure 2). An increasing number of interactions are shown to depend on rarely occurring 3-*O*-sulfated GlcNS units, as outlined below. Other rare sugar residues in HS are *N*-unsubstituted GlcN and 2-*O*-sulfated GlcA units, structures essentially in search of specific functional roles.

3.1.1. Anticoagulant activity

Elucidation of the structural basis behind the blood anticoagulation effect of heparin led to the first demonstration of a highly specific interaction with a target protein. Heparin was introduced in the clinic as an antithrombotic drug in the 1930s,^{24,25} at a time when no mechanistic information was available. Emerging awareness of an essential “heparin cofactor” protein in plasma led to the identification of AT,²⁶ a powerful inhibitor of serine proteinases involved in the blood coagulation

process. The binding of AT to heparin was shown to greatly accelerate proteinase inhibition, leading to the formation of AT-proteinase complexes devoid of procoagulant activity (Figure 4). The complexes are released from heparin, which can then activate new AT molecules in a catalytic fashion.^{27,28}

Only a fraction of commercial heparin (unfractionated heparin [UFH]) binds AT with high affinity, and accounts for essentially all anticoagulant activity of the starting material.^{29–31} This fraction was found to contain a pentasaccharide sequence with a previously undiscovered 3-*O*-sulfated GlcNS residue (Figure 4).^{32,33} The GlcNS3S unit was found to be essential for AT binding, hence for anticoagulant activity, along with the “common” units of the pentasaccharide sequence.

Interestingly, computational sorting of a virtual HS hexasaccharide library, involving > 46,000 structures, identified a sequence containing two rare consecutive GlcA2S-containing disaccharide units, associated with a high probability for interaction with heparin cofactor II.

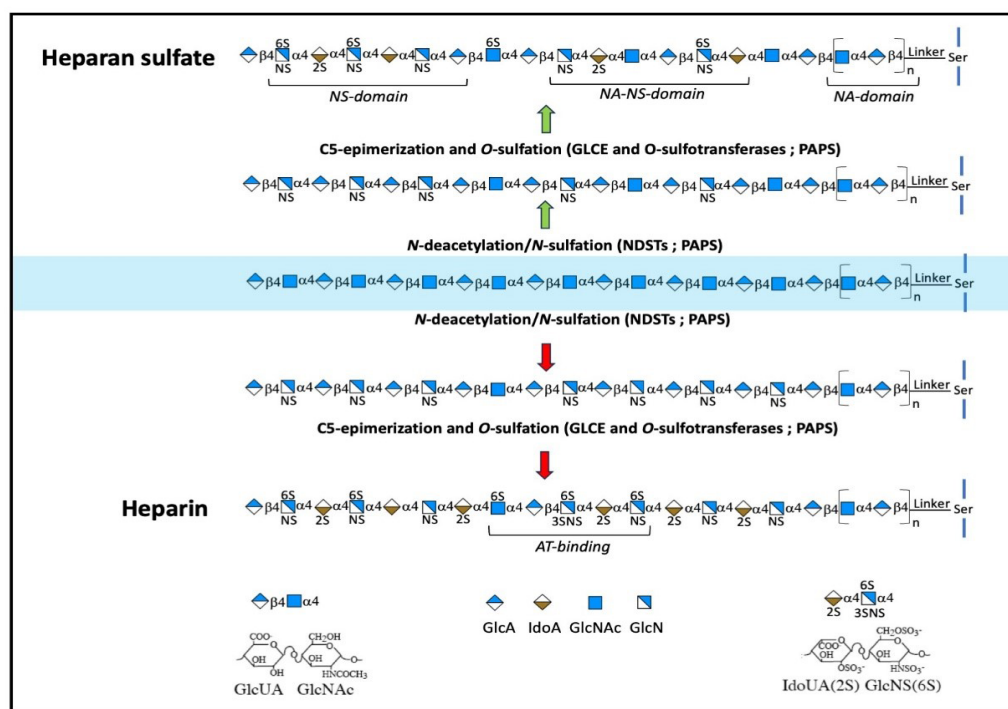


Figure 2. Biosynthesis of heparin and heparan sulfate. A precursor polymer of alternating GlcA and GlcNAc units is modified into heparin (arrows downward) or HS (arrows upward). The first modification reaction involves GlcNAc *N*-deacetylation/*N*-sulfation catalyzed by *N*-deacetylase/*N*-sulfotransferases (NDSTs). C5-epimerization of GlcA to IdoA residues is followed by 2-*O*-sulfation of IdoA and a few GlcA units. Lastly, GlcNAc/NS residues undergo 6-*O*- and, to a limited extent, 3-*O*-sulfation. Many of the enzymes occur in several isoforms, present in varying amounts in different cell types. Mammals express four NDSTs, three 6-*O*-sulfotransferases, and seven 3-*O*-sulfotransferases, while single genes encode the C5-epimerase (*GLCE*) and the 2-*O*-sulfotransferase. All sugar units shown in this scheme will be referred to as “common” units, except the “rare” 3-*O*-sulfated GlcNS unit (here, in the antithrombin [AT])-binding region of heparin; see also Section 3.1).

