Pharmacology of Heparin and Related Drugs

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Abstract

I. Introduction

II. History

III. Structure of Heparin and Heparan Sulfate
   A. Biosynthesis
   B. Monosaccharide Sequences in Heparin and Heparan Sulfate
   C. The Conformational and Dynamic Properties of Heparin (and Heparan Sulfate)
   D. The Low Molecular Weight Heparins
   E. Synthetic Heparin

IV. Molecular Interactions of Heparin
   A. Heparin Interactions with Small Molecules
   B. Heparin Interactions with Proteins
   C. Heparin and Cytokines, Growth Factors, Selectins, and Proteins of the Extracellular Matrix
      1. Four-helix bundle cytokines
      2. Chemokines
      3. Bone Morphogenetic Proteins and Their Antagonists
      4. Growth Factors
      5. Fibroblast Growth Factors
   D. Heparin, the Complement System, and Innate Immunity
   E. Other Heparin Binding Proteins
      1. Adhesion Molecules
      2. Snake Venom Cardiotoxins
      3. Eosinophil-Granule Major Basic Protein and Eosinophil Cationic Protein
      4. Annexin
      5. Thrombospondin
   F. Protein Aggregation in the Nervous System
   G. Interactions between Heparin and Pathogens
      1. Viruses
      2. Malaria
      3. Bacterial Heparin Lyases
   H. Heparin in Its Native Environment
      1. Interactions with Granule Contents
      2. Enzymes of Heparin Breakdown

V. Analysis
   A. Physicochemical Identification
   B. Molecular Weight Measurements for Heparin
   C. Spectroscopy of Heparin
   D. Physicochemical Quantification
   E. Sequence Analysis
      1. Disaccharide Analysis

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dx.doi.org/10.1124/pr.115.011247.
2. Sequence Determination .......................................................... 102
F. Detection of Contaminants and Impurities ................................. 103
VI. Mechanism of Action of Anticoagulant Activity ......................... 104
A. Antithrombin-Mediated Inhibition of Factor IIa (Thrombin) and Factor Xa .......................................................... 105
B. Antithrombin Inhibition of Other Coagulation Factors ................ 105
C. Other Heparin-Activated Serpins ............................................. 105
D. A Non-Serpin Inhibitor: Tissue Factor Pathway Inhibitor ............. 107
E. Measurement of Anticoagulant Activity of Heparin ..................... 107
1. Plasma-Based Assays .............................................................. 107
2. Purified System Assays ............................................................ 108
VII. Clinical Use as an Anticoagulant/Antithrombotic ....................... 108
A. Venous Thromboembolism ...................................................... 109
1. Treatment of Venous Thromboembolism ................................... 109
2. Venous Thromboembolism Prophylaxis in Medical Patients ........ 110
3. Venous Thromboembolism Prophylaxis in Surgical Patients ....... 110
B. Pregnancy ........................................................................... 111
C. Cancer ................................................................................ 112
D. Acute Coronary Syndromes .................................................... 113
E. Fondaparinux and Other Alternatives ....................................... 113
F. Adverse Reactions ................................................................. 113
1. Bleeding ............................................................................. 113
2. Heparin-Induced Thrombocytopenia ........................................ 114
3. Skin Lesions ....................................................................... 114
4. Osteoporosis ...................................................................... 115
G. Other Side Effects ................................................................ 115
1. Alopecia ............................................................................. 115
2. Elevation of Liver Enzymes ................................................... 115
3. Hyperkalemia .................................................................... 115
VIII. Non-Anticoagulant Effects of Heparin .................................... 115
A. Effects of Heparin on Inflammation and Inflammatory Mediators 116
B. Inflammatory Cellular Adhesion and Trafficking ....................... 117
C. Effects on Heparanase in Inflammation ................................. 118
IX. Non-Anticoagulant Effects of Heparin: Preclinical and Clinical Studies .......................................................... 119
A. Acute Inflammatory Reactions ................................................ 119
B. Human Inflammatory Diseases ................................................ 119
C. Cancer ................................................................................ 120
D. Would Healing and Tissue Repair .......................................... 120
E. Protraction of Labor .................................................................. 121
F. Endogenous Heparin ............................................................... 121
X. Heparin in Biomaterials and Regenerative Medicine .................. 122
A. Immobilized Heparin in Medical Devices and Regenerative Medicine .......................................................... 122

ABBREVIATIONS: APC, activated protein C; APP, amyloid precursor protein; APTT, activated partial thromboplastin time; AT, antithrombin; BACE, β-secretase; BMP, bone morphogenetic protein; CCP, complement control protein; CFH, complement factor H; COPD, chronic obstructive pulmonary disease; DVT, deep vein thrombosis; ECM, extracellular matrix; ECP, eosinophil cationic protein; EGF, epidermal growth factor; EM BP, eosinophil-granule major basic protein; FGF, fibroblast growth factor; FGF R, fibroblast growth factor receptor; GAG, glycosaminoglycan; gD, glycoprotein D; GO, graphene oxide; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HCIII, heparin cofactor II; HGF, hepatocyte growth factor; HHT, heparin-induced thrombocytopenia; HPLC, high-performance liquid chromatography; HPV, human papilloma virus; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; HSV, herpes simplex virus; ICAM, intercellular adhesion molecule; IdoA, L-iduronic acid; IgSF, immunoglobulin superfamily; IL, interleukin; LC, liquid chromatography; LMWH, low molecular weight heparin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MPS, mucopolysaccharidose; N-acetylated (also NAc); NDST, N-deacetylase-sulfotransferase; NMR, nuclear magnetic resonance; NOAC, novel oral anticoagulant; NS, N-sulfated; OPG, osteoprotegerin; OSCS, oversulfated chondroitin sulfate; OST, O-sulfotransferase; PCI, protein C inhibitor; PE, pulmonary embolism; PECAM, platelet endothelial cell adhesion molecule; PF4, platelet factor 4; PN, protease nexin; PRGP, peptidoglycan recognition protein; RANTES, regulated on activation normal T cell expressed and secreted; RI, refractive index; SAX, strong anion exchange; SDF, stromal-derived factor; SEC, size exclusion chromatography; SF, scatter factor; SPR, surface plasmon resonance; STEMI, ST-segment-elevation myocardial infarction; TFP, tissue factor pathway inhibitor; TGF, transforming growth factor; TOP, time of flight; TSPN, N-terminal domain of thrombospondin; UH, unfractionated heparin; VEGF, vascular endothelial growth factor; VTE, venous thromboembolism.
Abstract—Heparin has been recognized as a valuable anticoagulant and antithrombotic for several decades and is still widely used in clinical practice for a variety of indications. The anticoagulant activity of heparin is mainly attributable to the action of a specific pentasaccharide sequence that acts in concert with antithrombin, a plasma coagulation factor inhibitor. This observation has led to the development of synthetic heparin mimetics for clinical use. However, it is increasingly recognized that heparin has many other pharmacological properties, including but not limited to antiviral, anti-inflammatory, and antimetastatic actions. Many of these activities are independent of its anticoagulant activity, although the mechanisms of these other activities are currently less well defined. Nonetheless, heparin is being exploited for clinical uses beyond anticoagulation and developed for a wide range of clinical disorders. This article provides a “state of the art” review of our current understanding of the pharmacology of heparin and related drugs and an overview of the status of development of such drugs.

I. Introduction

The most salient structural feature of heparin for Louis B. Jaques, writing in Pharmacological Reviews in 1979, was its nature as a linear anionic polyelectrolyte (Jaques, 1979). The year 1979 was a critical period in the study of heparin (Petitou et al., 2003), and this article is an update several decades later of Jaques’ seminal review of the pharmacology of heparin. In 1979, several groups were working toward the identification of the specific sequence of monosaccharides in heparin with high affinity for antithrombin (AT) (Lindahl et al., 1979; Rosenberg and Lam, 1979; Choay et al., 1980), and investigations leading to the development of low molecular weight heparin (LMWH) products were well under way (Andersson et al., 1976; Lane et al., 1978). The study of the very close relative of heparin, heparan sulfate (HS), was about to expand (Gallagher et al., 1980; Sjöberg and Fransson, 1980) to reveal its nature as a multifunctional cell regulator (Turnbull et al., 2001). The years between then and now have seen the polyelectrolyte paradigm of Jaques (1979) change first to an emphasis on specific, detailed monosaccharide sequences, followed by a realization that neither structural model alone will explain all of the functions of heparin and the related molecule HS. Physiologically functional interactions of heparin with a large number of proteins (Table 1) range from the highly selective (AT) to the charge-density based (thrombin) (Li et al., 2004). There has also been a growing recognition in the intervening decades from Jaques original article of the “polypharmacy” exhibited by heparin and the wide array of conditions for which this drug is effective beyond the treatment of thrombosis. Jaques clearly identified in his 1979 review that heparin is also capable of many actions beyond anticoagulant activity. In this article, we update and expand on these ideas.

II. History

Heparin has been a successful anticoagulant for over 80 years, and it is on the World Health Organization list of essential drugs. Despite the availability of other anticoagulants such as LMWH, warfarin, and other newer direct thrombin and factor Xa (FXa) inhibitors, unfractionated heparin (UH) remains the therapeutic of choice, especially in surgical interventions such as cardiopulmonary bypass. The discovery and development of UH have been covered by a number of excellent reviews (Messmore et al., 2004; Barrowcliffe, 2012). The first description of heparin as an anticoagulant was recorded in a series of publications by Maurice Doyon in 1910 to 1911, when he investigated the effect of peptone on coagulability of blood in dog livers. Doyon subsequently summarized his finding in 1912 (Doyon, 1912). This was followed by the 1916 publication by Jay McLean, who set out to purify thromboplastic components in tissues from various organs, but copurified an “anticoagulant” fraction (McLean, 1916). The discovery of heparin was therefore incidental and it is interesting that its neutralizing antidote, protamine sulfate, was also discovered in a study designed to find a “long-acting” heparin (Chargaff and Olson, 1937). In 1918, William Howell, McLean’s supervisor, improved the method of purification and eventually named this anticoagulant “heparin” (Howell, 1918). Although the clinical potential of heparin was recognized in the 1920s, it was not until the 1930s that therapeutic preparations were available for clinical trials and the commercial development of heparin was led by Charles Best with the Connaught laboratory in Canada and Erik Jorpes with the Vitrum company in Sweden.

Jorpes and Bergström (1939) elucidated the main chemical structures of heparin in 1936, describing it as a polysulphuric ester of a polysaccharide composed of glucosamine and uronic acid. The finer and crucial structural features of heparin were published between the 1960s and 1980s. Cifonelli and Dorfman (1962) identified L-iduronic acid (IdoA) as the major uronic acid component. Johnson and Mulloy (1976) confirmed the polydisperse nature of heparin and found broad
TABLE 1

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<thead>
<tr>
<th>Serpins</th>
<th>Experiment</th>
<th>Heparin/HS</th>
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<tbody>
<tr>
<td>AT</td>
<td>Nitrous acid degradation, 3-O-sulfatase treatment, paper electrophoresis</td>
<td>High-affinity octasaccharide sequence containing pentasaccharide minimum motif; identification of 3-O-sulfated glucosamine (Lindahl et al., 1980)</td>
</tr>
<tr>
<td>Synthetic chemistry, NMR spectroscopy</td>
<td>High-affinity pentasaccharide sequence confirmed by synthesis, found to have high anti-Xa activity (Choay et al., 1983)</td>
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<td>Crystal structures of AT complexed with high-affinity synthetic pentasaccharide (1AZX.pdb, 1NQ8.pdb, 3EVJ.pdb, 2GD4.pdb, 1EO3.pdb)</td>
<td>Studies exploring in detail the interaction between AT and a synthetic high-affinity pentasaccharide (Jin et al., 1997; Johnson and Huntington, 2003; McCoy et al., 2003; Johnson et al., 2006b; Langdown et al., 2009)</td>
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</table>

| AT with FXa                    | Crystal structure of AT/FXa/synthetic pentasaccharide | Interactions between AT and two exosites on FXa (Johnson et al., 2006b) |
| AT with thrombin (FI)          | Crystal structure of AT/thrombin/heparin mimetic complex (1TBB.pdb) | Ternary complex, both AT and thrombin bound to same heparin molecule (Li et al., 2014) |
| Crystal structure of AT/thrombin/heparin mimetic complex (2BS5.pdb) | Native conformation of AT reactive center loop determined (Johnson et al., 2006a) |
| Crystal structure of AT/anhdrothrombin/synthetic heparin 16-mer (1SR5.pdb) | 16-mer saccharide just long enough to bridge AT heparin binding site and thrombin exosite 2 (Dementiev et al., 2004) |

| With FXa                      | Crystal structure of AT/FXa/heparin complex (3KCC.pdb) | Heparin-activated AT conformation interacts with FXa at both active site and exosite (Johnson et al., 2010) |

| PCI                           | Crystal structure with thrombin and heparin tetradecasaccharide (3B9F.pdb); crystal structure of cleaved PCI with heparin octasaccharide (3DY0.pdb) | Multiple binding modes of bridging heparin; electron density for the disaccharide at thrombin exosite II only (Li et al., 2008) |

| PN-1 protease nexin 1         | Crystal structure of PN-1 with heparin decamer (4D9Y.pdb) and PN-1/heparin/thrombin complex (4D97.pdb) | Heparin binds to helix H, rather than helix D as for AT (Li and Huntington, 2008) |

| Serine protease               | Crystal structure with heparin octasaccharide (1XMN.pdb) | Suggests a two-stage sequence of an initial binding event followed by conformational rearrangement to form productive complex (Li and Huntington, 2012) |

| Thrombin                     | Crystal structure with heparin octasaccharide | Octasaccharide can engage with exosite II of more than one thrombin molecule (Carter et al., 2005) |

| Growth factors               | Crystal structure of FGF-1/heparin complex (1AXM.pdb, 2AXM.pdb) | Heparin 4-mer to 6-mer (DiGabriele et al., 1998); stoichiometry FGF-1/heparin 2:1 |
| FGF-1                        | Crystal structure of FGF-1 with four disaccharides (3UD7, 3UD8, 3UD9, 3UDA) | Of 48 synthetic disaccharides, 4 were identified that bind to FGF-1 (Hu et al., 2012) |
| NMR structure of FGF-1 with a synthetic heparinoid hexasaccharide | FGF-1 interacts with the "one-sided" heparin mimetic: GlcNS-IdoA2S-GlcNAc6S-IdoA-GlcNS-IdoA5S (Muñoz-García et al., 2014) |

| FGF-1/FGFR2c                  | Crystal structure of FGF-1/FGFR2c/heparin (1EOO.pdb) | FGF-1/FGFR2c/heparin decamer 2:2:1 stoichiometry (Pellegri et al., 2000) |
| FGF-2                        | In complex with heparin tetrasaccharide (1BFp.pdb) and hexasaccharide (1BFc.pdb) | FGF2/heparin 1:1 stoichiometry (Faham et al., 1996) |
| In complex with three heparin disaccharides (4OEE.pdb, 4OEP.pdb, 4OEG.pdb) | Water-mediated interactions between disaccharide and protein (Li et al., 2008) |

| FGF-2/FGFR1                  | Crystal structure of FGF-2/FGFR1/heparin (1FOQ.pdb) | FGF-2/FGFR1/heparin decamer 2:2:2 (Schlesinger et al., 2000) |
| FGF-4, FGF-7, FGF-8b         | Affinity chromatography of HS fragments on immobilized FGF | All three FGFs bind to the same HS fragments with similar affinity; no evidence for differential binding to specific sequences (Kreuger et al., 2005) |

| FGFR                         | NMR spectroscopy and molecular modeling | Heparin induces dimerization of FGFR heparin binding domain without FGF (Nieto et al., 2013) |
| VEGF                         | SPR on immobilized heparin | Heparin octasaccharide minimum; the AT-binding motif not necessary (Zhao et al., 2012) |
| NMR titrations and molecular modeling | Interactions predicted involve N-, 2-O-, and 6-O-sulfates (Robinson et al., 2006; Jeong et al., 2013) |

| PDGF-AA                      | Filter-trapping and affinity chromatography | 6-8 monosaccharides minimum, NS domains containing both 2-O- and 6-O-sulfate groups (Feizi et al., 1997) |

| HGF/SF                       | X-ray crystallography of NK1 domain with tetrasaccharide (1GMN.pdb, 1GMO.pdb) | Binding to N domain critical for biologic activity (Lietti et al., 2001) |
| Chemokines                   | NMR of trapped dimer; titrations with octasaccharide | Heparin binds orthogonally to interhelical axis of disulphide-trapped dimer; octasaccharide is not long enough to span completely both heparin binding sites in the dimer (Poluri et al., 2013). |