Abbreviations: AT-binding: antithrombin-binding; *GLCE*: Glucuronyl C5-epimerase; NDSTs: *N*-deacetylase/*N*-sulfotransferases; PAPS: Sulfate donor 3'-phosphoadenosine 5'-phosphosulfate.

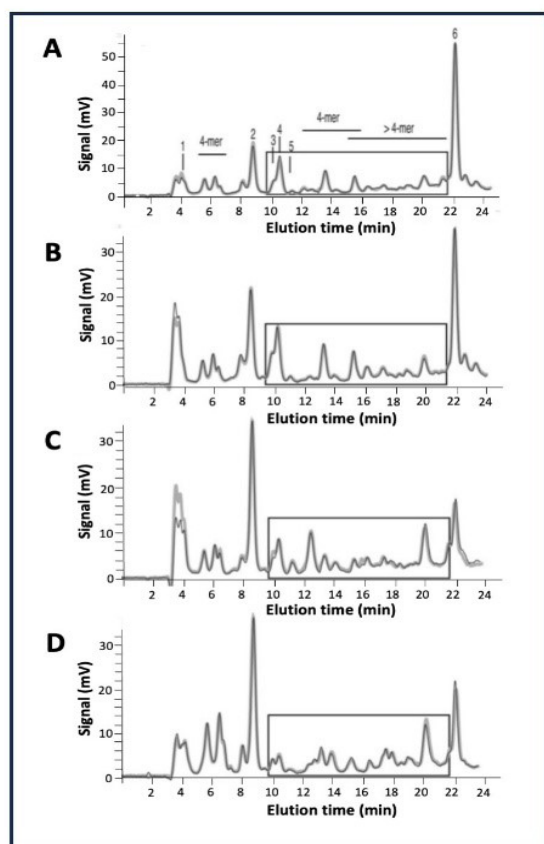


Figure 3. Fingerprints of heparan sulfate from different tissues of two essentially syngeneic mice. The experiment was initiated by the finding that N-sulfation patterns were virtually unaffected by deletion of the *Ndst2* gene; what about other structural parameters? HS isolated from the liver (A), kidney (B), lung (C), and bladder (D) of a wild type mouse and its *Ndst2*^{-/-} sibling were degraded by deamination (cleavage at N-sulfated GlcN residues), and the resultant oligosaccharides, differing with regard to size, hexuronic acid composition, and O-sulfate distribution, were fractionated by reversed phase ion-pair high-performance liquid chromatography. Each pattern contains superimposed images of the chromatograms from the two siblings, demonstrating close structural similarity within each tissue, in contrast to the distinct patterns observed between tissues. Notably, each pattern represents a mixture of HS species from different cells that occur in essentially fixed proportions in the tissue analyzed, and thus indicates average composition (see Section 2). Presumably, each cell type within the tissue contributes its own unique mixture of HS structures. How such mixtures are affected by variable external stimuli, deliberately avoided in the illustrated experimental setting, is a key question for future research. The boxed regions in the figure were in the original publication¹² enlarged and displayed together with the chromatograms shown here.

Like the structurally related AT, this serpin occurs at high levels in human plasma and targets thrombin but, contrary to AT, not factor Xa. Testing of the actual hexasaccharide, generated by chemical synthesis, revealed a ~250-fold activation of heparin cofactor II, as contrasted to virtually no activation of AT.³⁴ To our knowledge, no such structure has yet been identified in authentic HS.

3.1.2. Other effects related to the 3-O-sulfate group

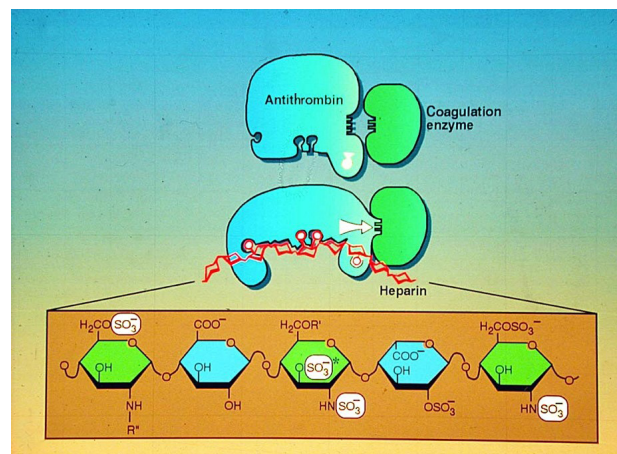


Figure 4. Structure and mode of action of the AT-binding pentasaccharide sequence in heparin. R', -H or -SO₃²⁻; R'', -SO₃²⁻ or -COCH₃. The binding of the pentasaccharide activates the serine proteinase inhibitor AT, resulting in enhanced inactivation of several enzymes required for thrombin generation, the final step of blood coagulation.

Notes: *indicates rare GlcNS3S structure; the arrow indicates the glycol structure of a GlcA residue susceptible to cleavage by periodate oxidation. The encircled sulfate groups are all critical for AT-binding.