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<table>
<thead>
<tr>
<th>Heparin-Binding Protein</th>
<th>Experiment</th>
<th>Heparin/HS</th>
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<tbody>
<tr>
<td>RANTES (CCL5)</td>
<td>SPR binding assays and molecular modeling</td>
<td>Minimum 14 monosaccharide units needed for high-affinity dimer complexed with heparin heptadecasaccharide modeled (Vives et al., 2002)</td>
</tr>
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<td></td>
<td>Crystal structure with disaccharides (1U4L.pdb, 1U4M.pdb)</td>
<td>Only disaccharides with 2-sulfation of uronic acid cocrystallized (Shaw et al., 2004)</td>
</tr>
<tr>
<td>IL-8 (CXCL8)</td>
<td>Filter-trapping binding assays</td>
<td>NS block of about 6 monosaccharide units within an approximately 22- to 24-mer sequence, separated by a region of ≥14 monosaccharide residues that may be fully NA (Spillmann et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>NMR titration</td>
<td>Heparin binding site on the C-terminal α-helix and proximal loop (Schlorke et al., 2012; Möbius et al., 2013)</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>Human marrow LTC-IC maintenance assays</td>
<td>An 8.3-kDa fragment consisting of two S domains separated by a short NA domain (Stringer et al., 2002)</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>NMR spectroscopy, ultracentrifugation</td>
<td>Disaccharide can support dimerization (McCornack et al., 2004)</td>
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<tr>
<td>CCR2 ligands: MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), Eotaxin-1 (CCL-11)</td>
<td>Filtration trapping and FTICR MS</td>
<td>Octasaccharides with at least 10 sulfates are required; MCP-1 and MCP-2 dimerize in presence of octasaccharide, the others remain monomeric (Yu et al., 2005)</td>
</tr>
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<td>Ion mobilization MS</td>
<td>MCP-1 can form two distinct dimers in interaction with heparin octasaccharide (See et al., 2013)</td>
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<td>Sedimentation equilibrium ultracentrifugation, affinity chromatography</td>
<td>Heparin binding site is a continuous ring of basic residues round tetramer (Lau et al., 2004)</td>
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<tr>
<td>SDF-1 (CXCL12)</td>
<td>NMR spectroscopy and crystal structure (2NWG.pdb)</td>
<td>Two distinct binding sites for the disaccharide on the CXCL12 dimer (Murphy et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>NMR titration with heparin dodecasaccharide</td>
<td>Heparin dodecasaccharide binds orthogonally to the dimer interface (Ziarek et al., 2013)</td>
</tr>
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<td>NMR titration, STD with a 13C-labeled semisynthetic octasaccharide; restrained molecular modeling</td>
<td>Heparin binding site and dimerization confirmed, NMR restrained and unrestrained molecular modeling in good agreement (Laguri et al., 2011)</td>
</tr>
<tr>
<td>PF4 (CXCL4)</td>
<td>Filter-trapping assays, protection of HS fragment against enzyme</td>
<td>9-kDa fragment, two S domains with short linking NA domain (Stringer and Gallagher, 1997)</td>
</tr>
<tr>
<td>I-TAC (CXCL11)</td>
<td>Affinity chromatography, NMR spectroscopy, molecular modeling</td>
<td>Two binding modes identified with differing affinities (Severin et al., 2010)</td>
</tr>
<tr>
<td>Cytokines</td>
<td>ELISA binding and competition assays</td>
<td>Full-size heparin (17kDa) required for maximum ability to compete with mAb (Najjam et al., 1998)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Heparin affinity chromatography, competition assays on immobilized IL-4</td>
<td>S domains of HS required for interaction (Lortat-Jacob et al., 1997)</td>
</tr>
<tr>
<td>IL-4</td>
<td>ELISA competition assays</td>
<td>N- and 2-O-sulfation required but not 6-O-sulfation (Mummery and Rider, 2000)</td>
</tr>
<tr>
<td>IL-6</td>
<td>SPR with heparin oligosaccharides and other GAGs</td>
<td>Heparin binds with higher affinity to human than to murine IL-7 (Zhang et al., 2012b)</td>
</tr>
<tr>
<td>IL-7</td>
<td>STD-NMR and transferred NOE techniques</td>
<td>Sulfated disaccharide will bind, but minimum hexasaccharide required for high affinity (Kunze et al., 2014)</td>
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<tr>
<td>IL-10</td>
<td>ELISA competition assays</td>
<td>Affinity increases evenly with length of oligosaccharide (Hasan et al., 1999)</td>
</tr>
<tr>
<td>IL-12</td>
<td>Affinity chromatography</td>
<td>pH-dependent binding to helix C of GM-CSF due to histidine involvement (Sebollela et al., 2005)</td>
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<td>GMC-SF</td>
<td>NMR titration</td>
<td>Octasaccharide minimum length for binding (Vanhaverbeke et al., 2004); two cationic sites on the protein surface involved (Saesen et al., 2013)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>SPR binding assays</td>
<td>12–14 monosaccharide units, 2-O- and N-sulfate groups (Sadir et al., 2001)</td>
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<tr>
<td>Morphogens</td>
<td>NMR titration and molecular modeling (2K8P.pdb)</td>
<td>Binding site can accommodate a heparin decamer (Veverka et al., 2009)</td>
</tr>
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<td>Sclerostin</td>
<td>Displacement of noggin from CHO cell surface; immunoprecipitation</td>
<td>Prefers 10 or more monosaccharide units, with fully sulfated 8 domains (Viviano et al., 2004)</td>
</tr>
<tr>
<td>Noggin</td>
<td>Crystal structure with heparin 18-mer (4C4N.pdb) and with chondroitin 4-sulfate (4C4M.pdb)</td>
<td>Heparin 15-mer required to induce dimerization; orientation of GAG chains in complex depends on sulfation pattern (Whalen et al., 2013)</td>
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<tr>
<td>Sonic hedgehog</td>
<td>Molecular modeling (docking)</td>
<td>A conserved heparin binding site identified in RGD integrins but not in non-RGD integrins (Ballut et al., 2013)</td>
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<td>Heparin binding:</td>
<td>ELISA assays, cell adhesion assays</td>
<td>Hexasulfated tetrasaccharide (Nelson et al., 1993)</td>
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<td>adhesion molecules</td>
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<td>Integrins (αvβ3)</td>
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<td>P-selectin, L-selectin</td>
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Table 1—Continued

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<tr>
<th>Pharmacology of Heparin and Related Drugs</th>
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<tr>
<td><strong>PECAM-1</strong></td>
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<tr>
<td><strong>ECM proteins</strong></td>
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<td><strong>Fibronectin</strong></td>
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<td><strong>Thrombospondin</strong></td>
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<td><strong>Complement system and innate immunity</strong></td>
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<td><strong>Factor H</strong></td>
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<td><strong>Human β-defensin 2</strong></td>
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<td><strong>Cytotoxic mediators</strong></td>
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<td><strong>ECP</strong></td>
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<td><strong>EMBP</strong></td>
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<td><strong>Glucuronyl C5-epimerase</strong></td>
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<td><strong>2-OST</strong></td>
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<td><strong>3-OST-1</strong></td>
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<td><strong>3-OST-3</strong></td>
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<td><strong>Neutrophil elastase</strong></td>
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<td><strong>Tryptase</strong></td>
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<td><strong>Bacterial heparinase I</strong></td>
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<td><strong>Microbial adhesins</strong></td>
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<td><strong>Heparin-binding hemagglutinin of Mycobacterium tuberculosis</strong></td>
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<td><strong>Decorin-binding protein of Borrelia bergdorferi</strong></td>
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<td><strong>Histone-like protein Hlp</strong></td>
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<td><strong>Viral proteins</strong></td>
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<td><strong>HIV-1 gp120</strong></td>
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<td><strong>HPV18 capsid</strong></td>
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<td><strong>FMDV capsid</strong></td>
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<td><strong>gD glycoprotein of HSV</strong></td>
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<td><strong>Cyclophilin B</strong></td>
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molecular weight ranges for commercial heparins. Johnson et al. (1976) further prepared high and low molecular weight fractions of heparin and found that when injected subcutaneously into volunteers, the low molecular weight fractions showed a longer half-life by anti-Xa assay than the parent heparin and the high molecular weight fractions. This landmark study led to the development of LMWHs for clinical use in the late 1970s. Höök et al. (1976), Andersson et al. (1976), and Lam et al. (1976) independently found that only one-third of heparin binds to AT with high affinity and is responsible for the anticoagulant action of heparin. Another milestone was the discovery of an octasaccharide—sulfated chondroitin sulfate (OSCS) in 2008, and this has led the US Food and Drug Administration to consider the reintroduction of bovine heparin for clinical use in the U.S. market (http://www.fda.gov/Advisory-Committees/CommitteesMeetingMaterials/Science-Board/totheFoodandDrugAdministration/ucm399395.htm); thus, the source of clinical heparin has come full circle. It is well known that not all heparin preparations are the same, because in addition to species differences, there are also structural and activity disparities between tissue sources. It is not clear whether bovine and porcine heparins behave similarly in clinical settings, and there have been reports that bovine heparin may not be as effective and may also have induced more bleeding events than porcine heparin (Aquino et al., 2010; Tovar et al., 2013; Santos et al., 2014). Therefore, there will be interest in the comparison of the structure and function relationship of bovine and porcine heparin once bovine heparin is reintroduced.

The adulteration of heparin with OSCS in 2008 has been a highly publicized incident (Guerrini et al., 2008;
Fig. 1. Structures in heparin and other glycosaminoglycans. (A) An octasaccharide with high affinity for antithrombin isolated using nitrous acid depolymerization of heparin (Lindahl et al., 1980), containing the essential pentasaccharide sequence shown in blue. (B) The major repeating
Kishimoto et al., 2008; http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2008/ucm116858.htm). The existing pharmacopeial methods to assay the activity of natural heparins, especially the nonspecific potency assay, proved inadequate to detect this unexpected contaminant. This led to the rapid revision of pharmacopeial monographs for heparin and LMWHs. New physicochemical methods such as nuclear magnetic resonance (NMR) and strong anion exchange (SAX) high-performance liquid chromatography (HPLC) methods were quickly devised and implemented. The potency assay based on the clotting of sheep plasma was replaced with an assay that employs purified reagents and is dependent on measurement of heparin's interaction with AT. The revision of pharmacopeial monographs is still ongoing; interestingly, the detection of bovine heparin as a contaminant in porcine heparin products has been a recent focus for regulators and pharmacopeias.

Jaques (1979) declared that heparin is a unique class of biochemical compound and heparins should be named “linear anionic polyelectrolytes,” rather than an “anticoagulant.” Recent clinical developments support Jaques’ concept because there are a number of ongoing clinical trials on heparin, LMWHs, and related polysaccharides that are unrelated to its anticoagulant action. These include, but are not limited to, the treatment of chronic obstructive pulmonary disease (COPD), protraction of labor, malaria, and cancer (Table 2).

III. Structure of Heparin and Heparan Sulfate

The names heparin and heparan sulfate are not especially rational; they came into use for historical, rather than scientific, reasons. HS was first described in 1948 as heparin monosulfuric acid and was initially thought of as a low-sulfated heparin species (Jorpes and Gardell, 1948). When HS was eventually shown to be a widespread and functional component of the cell surface (Gallagher et al., 1986), the tables were turned: heparin is now considered to be a member of the HS family of glycosaminoglycan (GAG) polysaccharides (Casu and Lindahl, 2001).

Both heparin and HS polysaccharides are found attached to a protein core to form proteoglycans. Heparin is confined to mast cell granules (see section IV), whereas HS is a ubiquitous component of cell surface and extracellular matrix (ECM) proteoglycans. Structurally they are made up of identical repeating disaccharide components, but in widely differing proportions. Heparin is, by and large, more highly sulfated than HS and will usually outcompete HS for binding to protein ligands. HS is involved in embryonic development, inflammation, immune defense, and cell growth (Xu and Esko, 2014), and the ability of heparin to interfere in these processes or mimic the action of HS forms the molecular basis for several potential therapeutic uses of heparin and heparin-like compounds (Lever and Page, 2012).

A. Biosynthesis

The biosynthesis of heparin was summarized recently (Esko et al., 2009; Carlsson and Kjellén, 2012; Kreuger and Kjellén, 2012). The biosynthetic pathways for heparin and HS are almost identical and are summarized in Fig. 2. GAGs are built up directly onto a protein backbone. Whereas one or more HS chains may be added to serine residues on a number of proteoglycans, heparin has a single core protein, serglycin, carrying numerous heparin chains, highly sulfated chondroitin chains, or both (Kolset and Tveit, 2008; Rönberg et al., 2012). The initial stages of heparin biosynthesis are taken up with the assembly of a linker region common to all GAGs [β-d-Gal-(1→3)-β-d-Gal-(1→4)-β-d-Xyl-1-Ser], followed by the enzymes that commit to either the galactosaminoglycan or glucosaminoglycan family. Then, as for HS, chain elongation of heparin involves the addition of alternating β-d-GlcA (GlcA) and α-d-GlcNAc (GlcNAc) monosaccharide residues by the exostosin enzyme isoforms.

Conversion of this initial polymer (termed heparan, or more appropriately N-acetyl heparosan; a repeating disaccharide structure shown in Fig. 1B) to heparin is completed by a set of postpolymerization enzymes, initially the N-deacetylsulfotransferase (NDST) isoform 2 or NDST2. The equivalent enzyme in HS biosynthesis is NDST1, and this stage is one of the most marked differences in the two biosynthetic systems. It is in the action of the NDST enzymes that the eventual domain structure of HS is laid down (Sheng et al., 2011). While the chain is elongated from the reducing to nonreducing end, the NDSTs work in the opposite direction, in a processive manner, creating N-sulfated (NS) regions of the polysaccharide separated by N-acetylated (NA; also NAc) regions (Carlsson et al., 2008). The regulation of the action of NDSTs in determining the lengths of NS and NA regions is not well understood, but the NDST2 isoform in mast cells produces long NS regions, separated by short NA regions, in contrast with the short NS regions introduced by NDST1 in HS.