Following the discovery of the 3-O-sulfate group in AT-binding heparin, attempts to isolate and genetically define the relevant 3-O-sulfotransferase revealed no fewer than seven distinct isoforms,³⁵ which in turn prompted a search for additional 3-O-sulfated HS sequences with novel functions.³⁶ Resulting findings could be associated with distinct HS species and cells of origin. Functional properties associated with 3-O-sulfate-substituted HS sequences varied widely, and included co-receptor roles for viral entry into host cells, regulation of neural and salivary gland development, enhancement of tau internalization into cells, and others.³⁷ Notably, the 3-O-sulfation of a GlcNS residue surrounded on both sides by IdoA units has been implicated in the control of HS chain conformation, promoting change from linear to compact topologies.³⁸ A synthetic approach toward the production of structurally diverse HS oligosaccharides with and without 3-O-sulfate groups was employed to prepare 27 hexasaccharides that were printed as a glycan microarray to examine ligand requirements of a wide range of HS-binding proteins.³⁹

3.2. Interactions based on “common” sugar units

The multitude of HS populations suggests that many or most of the heparinome proteins bind to more than one HS motif, as actually demonstrated for members of the fibroblast growth factor family (Figure 5A).^{40,41} Is the regulation of HS biosynthesis, at the same time strict

and nebulous (Section 2), designed to enable controlled, graded modulation of protein binding and resultant functional responses? This futuristic concept, yet to be experimentally verified, was elaborated in a recent review¹¹ and is illustrated in Figure 5B. Two conjectured proteins interact with two slightly different HS sequences, with inverse relative affinities. Both HS sequences express patterns of sulfate groups involved in interaction with target basic amino-acid residues, along with redundant (but tolerated) groups. Subtle shifts in the proportions of 2-O- and 6-O-sulfation will modulate protein binding preferences. A “critical” sequence, involving only residues indispensable for binding of a particular protein ligand, is rarely created as such but rather as part of more extensive polymer modification. Figure 5C illustrates how variably generated HS sequences might regulate selective, graded modulation of protein binding in control of distinctly shaped gradients of two morphogens along differentiating cells. Importantly, HS-interacting proteins generally bind heparin with high affinity (Figure 5B), hence the term “hidden sequence dependence” to indicate that spatially accessible critical groups are obscured by redundant sulfate groups.^{11,42} The phenomenon is sometimes, in an oversimplified manner, referred to as “nonspecific charge interaction.”

Special structural conditions apply when a single HS chain interacts with two distinct polypeptide sites, located either on the same protein (for example, endostatin) or on two distinct proteins (AT and thrombin). Several examples of such interactions, involving two separate NS-domains, have been described in a previous review.⁴²

4. Heparanase, an endogenous modulator of heparinome activities

Studies on heparin metabolism in neoplastic murine mast cells showed rapid degradation of the initial biosynthesis product, the >900 kDa PG serglycin, to ~15 kDa fragments (UFH size).⁴³ A glycosidase involved, initially termed “heparinase,” was identified as an endo- β -glucuronidase.⁴⁴ Subsequent studies showed that the enzyme is not restricted to mast cells but occurs in various cell types, with HS as its primary target. It was renamed heparanase (HSase), cloned, and found to have a profound influence on HS metabolism and pathophysiology.⁴⁵ The following examples illustrate the diverse pathophysiological roles of HSase.

4.1. Cancer

HSase expression is increased in virtually all types of cancer, and the enzyme has emerged as a major regulator of cancer, inflammation, and other disease processes. HSase

accomplishes this by degrading HS chains in control of the abundance and location of HS-binding growth factors and cytokines, thereby influencing multiple signaling pathways that regulate cell behavior.^{46,47} Furthermore, passage of cells from blood into the extravascular compartment is facilitated by HSase-mediated degradation of basement membrane HS.⁴⁸ In contrast to HSase, which generally promotes cancer progression, the close homolog HSase-2 lacks enzymatic activity and instead promotes cellular processes that together result in tumor suppression.⁴⁹

4.2. Appetite regulation

The heparan sulfate proteoglycan (HSPG) syndecan-3 serves as a co-receptor for binding of agouti-related peptide (AgRP) to the dual-acting, hypothalamic melanocortin receptor MC4R, resulting in appetite stimulation (Figure 6A, upper panel).⁵⁰ Interaction is normally interrupted by satiety-induced protease cleavage of the syndecan-3 core protein, leading to the release of AgRP and instead binding of α -melanocyte-stimulating hormone (α -MSH), and appetite suppression. The role of HSase in the regulation of food intake, body weight, and body composition was studied in genetically modified mice that either lacked or overexpressed the enzyme.⁵¹ HSase knockout mice showed increased food intake and maturity-onset obesity, with up to a 40% increase in body fat. By contrast, mice overexpressing HSase showed the opposite phenotype essentially, with reduced fat mass, indicating increased α -MSH signaling (Figure 6A, lower panel). These results implicate HSase in energy balance control via the central melanocortin system, acting as a negative modulator of AgRP signaling at MC4R, through cleavage of HS chains presumably linked to syndecan-3. Would it be possible to design an appetite-reducing HS oligosaccharide (or mimetic) that efficiently binds AgRP and prevents its interaction with syndecan-3-bound HS?