The next enzyme in line is the C5-epimerase, specific for β-d-GlcA residues glycosylated at the 4-position with NS glucosamine (Hagner-McWhirter et al., 2004). The
<table>
<thead>
<tr>
<th>Condition</th>
<th>Intervention</th>
<th>Purpose</th>
<th>Phase</th>
<th>Trial Identifier(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiphospholipid syndrome and pregnancy</td>
<td>Heparin, enoxaparin</td>
<td>Treatment of recurrent pregnancy loss associated with antiphospholipid syndrome</td>
<td>2</td>
<td>NCT01051778</td>
</tr>
<tr>
<td>Assisted reproductive technology</td>
<td>Enoxaparin</td>
<td>Topical application of heparin to improve intracytoplasmic sperm injection</td>
<td>2</td>
<td>NCT02325479</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>Heparin</td>
<td>Evaluate if topically applied heparin aids construction of primary arteriovenous fistulas</td>
<td>2</td>
<td>NCT01382888, 2011-000455-16</td>
</tr>
<tr>
<td>Inhalation burns, smoke inhalation injury</td>
<td>Heparin</td>
<td>Efficacy of nebulized heparin on lung injury score in inhalation burn injury over normal care (HEPBURN)</td>
<td>2</td>
<td>NCT01773083, 2012-003289-42</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Tinzaparin/enoxaparin</td>
<td>LMWH can inhibit tumor growth and metastasis and enhance survival of patients</td>
<td>3</td>
<td>NCT00475098, NCT00771563, 2007-007696-16</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Enoxaparin, heparin</td>
<td>Treatment of inflammation in intraocular lens implantation, cataract surgery, and chronic glomerulonephritis</td>
<td>3 and 4</td>
<td>NCT00986076, NCT00001311, 2005-002989-11</td>
</tr>
<tr>
<td>Adenocarcinoma of the colon</td>
<td>Tinzaparin</td>
<td>LMWH reduction of metastases and recurrence in patients as seen in animal models</td>
<td>3</td>
<td>NCT01455831</td>
</tr>
<tr>
<td>Breast, colorectal, lung, prostate and veno-occlusive cancers</td>
<td>Dalteparin, nadroparin, enoxaparin</td>
<td>Assess whether LMWH beyond inhibition of thrombosis improves quality of life/survival rate over standard chemotherapy</td>
<td>2 and 3</td>
<td>NCT00003674, NCT00718354, NCT00717935, 2005-005336-27, 2007-002608-16</td>
</tr>
<tr>
<td>Supratentorial glioblastoma multiforme</td>
<td>Dalteparin</td>
<td>Heparin may stop the growth of cancer stopping blood flow and blocking enzymes in tumor growth</td>
<td>2</td>
<td>NCT00028678</td>
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<tr>
<td>Vulvodynia</td>
<td>Enoxaparin</td>
<td>Heparin may stop the growth of cancer slowing blood flow and blocking enzymes in tumor growth</td>
<td>2</td>
<td>NCT00874484</td>
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<td>Ulcerative colitis</td>
<td>Deligoparin</td>
<td>Ultra-LMWH may help to reduce inflammation in ulcerative colitis</td>
<td>2 and 3</td>
<td>NCT00033943, 2006-001782-42</td>
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<td>Diabetic foot ulcers</td>
<td>Dalteparin</td>
<td>Treatment on chronic foot ulcers due to peripheral arterial occlusive disease in patients with diabetes</td>
<td>2 and 3</td>
<td>NCT00765063, NCT00662831</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Dalteparin</td>
<td>To improve morbidity and mortality when used in conjunction with standard therapy</td>
<td>2</td>
<td>NCT00239980</td>
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<tr>
<td>Pediatric solid tumors, acute myeloid leukemia</td>
<td>ODSH</td>
<td>Treatment with heparin to increase platelet recovery time after chemotherapy</td>
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<td>NCT02164097, NCT2056782</td>
</tr>
<tr>
<td>Metastatic pancreatic cancer</td>
<td>ODSH, dalteparin</td>
<td>Determine if ODSH or dalteparin is efficacious in patients receiving normal therapy</td>
<td>2</td>
<td>NCT01461915, NCT00462582</td>
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<tr>
<td>Metastatic kidney cancer</td>
<td>Tinzaparin</td>
<td>Prevention of growth in inoperable cancer</td>
<td>1 and 2</td>
<td>NCT00293501</td>
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<tr>
<td>Burns</td>
<td>Heparin</td>
<td>Assess analgesic effect of heparin in topical and parenteral treatment</td>
<td>2</td>
<td>2007-004304-12</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Heparin</td>
<td>Nebulized heparin on easing cystic fibrosis</td>
<td>2</td>
<td>2007-006276-11</td>
</tr>
<tr>
<td>Pulmonary conditions</td>
<td>Heparin, desulfated heparin</td>
<td>Improve lung function in obstructive pulmonary conditions</td>
<td>2</td>
<td>2006-006375-32, 2010-024168-16</td>
</tr>
<tr>
<td>Acute chest syndrome</td>
<td>Heparin</td>
<td>Improve clinical outcome, and decrease hospitalization</td>
<td>2</td>
<td>NCT02089893</td>
</tr>
<tr>
<td>Labor Microalbuminuria</td>
<td>Non-anticogualant LMWH Sulodexide (LMWH and dermatan sulfate)</td>
<td>Reducing prolonged labor and treatment microalbuminuria in type 2 diabetes</td>
<td>2 and 3</td>
<td>2006-005859-20, 2005-003158-91</td>
</tr>
</tbody>
</table>

Data are from the US National Institutes of Health ClinicalTrials.gov database (http://www.clinicaltrials.gov) and the EU Clinical Trials Register (http://www. clinicaltrialsregister.eu) in December 2014. ODSH, O-desulfated heparin.
The crystal structure of this enzyme (the sequence from *Danio rerio*) in complex with a heparin oligosaccharide was recently solved (4PXQ.pdb) and compared with the unbound enzyme (4PW2.pdb) (Qin et al., 2015). The sequence GlcNAc–IdoA is therefore not found in mammalian HS or heparin. Closely associated with the epimerase is the IdoA–2-0-sulfotransferase (2-OST) (Qin et al., 2015), which as its name implies adds sulfate to the 2-position of the newly formed IdoA residue to give IdoA2S; rarely, it can also add 2-O-sulfate to β-D-GlcA. 2-O-sulfated GlcA was found in nuclear HS as long ago as 1986 (Fedarko and Conrad, 1986) and has also been seen in highly sulfated heparin sequences (Yamada et al., 1995). A recent crystal structure of the *Gallus gallus* 2-OST complexed with a hexasaccharide has appeared (4NDZ.pdb).

The 6-OSTs complete the set of enzymes that make up the major repeat unit of heparin. There are three

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**Fig. 2.** Biosynthesis. A simplified scheme outlining the consecutive stages of heparin biosynthesis, based on several sources (Esko et al., 2009; Carlsson and Kjellén 2012; Kreuger and Kjellén, 2012). A hypothetical heparin sequence is shown, containing the pentasaccharide sequence with high affinity AT. For the initial GlcA-GlcNAc disaccharide repeats in heparin, n = 1 or 2; for HS, n = 5–10. 2S, 2-O-sulfated IdoA; 3S, 3-O-sulfated GlcN; 6S, 6-O-sulfated GlcN; EXT, exostosin (HS/heparin polymerase complex); EXT1, initial N-acetyl glucosaminyl transferase; GalT, galactosyltransferase; GlcAT, glucuronosyltransferase; NS, N-sulfated GlcN; PAPS, 3′-phosphoadenosine-5′-phosphosulfate (sulfate donor); XylT, xylosyltransferase.

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**Key**
- GlcN
- GlcNAc
- GlcA
- IdoA
- Gal
- Xyl

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86 Mulloy et al.
isoforms, all of which are capable of acting on both NS and NA glucosamine (Smeds et al., 2003). The resulting trisulfated disaccharide repeating structure is shown in Fig. 1C. In the case of HS, a set of sulfatase enzymes, Sulf1 and Sulf2, act extracellularly, after the transport of HS to the cell surface. These enzymes trim some of the 6-O-sulfates from HS, contributing to distinctive organ-specific HS structures (Nagamine et al., 2012). Because heparin is not a cell surface GAG, it is not (as far as we know) modified by the sulfatases.

A rare modification with particular significance for heparin is the 3-O-sulfation of occasional GlcNS mono-saccharide residues. Of the multiple isoforms of the 3-OST enzyme, it is 3-OST-1 that forms the central structural motif in the AT-binding sequence of heparin shown in Fig. 1A (Shworak et al., 1999). In addition to the importance of this heparin/HS modification in anti-coagulation (see below), it is also clear that 3-OST-1 products are involved in inflammation (Shworak et al., 2010) and reproduction (de Agostini, 2006; de Agostini et al., 2008). A high-resolution crystal structure of 3-OST-1 with a synthetic heptasaccharide (Moon et al., 2012) provided detailed insights on the induced oligosaccharide conformations on initial binding to this enzyme, and comparisons with the earlier structure of 3-OST-3 (Moon et al., 2004) allow insight into the structural selectivity of 3-OST isoforms. 3-OSTs can be divided into two groups: the AT-binding group, 3-OST-1 and 3-OST-5, which require GlcA before the modified GlcN; and the glycoprotein D (gD) group, 3-OST-2, 3-OST-3, 3-OST-4, and 3-OST-6, which require IdoA2S in that position (Thacker et al., 2014). Of these, the evidence that DNA hypermethylation of 3-OST genes is associated with several common cancers is of particular interest (Miyamoto et al., 2003; Shivapurkar et al., 2007). 3-OST-3 can act on N-unsubstituted glucosamine, generating a sequence capable of interaction with the herpes simplex virus (HSV) gD (Liu et al., 2002); in addition, 3-OST-3b can produce the sequence that binds to cyclophilin (Vanpouille et al., 2007).

**B. Monosaccharide Sequences in Heparin and Heparan Sulfate**

The biosynthetic scheme outlined above and in Fig. 2 yields heparin and HS sequences that are neither wholly determined (in the sense that peptides are) nor random. Both cell surface HS and mast cell heparin are block copolymers of NA and NS domains; these are respectively stretches of unmodified polysaccharide with alternating GlcA and GlcNAc, the NA domains (Fig. 1B), and stretches in which the GlcNAc has been transformed to GlcNS, allowing the epimerization of GlcA to IdoA and its conversion to IdoA2S; these are the S domains, or NS domains (Fig. 1C) (Esko and Selleck, 2002). The boundaries between NS and NA domains are not necessarily tidy, and they may contain intermediate sequences; the isoforms of 6-OST can also produce GlcNAc6S as well as GlcNS6S (Smeds et al., 2003). In HS, the NA/NS sequences may quantitatively equal or exceed the NS domains (Maccarana et al., 1996). The effect of sequence on function can therefore operate at several levels: the overall degree of sulfation of the molecule (highly sulfated heparin versus less sulfated HS), the sizes of individual domains within a molecule (large NS domains in heparin, large NA domains in HS) and the fine structure, or specific monosaccharide sequences.

**C. The Conformational and Dynamic Properties of Heparin (and Heparan Sulfate)**

The overall shape of a polysaccharide is dependent on both the linkage conformation, between residues, and the conformations of the monosaccharide residues themselves. This is an important difference between peptides and saccharides; in peptides, the α-amino acid residues have side chains that are not incorporated into the polymer backbone. Many polysaccharides are flexible molecules with a high degree of internal mobility (Rutherford et al., 1994; Yang et al., 2013). For this reason, the solution dynamics of polysaccharides are important to their properties and physiologic functions. The conformational properties of heparin and HS are complex, and the past decades have seen numerous experimental and theoretical studies to better understand these properties (Mulloy and Forster, 2000; Skidmore et al., 2008; Rudd et al., 2010a).

For the GAGs, heparin, and HS, conformational properties vary between the two major domain types. The NA domains contain alternating α-1→4 and β-1→4 linkages between gluco-series hexopyranoses. The disaccharides maltose [α-D-Glc-(1→4)-D-Glc] and cellobiose [β-D-Glc-(1→4)-D-Glc] are the two best studied model compounds for these linkage types, and recent studies of their conformational properties imply that the polysaccharide might adopt a relatively extended shape capable of some conformational heterogeneity (Perić-Hassler et al., 2010). Both β-d-GlcA and α-d-GlcN predominantly adopt the 4C1 conformation of the pyranose ring, as demonstrated by 1H NMR studies of scalar coupling constants between vicinal protons. Recent studies have, however, raised the possibility that a significant deviation from this conformation is a possibility in some circumstances (Hricovini, 2011; Sattelle and Almond, 2011). Two recent studies, one using 15N NMR and one using analytical ultracentrifugation (Mobli et al., 2008; Khan et al., 2012, 2013), indicate that the NA domains have sufficient flexibility to allow adoption of a wide variety of curved and bent configurations.

Replacement of NA with NS groups and epimerization of GlcA to IdoA generates S domains with different conformational properties, made up of the repeating disaccharide in Fig. 1C. In summary, flexibility of the glycosidic linkages is reduced, and that of the uronic acid is increased. The solution conformation of the
heparin (and HS) S-domain structure, as determined by NMR spectroscopic methods (Mulloy et al., 1993), is remarkably similar to the solid-state conformation determined by fiber diffraction (Atkins and Nieduszynski, 1975). The molecular models based on the NMR data (1HPN.pdb) have an average axial periodicity of about 1.7 nm, within the range of 1.65–1.73 nm found in the fibers (Fig. 3A). Using the same NMR methodology, solution structures of several systematically modified heparin samples were also obtained (Mulloy et al., 1994). Neither variations in sulfation positions, nor replacement of \(N\)-sulfate by \(N\)-acetyl, caused major changes in the glycosidic linkage conformation, but the iduronate ring conformational equilibrium was affected strongly by replacement of \(N\)-sulfate by \(N\)-acetyl.

The NMR studies on which the solution conformation was based relied heavily on quantitative measurements of the nuclear Overhauser effect, a relaxation-related parameter that is dependent on short distances between protons. This measurement usually assumes that the molecule studied behaves as if it were a sphere, tumbling isotropically in solution. In the course of the NMR conformational studies, it became clear that heparin did not behave in this way (Forster et al., 1989; Mulloy et al., 1993); the quantitative nuclear Overhauser effect values could not be interpreted unless it was assumed that heparin tumbled as an “asymmetric top,” specifically as if the shape of the heparin molecules was more like a cylinder than a sphere. Analytical ultracentrifugation and X-ray scattering results were consistent in this assessment (Pavlov et al., 2003) and indicated a semirigid cylindrical structure for heparin oligosaccharides, with flexibility increasing as oligosaccharide length increases (Khan et al., 2010).

For heparin, largely composed of S domains, the overall effect is of a semiflexible rod-like shape within which patterns of presentation of sulfates change to and fro on an approximately microsecond timescale. These S domains are interrupted by more flexible, unsulfated NA domains. The NA domains dominate the structure of HS, which can consequently be expected to have more overall flexibility than heparin and to be able to present short S domains and NA/NS regions (Maccarana et al., 1996) in space in such a way that a single HS molecule can interact with more than one suitable binding site, perhaps on a multimeric protein (Khan et al., 2012; Stringer et al., 2002), or two separate proteins, such as AT and thrombin (see below). The combination of two S domains separated by one NA domain (Murphy et al., 2004) has been termed the “SAS” motif (Kreuger et al., 2002).

The dynamic complexities of heparin structure, involving limited rotation around glycosidic linkages and mobile IdoA residues, require theoretical analyses to interpret experimental data; a single “average” structure derived from experimental data is not enough. Investigations of the dynamic aspects of the iduronate ring conformation used NMR data (\(^1\)H–\(^1\)H coupling constants) for both sulfated and unsulfated iduronate (Sanderson et al., 1987; Ferro et al., 1990). A force-field study provided a rationalization of these data in terms of a conformation equilibrium between \(^1\)C\(_4\) chair and skew-boat forms for iduronate, whether sulfated or not, in heparin/HS sequences (Ferro et al., 1990). Molecular dynamics of unsulfated, internal iduronate (Forster and Mulloy, 1993) indicated that the skew-boat \(^2\)S\(_0\) conformation might in fact be an average of several related, rapidly interconverting boat and skew-boat forms; however, more recent molecular dynamic studies of sulfated iduronate have not identified any such pseudorotational transitions (Gandhi and Mancera, 2010).

D. The Low Molecular Weight Heparins

The key observations that led to the development of LMWH products were made in the mid-1970s, when
high and low molecular weight fractions of heparin prepared in the laboratory of E.A. Johnson were injected subcutaneously into healthy volunteers (Johnson et al., 1976). The plasma anti-Xa activity of the low molecular weight fraction (molecular weight around 9000) persisted for several hours longer than that of the high molecular weight fraction or of the parent material.

All the LMWHs are prepared from UH that meets the appropriate requirements for clinical use (European Pharmacopoeia, 2015). The main methods of depolymerization used are enzymic β-elimination, base-catalyzed chemical β-elimination, and nitrous acid treatment (Linhardt and Gunay, 1999).

Heparinase I, the enzyme used to generate the LMWH tinzaparin, can cleave the AT-binding pentasaccharide (Shriver et al., 2000b); the GlcNS3S6S-IdoA2S linkage is particularly susceptible (Xiao et al., 2011). This may explain why oligosaccharide subfractions of equivalent lengths fractionated from tinzaparin and enoxaparin have differing anti-Xa activities (Bisio et al., 2009; Schroeder et al., 2011). Depolymerization in the production of LMWH causes some loss of the AT-binding sequence. However, the ultra-LMWH semuloparin, prepared using a phosphazene-based depolymerization, is comparatively enriched in the pentasaccharide (Viskov et al., 2009b) and even contains dodecasaccharides with two adjacent pentasaccharide sequences (Viskov et al., 2013) and pentasaccharides containing two 3-O-sulfated glucosamine residues (Guerrini et al., 2013).

The minor structural changes introduced in the manufacture of LMWHs can be significant in identifying characteristics of a specific product as well as influencing the anticoagulant action of the product (Guerrini et al., 2010). This is an important consideration for the introduction of biosimilar LMWH products. In addition to the development of follow-on versions of existing products, advances are still being made in production techniques for new LMWHs (Fu et al., 2014).