4.3. Amyloidosis

Amyloidosis is characterized by tissue deposition of proteins prone to aggregation into fibrillar, insoluble forms. This group of serious diseases is characterized by structurally unrelated amyloid proteins that share the ability to bind HS.⁵²⁻⁵⁴ The constant occurrence of HSPGs in amyloid deposits is ascribed to the ability of the constituent HS chains to promote fibrillogenesis (Figure 6B, upper panel). The importance of HS chains in this process was underpinned in studies of inflammation-related amyloid A amyloidosis in HSase-overexpressing mice, which showed dramatic attenuation of amyloid deposition in enzyme-expressing organs (Figure 6B, lower panel).⁵⁵ These findings suggest that HS oligosaccharides, designed to interact with the appropriate amyloid mono- or oligomers, may

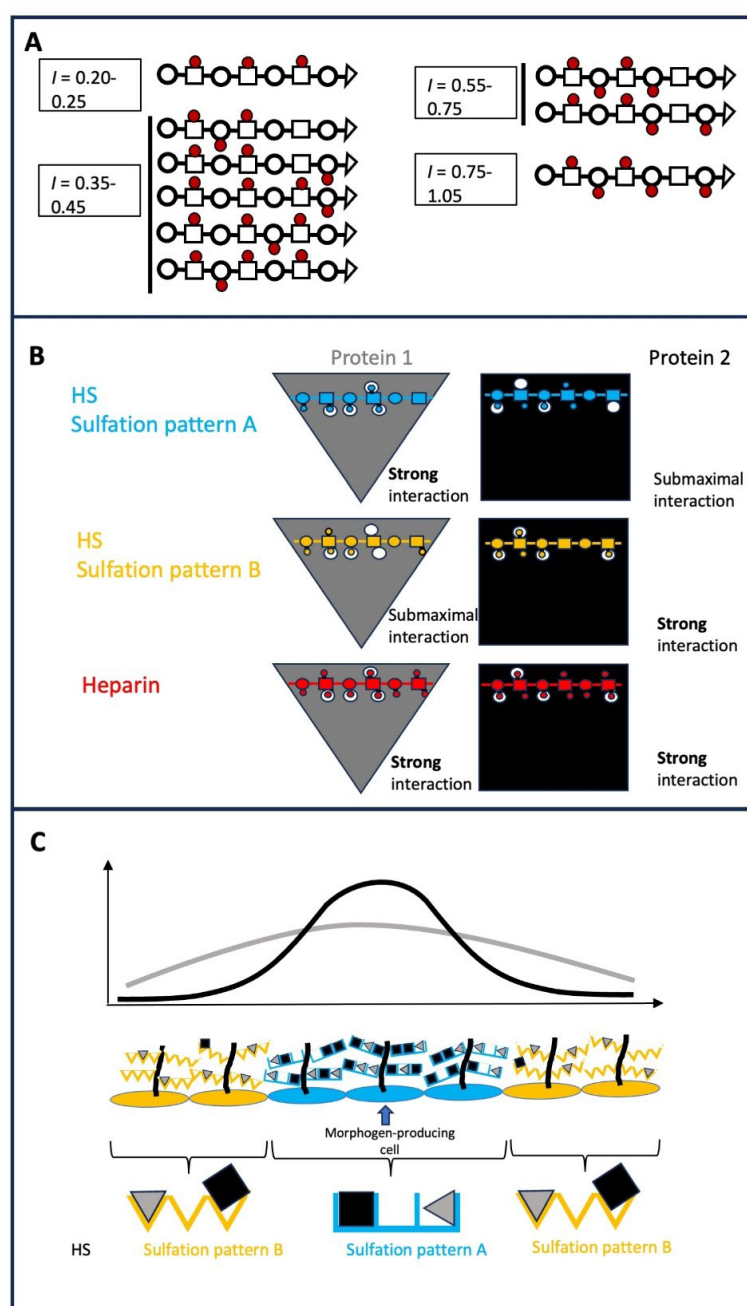


Figure 5. Modulation of protein binding preferences by subtle changes in heparan sulfate (HS) structure. (A) Structures of *in vitro*-generated HS-like octasaccharides recovered by salt-elution (ionic strength of eluted fractions indicated by *I* values) from immobilized fibroblast growth factor (FGF) 1. The growth factor was challenged with a radiolabeled octasaccharide library generated by partial enzymatic *O*-sulfation of partially *O*-desulfated heparin octamers. Fractions recovered at low ionic strength, yet higher than physiological, from a corresponding experiment with FGF-2 displayed structures (not shown) largely deviating from those eluted off immobilized FGF-1, thus reflecting different requirements for *O*-sulfate groups in growth factor binding.⁴⁰ Notes: Void circles: IdoA units; Squares: GlcNS residues; Red-filled circles: IdoA2S or GlcN6S *O*-sulfate groups. (B) Two hypothetical proteins (grey and black) interact with two distinct HS domains (blue and yellow) of slightly different composition. White circles indicate binding sites in the proteins for sulfate groups conducive to interaction. Four sulfate groups of the blue HS domain interact with the grey protein, resulting in a strong interaction, whereas binding to the black protein engages only two sulfate groups. Conversely, the yellow HS domain binds more strongly to the black than to the grey protein. The extensively sulfated, red heparin domain binds strongly to both proteins. (C) The HS domains presented in (B) are used to illustrate how differential HS binding might affect the formation of morphogen gradients along an endothelial surface, generated by two proteins produced by the central cell. This cell and its closest neighbors synthesize "blue" HS, whereas the more distant cells produce "yellow" HS.