**E. Synthetic Heparin**

Heparin interacts with the coagulation system in numerous ways, but the interactions with AT that inhibit the action of thrombin and FXa are those that are uniquely characteristic of heparin and were obvious initial targets for the development of synthetic heparin mimetics. The realization that there existed in heparin an oligosaccharide sequence with high affinity and specificity for the serpin AT provided a clear, if challenging, target for synthetic chemists. The essential pentasaccharide at the heart of this sequence (Fig. 1A) was synthesized independently by two groups (Choay et al., 1983; van Boeckel et al., 1985). van Boeckel and Petitou (1993) have summarized the subsequent detailed work on structural variants of the pentasaccharide, to assess the exact requirements for high-affinity binding to AT.

Total synthesis of the essential pentasaccharide (Fig. 1A) confirmed its structure, and was a step on the road toward synthetic heparin mimetics such as the pentasaccharide fondaparinux (Bauer et al., 2002). The advantages of these synthetic compounds are simplified pharmacodynamics and reduction of those side effects that depend on the presence of other structures in heparin or depend on particularly large molecular size (Petitou et al., 1999). In this article, Petitou et al. give a clear account of the various extended synthetic oligosaccharides investigated in the process of designing a thrombin inhibitor with reduced capacity to bind to platelet factor 4 (PF4). The authors include a molecule with the thrombin binding extension on the reducing side of the pentasaccharide, which has little activity, and they were able to show that a neutral linker between AT and thrombin binding regions was acceptable for binding and activity, because the linker does not directly interact with either protein. Both of the sulfated regions are too small to interact with PF4.

Synthetic constructs have been designed that address the problem of an antidote. The compound EP42675 is a fondaparinux analog conjugated via a spacer to a direct thrombosis inhibitor. EP217609 has biotin covalently linked to the spacer allowing the use of avidin as an antidote. However, neither of these compounds has made it to the market (Petitou et al., 2009).

Synthetic chemistry is potentially able to generate oligosaccharides of defined and homogeneous structure in sufficient amounts for structural biology studies. An obvious initial problem is the choice of synthetic target, given the very large number of possible heparin sequences. Noting that heparin has a rod-like solution conformation, and that this conformation changes only slightly with patterns of sulfate substitution, a group of synthetic carbohydrate chemists has produced a set of compounds that explore the interaction between heparin/HS and the fibroblast growth factors (FGFs). Oligosaccharides have been produced that have the IdoA-GlcN backbone of heparin, with either full heparin-like sulfation, or a pattern that results in sulfates down one side only of the heparin molecule (Fig. 3). The first such “one-sided” heparin-like oligosaccharide, an octamer (de Paz et al., 2001), was found to be effective in activating FGF-1, indicating that the trans-dimeric FGF-1/heparin structure may not be necessary for activation (Angulo et al., 2004). The solution conformations and dynamics of the oligosaccharides have been characterized in some detail, and as expected, they behave in a highly anisotropic manner in solution, characteristic of heparin sequences (Angulo et al., 2005).

Automated solid-state synthesis of oligosaccharides is now well established (Seeberger and Werz, 2007) and has been applied to heparin/HS sequences for use in microarray techniques to analyze heparin-protein interactions (Noti et al., 2006; Yin and Seeberger, 2010). The
scale on which heparin-like molecules can be synthesized has been a particular challenge. Another synthetic strategy has proved capable of delivering grams of synthetic heparin oligosaccharides by an iterative procedure using disaccharide (Hansen et al., 2013a,b) or tetrasaccharide (Hansen et al., 2013a) building blocks. The enabling technology in this strategy was the development of plentiful iduronate-containing precursors (Hansen et al., 2012).

A route toward heparin mimetics structurally allied with natural heparin/HS has been explored through the use of the capsular polysaccharide from *Escherichia coli* strain K5. This polysaccharide has the same structure as heparosan and may be modified both chemically and enzymatically to yield heparinoids with or without anticoagulant activity (Lindahl et al., 2005; Oreste and Zoppetti, 2012). Non-anticoagulant heparinoids are particularly promising in applications in which potent anticoagulant activity is not needed, and compounds of this type have been shown to inhibit cell infection with viruses (Pinna et al., 2008; Vervaeye et al., 2013) and to interact with the vascular endothelial growth factor (VEGF) receptor antagonist gremlin (Vervaeye et al., 2013).

To generate synthetic heparin with controllable structural and functional profiles, a different combined chemoenzymatic approach was also recently developed (Xu et al., 2011; Chappell and Liu, 2013; DeAngelis et al., 2013). The enzymes of heparin/HS biosynthesis, expressed in *E. coli* or insect cells, may be used for postpolymerization transformations of a heparosan-like polymer made using bacterial enzymes. This allows the incorporation of N-trifluoroacetetylglucosamine rather than N-acetylgalactosamine, and therefore easy chemical deacetylation and re-N-sulfation if necessary.

### IV. Molecular Interactions of Heparin

#### A. Heparin Interactions with Small Molecules

Heparin binds a wide variety of basic small molecules. Heparin is found naturally in mast cell granules, where it interacts with histamine; this interaction has been analyzed in some structural detail and is highly ordered and specific, with the imidazolium ring of protonated histamine located between two IdoA2S residues by hydrogen bonds between the histamine NH and IdoA carboxylates (Chuang et al., 2000). Histamine alters the profile of oligosaccharides resulting from depolymerization with heparinase I (Chuang et al., 2001, 2002).

Heparin binds to basic dyes such as methylene blue, azure A, and toluidine blue (Shrimer and Sasisekharan, 2013), brilliant cresol blue (Zhang et al., 2002), and pinacyanol chloride (Nandini and Vishalakshi, 2010), altering the absorbance maximum (Templeton, 1988) (the “metachromatic” effect), and this property can be used in a quantitative assay or in histology (Templeton, 1988), as well as for visualization of bands in gel electrophoresis of GAGs (Volpi and Maccari, 2002). For the most part, these interactions are susceptible to disruption at ionic strengths in the physiologic range, and they thus cannot be used for direct heparin assays in serum or plasma; however, the recently described Mallard blue [named after the steam locomotive (Hale, 2005), not the duck], and Heparin Red (Szelke et al., 2010) give improved affinity for heparin and can be used to measure heparin concentrations in serum directly (Bromfield et al., 2013a), in contrast with older methods that require prior chromatographic isolation of heparin (Jaques et al., 1990).

#### B. Heparin Interactions with Proteins

The high degree of sulfation of heparin makes it one of the most strongly anionic biologic macromolecules. Heparin will therefore interact to some extent with any basic molecule it encounters. Many proteins display basic amino acid residues on their surfaces, sometimes clustered into patches of positive charge that form so-called heparin binding domains. Because heparin itself is located in mast cell granules, liberated into the surrounding tissue only briefly on degranulation (Green et al., 1993; Wang and Kovanen, 1999), it is likely to come into contact with only the subset of proteins found in mast cell granules, the immediate ECM and possibly the circulation, as elevated levels of heparin-like material have been measured in patients with allergic diseases who undergo regular mast cell degranulation (Lasser et al., 1987). Interestingly, such patients also have a mild hemostatic defect consistent with elevated heparin levels in the circulation (Szczeklik et al., 1991). However, the ECM is rich in GAGs, including HS; thus, proteins in this milieu that have evolved HS binding sites will inevitably also bind heparin when it is present, because the same structures are present in both. Indeed, many of the therapeutic applications of heparin are based on modifying the physiologic functions of HS.

Even the simplest of interaction models, that of heparin interacting with polylysine, can be analyzed in structural terms. It had been understood for many years that heparin is capable of inducing α-helical conformation in basic homopolypeptides (Gelman and Blackwell, 1973; Stone, 1977). Circular dichroism can be used effectively to monitor the induction of regular secondary structure in polypeptides (Wawrzynczak et al., 1988), and this technique was used to show that heparin interacted equally well with both poly(D-lysine) and poly(L-lysine) (Mulloy et al., 1996). Such studies demonstrated that there was no intertwining of the two linear polymers; heparin and polypeptide simply lined up alongside one another, with the periodicity of sulfate clusters in heparin closely matching that of the α-helix in polylysine.