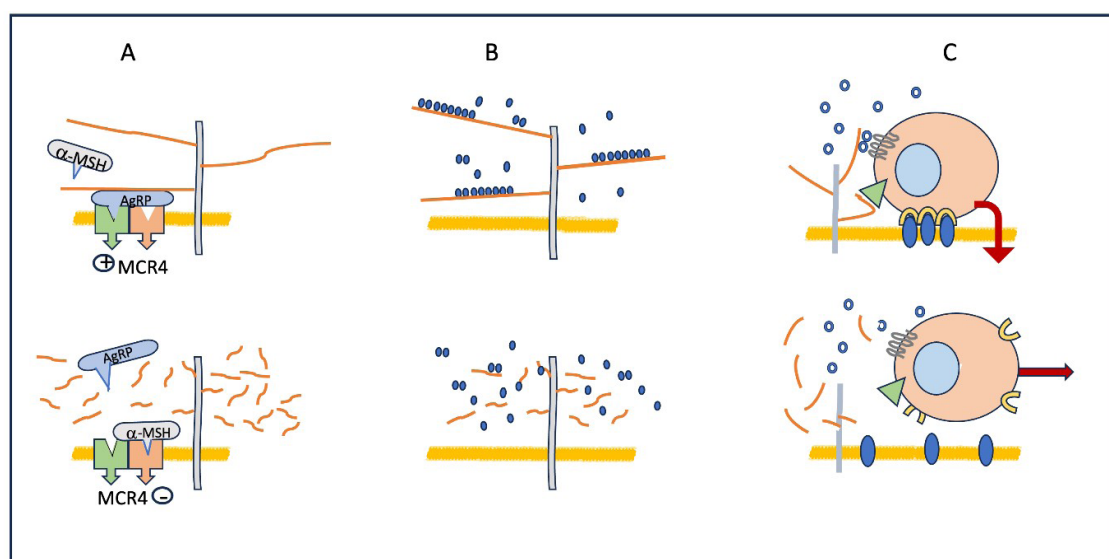


Figure 6. Selected examples of pathophysiological effects of HSase action. (A) Role of HSase in appetite regulation. The dual-acting hypothalamic receptor, MC4R, is controlled by two proteins: AgRP, which stimulates, and α-MSH, which depresses appetite. The binding of AgRP to the receptor requires interaction with a proteoglycan-bound heparan sulfate (HS) chain. The bound AgRP blocks access of α-MSH to the receptor (upper panel). Fragmentation of the HS chain by HSase releases AgRP and allows receptor binding of α-MSH (lower panel). (B) HS chains bind amyloid mono- and oligomers, thereby promoting amyloid fibrillization (upper panel). Fragmentation of HS prevents fibril formation (lower panel). (C) Anti-inflammatory effect of HSase in the brain. The upper panel illustrates tethering of a monocyte to the vascular endothelium through interaction between L-selectin (green triangle) and immobilized HS. Cytokines released by inflammatory stimulus bind to HS and activate a specific receptor (CCR2), resulting in monocyte adhesion to endothelium through interaction of endothelial ICAM-1 with monocyte ICAM-1 receptors. The monocytes then transmigrate through the endothelial barrier into the extravascular domain, where they differentiate into activated macrophages. Fragmentation of HS by HSase disrupts the initial phases of this process (lower panel), thereby preventing monocyte diapedesis and attenuating neuroinflammation.

have therapeutic potential. Is the noted delayed onset of Alzheimer's disease associated with heparin therapy⁵⁶ due to displacement of aggregating amyloid from endogenous HS?

4.4. Inflammation

HSase is strongly upregulated in various inflammatory diseases.⁵⁷ The enzyme regulates gene expression, activates immune cells, and stimulates signal transduction pathways via catalytic and non-catalytic activities, effects that together drive inflammatory responses. However, there is no universal mechanistic role for HSase in inflammatory conditions. Overexpression of HSase thus attenuated neuroinflammation induced either by lipopolysaccharide stimulation or by localized cerebral microinjection of amyloid-β aggregates in mice.⁵⁸ The neuroinflammation normally involves transfer of generated HS-bound chemokines to the luminal side of microvessels, where they stimulate monocytes to exit the vessel and transform into macrophages (Figure 6C, upper panel). HSase overexpression resulted in degradation of HS chains, perturbation of monocyte diapedesis (Figure 6C, lower panel), and attenuation of neuroinflammation and macrophage-mediated amyloid degradation. Notably, this

effect of HSase on inflammation has so far been observed only in the brain. More generally, the potential effects of administered heparin-related oligosaccharides on cellular diapedesis should be considered, particularly their capacity to disrupt the HS-protein interactions involved.

5. Heparinome-related drugs

A number of drugs, either well-established in clinical practice or under development, are based on structural information pertaining to the AT-binding pentasaccharide sequence of heparin. More recent attention is devoted to oligosaccharide constructs, specifically tailored to provide optimal interaction with selected protein ligands.

5.1. Heparin-derived drugs with anticoagulant activity

5.1.1. Low-molecular-weight heparin

Heparin is commercially isolated from animal sources, primarily (but not exclusively) pig intestinal mucosa. Current annual production exceeds 100 tons and requires over a billion pigs. The polydisperse primary product averages $\sim 15 \times 10^3$ Da, corresponding to ~ 50 monosaccharide units (commonly referred to as UFH).

From the outset, both the treatment of thrombosis and thromboprophylactic use were fraught with problems, particularly with dose selection, as underdosing increased thrombotic risk, whereas overdosing increased bleeding risk. Protamine sulfate, a basic protein with high affinity for heparin, was introduced as a means of instantaneously neutralizing overdosed heparin.⁵⁹

Attempts to develop a heparin product of reduced chain length were facilitated by the discovery of the specific AT-binding pentasaccharide sequence (Section 3.1.1). Random, partial chemical cleavage using nitrous acid generated a low-molecular-weight heparin (LMWH), termed dalteparin, with an average $\sim 4\text{--}5 \times 10^3$ Da.⁶⁰ The anticoagulant properties were essentially similar to those of UFH, except for a size-dependent change in target spectrum, particularly favoring factor Xa inhibition.⁶¹ Significant functional differences compared to UFH included high bioavailability after subcutaneous injection (around 90%), increased plasma half-life (~ 4 h compared to ~ 2 h for UFH), and predictable dose/response relations.⁶² Together, these properties led to the rapid replacement of UFH with LMWH and rationalized clinical practice. Other LMWH products (ardeparin, parnaparin, enoxaparin, tinzaparin) were introduced, based on alternative depolymerization techniques and with essentially similar clinical properties.

5.1.2. Fondaparinux

The AT-binding pentasaccharide was initially recovered as part of larger oligosaccharides³³ following partial deaminative cleavage of UFH, but was also generated by chemical synthesis, successively optimized into a commercial drug, fondaparinux (Arixtra), with distinct anti-factor Xa activity.^{63,64} Arixtra offered excellent bioavailability and dose–response prediction, in addition to eliminating the potential risk of animal production sources, but also raised novel problems. While plasma half-life following subcutaneous injection of Arixtra was further increased compared to LMWH, the reduced polyanionic properties were found to largely preclude neutralization by protamine.⁶⁵ The diverging half-lives were explained by the combined effects of two elimination routes for polyanions, one involving a receptor on liver endothelial cells featuring increasing uptake efficiency with increasing polyanion size. This mechanism is balanced by filtration through the kidneys.^{66,67}

5.2. Heparin-derived drugs devoid of anticoagulant activity

5.2.1. Tafoxiparin, an obstetrical drug candidate

Long-term treatment of pregnant women with therapeutic doses of LMWH to prevent thromboembolic disease

was, unexpectedly, found to shorten delivery time.⁶⁸ Anticoagulant activity is undesirable in the obstetric setting, which prompted the development of an anticoagulant-deactivated product suitable for obstetric treatment. Key to this process was the structure of the AT-binding pentasaccharide sequence in heparin, containing a GlcA residue with a 2,3-glycol structure amenable to periodate oxidation and subsequent chain cleavage (Figure 4). The product, tafoxiparin, was found to increase oxytocin-induced myometrial contractile activity of uterine biopsies in an *in vitro* contractility assay. Furthermore, tafoxiparin increased the secretion of interleukin-8 in a primary cervical cell culture, pointing to a possible effect in the inflammatory reaction required for the cervical ripening process.^{69,70} Phase II studies indicated that subcutaneous treatment with tafoxiparin of term pregnant women planned for labor induction resulted in both cervical ripening and onset of labor. Tafoxiparin acts by a new, but not fully understood, safe mechanism without fetal or maternal side effects.⁷¹ The drug is being advanced toward phase III clinical evaluation.

5.2.2. Sevuparin for treatment of malaria

Malaria caused by *Plasmodium falciparum* is particularly serious in children, often manifested by cerebral ischemia associated with high mortality. The excessive binding of parasite-infected erythrocytes to the vascular endothelium (cytoadherence) and to other erythrocytes (rosetting) was therefore initially interpreted as a thrombotic disorder for which heparin might be beneficial.⁷² Such treatment was frequently complicated by intracranial bleeding and was therefore abandoned. More recent work revealed that the condition, rather than a coagulopathy, is due to binding of PfEMP1, a parasite-encoded erythrocyte membrane protein, to host receptors on endothelial and red blood cells.⁷³ These receptors were identified as cell-surface HS chains, and the effect of heparin treatment was thus ascribed to interference with PfEMP1-HS interaction (Figure 7).⁷⁴ The binding of PfEMP1 was shown to require ≥ 12 -mer highly sulfated heparin sequences but no AT-binding domain. This information was more recently exploited in the generation of sevuparin, another LMWH devoid of anticoagulant activity. A phase I/II study involving short intravenous infusions to patients with uncomplicated *P. falciparum* malaria showed transient de-sequestration of infected erythrocytes.⁷⁵

5.2.3. Heparanase inhibitors for cancer treatment

Heparin inhibits HSase enzymatic activity through binding to the HS-binding domain of the enzyme.⁷⁶ The effect depends on a high degree of sulfate substitution, but not on anticoagulant activity. This information has led to extensive

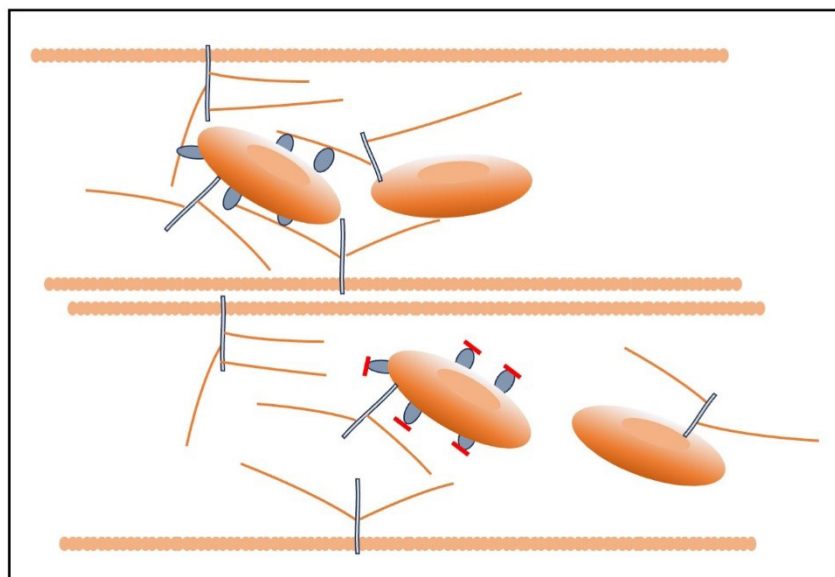


Figure 7. Cytoadherence and rosetting of erythrocytes due to interactions of vascular heparan sulfate (HS). Erythrocytes infected with *Plasmodium falciparum* express surface-attached PfEMP1 protein (blue ellipsoids) that interacts with HS chains on heparan sulfate proteoglycans located on other erythrocytes (rosetting) or on the vascular endothelium (cytoadherence; upper panel). Heparin fragments, devoid of anticoagulant activity, displace HS by competitive binding, thereby precluding cell aggregation (lower panel).

attempts to generate HSase inhibitors of different kinds, in particular, various heparin mimetics, polysaccharides, but also oligosaccharides, generally sulfated but in some cases based on glycol backbones unrelated to heparin.^{77,78} The inhibitors are primarily of interest as cancer therapeutics, due to the promoting effect on tumor invasion and metastasis of HSase-mediated degradation of HS in the extracellular matrix. In addition, heparin-related structures may attenuate various selectin-mediated interactions between cancer cells and platelets, endothelium, and leukocytes.⁷⁹ Various anti-HSase antibodies have also been applied. A word of caution is warranted considering the unexpected beneficial effects of HSase in certain clinical scenarios (for instance, the inhibitory effect of HSase on amyloid fibril generation illustrated in Figure 6B). Can context-dependent modulation become a realistic alternative to overall systemic inhibition of HSase activity?

5.3. Combined anticoagulant and non-anticoagulant effects

Multifactorial effects of heparin were recently exploited in connection with the COVID-19 pandemic. One of the well-known complications of this disease is acute lung injury, caused by the virus itself and virus-induced inflammation. During the COVID-19 pandemic, LMWH was widely used in hospitalized and critically ill patients to reduce thrombotic complications and potentially modulate

inflammatory responses (the “cytokine storm” that often precedes the development of pulmonary microthrombi and vascular leakage). The involvement of proteins bound to cell-surface HS is striking.⁸⁰ Anti-inflammatory effects of heparin involve displacement of cytokines and selectins from HSPGs expressed on endothelial cells, where they control the recruitment of immune cells during inflammation (Figure 6C, upper panel).⁸¹ In addition, heparin is an inhibitor of HSase that has been attributed key roles in many inflammatory conditions (Section 4.4). Moreover, binding of a viral coat protein to cell-surface HS often initiates viral entry and infection. The interaction of heparin with the receptor-binding domain of the SARS-CoV-2 spike S1 protein suggests a potential way to prevent viral adhesion.⁸²

The majority of potential effects of administered heparin, or heparin derivatives, discussed so far (apart from anticoagulation) have involved displacement of proteins from endogenous HS ligands, thus interrupting signaling or other processes depending on the interaction. In certain contexts, the added heparin may instead take over the role of HS. This option was found to prevail in experiments involving Swiss 3T3 fibroblasts that had been treated with chlorate to deplete their endogenous HS of sulfate groups (as illustrated in Figure 8C). These cells no longer responded to added fibroblast growth factor 2 by receptor signaling unless supplemented with exogenous heparin.⁸³

6. Tailored oligosaccharides: Future drugs?

Scattered observations in the literature point to mechanisms, yet poorly understood, to explain the beneficial effects of heparin in various pathological conditions. Such observations were generally accidental, secondary to antithrombotic therapy, as seen in cases of ulcerative colitis⁸⁴ or cancer.⁸⁵ One plausible mechanism is heparin-mediated displacement of a protein ligand from an endogenous HSPG, as illustrated in Figure 8 (where an oligosaccharide substitutes for heparin polymer; see also proposals in Section 4). Inhibition of experimental melanoma metastasis by heparin has been ascribed to the blocking of VLA-4 integrin and P-selectin.⁸⁶ More deliberate surveys are likely to reveal further related scenarios.

In these and similar situations, treatment with heparin, preferably as oligosaccharides devoid of anticoagulant activity, may be of clinical value. However, owing to the predicted broad interaction spectrum (see Section 3.2), long-term treatment with fully sulfated heparin or oligosaccharides is undesirable. Instead, once a key protein mediator has been selected, an oligosaccharide (or mimetic) designed for efficient, maximally selective—rather than maximally strong—interaction should be generated, in accord with the concept illustrated in Figure 5. Identification of sulfate groups of critical importance to interaction will require access to elaborate oligosaccharide libraries as well as advanced analytical techniques. Results should provide a basis for the generation of lead compounds by organic or chemoenzymatic synthesis.

Omics-oriented research generally requires efficient

structural analysis of implicated polymers, such as polynucleotides or polypeptides. Conversely, readily available synthetic products are routinely required for various purposes. Glycobiological research is notoriously hampered in these regards. The lack of automated analytical procedures has been an obvious obstacle in HS-oriented studies, further complicated by the structural heterogeneity of potential target saccharides. Likewise, the generation of even short, selectively sulfated HS sequences remains challenging and is still within reach of only a limited number of specialists. Nevertheless, examples of recent advances are notable. Computational surveys thus identified unique carbohydrate sequences selectively targeting HS-binding protein(s).³⁴ Computational tools were further applied to the selection of heparin-binding proteins capable of interaction with the lipopolysaccharide component of Gram-negative bacteria, allowing them also to act as antimicrobial proteins.⁴ Chemoenzymatic methods were used to generate HS oligosaccharide libraries/microarrays for analysis of protein binding⁸⁷ or determination of substrate specificities of 3-O-sulfotransferases.⁸⁸ Refined mass-spectrometry techniques have been developed for compositional and sequence analysis of HS oligosaccharides.^{37,89} Recent development predicts nanopore-based sequencing of single HS chains^{90,91} (applicability complicated by the inevitable heterogeneity of HS samples from natural sources?). While these and other technical advances are prerequisites for detailed mapping of the heparinome, a comprehensive overview of associated intracellular downstream signaling networks will be essential for a deeper understanding of HS pathophysiology.⁹²

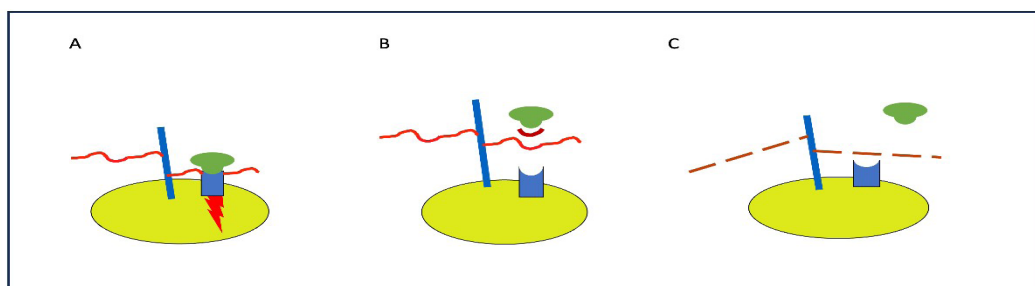


Figure 8. Interference with heparan sulfate (HS)–protein interactions. The selected model represents cell-surface receptors for signaling proteins (growth factors, cytokines, or others) that depend on HS for interaction (A). A competing oligosaccharide will prevent binding of the protein to HS, thus to the receptor (B). Similar effects may be achieved by HSase-catalysed degradation of HS, or by interference with HS biosynthesis (C). The illustrated mechanism is not restricted to signaling proteins but may apply to a variety of ligands, such as extracellular-matrix proteins, cytokines, selectins, viral capsular proteins, and others.

7. Concluding remarks

Routine clinical applications of heparin have so far been largely restricted to antithrombotic therapy, based on

interaction with AT and resultant inhibition of the serine proteinases involved in blood coagulation. This review describes how the identification of the AT-binding pentasaccharide sequence has provided information

essential to the development of two drugs. One of these, dalteparin (and other LMWHs), has enabled substantially improved clinical management. The other, tafoxiparin, is devoid of anticoagulant activity and points to novel therapeutic possibilities in obstetrics. Notably, the distinguishing structural feature of the AT-binding pentasaccharide, the GlcN 3-O-sulfate group, has more recently been implicated in various HS structural settings, in some cases associated with distinct physiological functions. HS-protein interactions so far lacking detailed structural information have been associated with disease conditions as diverse as COVID-19 infection, amyloidosis, and malaria. Indications of strictly regulated HS biosynthesis point to gradual modulation of HS-protein interactions, based on subtle changes in sulfation patterns.

Scattered information regarding unexpected clinical effects of heparin treatment presumably reflects interference with interactions between various HS structures and protein ligands. Still, few HS-based drugs have reached clinical use. Drug development will require identification of these proteins and subsequent optimization of relevant HS structures, thus posing novel challenges regarding analytical as well as synthetic techniques. Progress has undoubtedly been hampered by these challenges, but continued methodological advances are likely to alleviate them. Future work will be required to address issues such as selectivity, pharmacokinetics, including tissue distribution, clinical efficiency, and drug manufacturing optimization.

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