The structural biology of heparin-protein complexes is based on both experimental and theoretical approaches.
There are, at the time of writing, over 35 crystal structures of heparin-protein complexes in the Protein Data Bank (PDB) (www.rcsb.org). Many of these experiments use heparin oligosaccharides as model compounds for HS S domains; another portion involves synthetic oligosaccharides based on the AT-binding pentasaccharide. Solution studies of complexes, largely dependent on NMR spectroscopy, are able to identify heparin binding sites on the protein surface but can say relatively little about the detailed orientation of the heparin molecule with respect to these sites, although the combination of NMR with molecular modeling can be fruitful (Pomin, 2014). Table 1 lists a number of examples of heparin/protein complexes for which some structural information is available; this table cannot be comprehensive but is intended to give an indication of the sheer number and variety of heparin/HS binding proteins.

A comprehensive account of heparin-binding proteins, published by Conrad (1997), regarded AT as the prototype for heparin-protein interactions in general. A specific sequence in heparin, containing at its core an essential pentasaccharide structure, is necessary for a high-affinity heparin-AT interaction leading to enhanced inhibition of serine proteases in coagulation (Fig. 1A). In the years since the discovery of this structure, at about the same time that Louis B. Jaques’ original review was published (Jaques, 1979; Petitou et al., 2003), no other example of such a selective sequence in heparin, HS, or any other GAG, has been described. Despite this, there is equally no doubt that the fine structure of HS is physiologically important and finely controlled; it results, for example, in distinctive and consistent compositional patterns in HS from different mouse tissues (Ledin et al., 2004). Within the same tissue, phage display antibodies have shown that epitope structures have a defined topological tissue distribution, confirming the strict regulation of HS biosynthesis (Dennissen et al., 2002). In addition, genetic manipulation experiments have shown that the consequences of failure to express enzymes of HS postpolymerization modification are not all equivalent; for example, the phenotype of mice lacking HS 2-OST (Bullock et al., 1998) is not the same as that of mice lacking HS 6-OST (Bullock et al., 1998). More comprehensive discussion of HS interactions with proteins can be found in recent reviews (Lindahl and Li, 2009; Lindahl and Kjellén, 2013; Xu and Esko 2014).

Similarly, the search for a specific protein sequence or structure with special affinity for heparin has not yielded a single answer either in terms of sequence, secondary, or tertiary structure. The identification of the Cardin and Weintraub (1989) XBBXBX and XBBBXXBX (where B is a basic amino acid and X is a hydrophobic amino acid) “consensus” sequences for heparin binding, and further elaborations, gave rise to a productive period in the location of heparin binding sites and domains on numerous proteins. In the same study, Cardin and Weintraub also introduced the concept of secondary structure periodicity, in which noncontiguous basic amino acids may be brought together on the same “side” of an α-helix or β-strand, forming a concentrated basic patch capable of binding to GAGs.

A number of alternative heparin binding sequences were also proposed: LIGRK and LIGRR in fibronectin and heat-shock proteins (Hansen et al., 1995), TXXBXXTBXXXTBB (Hileman et al., 1998) in growth factors, and XKKXXXXKRXX in a β-sheet three-finger loop in cobra cardiotoxin (Vyas et al., 1997). Although the proposed consensus sequences for heparin binding are neither necessary nor are they sufficient to define a heparin binding site, the concept has been and remains productive (see recent articles on Sonic hedgehog by Chang et al., 2011; Farshi et al., 2011). Sequence-based binding motifs are attractive because they can be used to search sequences and databases, but it must be kept in mind that searches of this type can always yield false negatives and false positives.

The mode of binding of heparin to the proteins through which it exerts its physiologic and pharmacological properties is therefore highly variable, from the strongly specific interaction of a defined oligosaccharide sequence with AT to the assembly of multimeric protein complexes, dependent more on the polyanionic nature of heparin than its fine structure (Imberty et al., 2007; Seyrek and Dubin, 2010). It is necessary to understand the specificity and affinity of interactions between proteins and heparin/HS to design new heparin-based drugs (Coombe and Kett, 2005), especially where heparin is acting as an HS mimetic, in which the length and spacing of S and NA domains must be considered, as well as the detailed sequence (Kreuger et al., 2006).

As sufficient computing power became readily available, it proved possible to perform searches for heparin binding sites on protein surfaces by a number of theoretical molecular modeling techniques, using the three-dimensional structure of proteins rather than merely the sequence. The development of drug discovery techniques using computational chemistry also provided a number of tools for ligand-protein interaction studies, of which a frequently used example is the technique of docking. In this technique, the optimum (lowest energy) position and orientation of a ligand probe with respect to a protein structure is identified. Calculations of this kind can take place at high resolution, adjusting the “pose” of a ligand within its known binding site, or they can be used to survey the entire surface of a protein for likely binding sites. In the case of heparin-protein interactions, the latter of these models is the more common, and several different protocols have been developed (Bitomsky and Wade, 1999; Sadir et al., 2001; Forster and Mulloy, 2006; Gandhi et al., 2008; Mottarella et al., 2014).
Molecular modeling can be used to aid in the design of experiments and to help interpretation of experimental results. Docking calculations can be useful to visualize the implications of mutagenesis experiments (Knappe et al., 2007) or changes in the NMR spectrum of a protein on binding to heparin (Veverka et al., 2009). Where the main interest lies in identifying a heparin binding site, the calculations need not provide a full simulation of the protein-ligand complex in solution and cannot be relied on for the exact orientation of the ligand at the binding site (Forster and Mulloy, 2006). However, where detailed analysis of the interaction is required, greater care must be taken to accommodate complexities such as the conformational plasticity of the iduronate residue (Samsonov and Pisabarro, 2013) and the role of solvent in the interaction (Samsonov et al., 2011). A strategy for high-throughput screening of heparin/HS sequences has also been developed using docking calculations (Raghuuraman et al., 2006).

C. Heparin and Cytokines, Growth Factors, Selectins, and Proteins of the Extracellular Matrix

The survey of heparin-protein interactions in the text that follows, and in Table 1, cannot hope to be comprehensive and concentrates on those systems of most immediate relevance to the pharmacology of heparin, rather than the full spectrum of HS structure/function relationship.

The numerous small, soluble proteins present in the spaces between cells, whether they are cytokines, morphogens, growth factors, or morphogen/growth factor inhibitors, encounter cell surface and matrix-bound HS at relatively high concentrations; for many of them, modulation of diffusion by HS binding is key to their function (Yan and Lin, 2009). Structures of heparin and heparin-like molecules are of considerable interest for functional and pharmacological reasons. The numerous small, soluble proteins present in the spaces between cells, whether they are cytokines, morphogens, growth factors, or morphogen/growth factor inhibitors, encounter cell surface and matrix-bound HS at relatively high concentrations; for many of them, modulation of diffusion by HS binding is key to their function. Structures of heparin and heparin-like molecules are of considerable interest for functional and pharmacological reasons.

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Some systematic studies of chemokine binding to heparin structures have taken place. A mass spectrometric study (Yu et al., 2005b) identified heparin octasaccharides with 11 and 12 sulfates as able to form complexes with several chemokine receptor 2 ligands, inducing dimerization in some chemokines [monocyte chemoattractant proteins (MCPs) MCP-1/CCL2, MCP-2/CCL8], but not others [MCP-3/CCL7, MCP-4/CCL13, eotaxin/CCL11]. A heparin microarray study of eight chemokines (de Paz et al., 2007) provided the interesting results that the macrophage inflammatory protein (MIP)-3b/CCL19 did not bind and stromal-derived factor (SDF)-1/CCL12 bound weakly to highly sulfated heparin sequences, although other studies have characterized the SDF-1/heparin complex in some detail (Ziarek et al., 2013).

On activation, platelets release a number of heparin-binding chemokines such as PF4, MIP-1α/CCL3, MCP-3/CCL7, IL-8/CXCL8, and regulated on activation normal T cell expressed and secreted (RANTES)/CCL5 (Boehlen and Clemetson, 2001). Because PF4 interacts with heparin to form the antigenic aggregates that give rise to heparin-induced thrombocytopenia (HIT), a side effect of anticoagulant therapy with heparin, the molecular basis of this interaction has been studied in some detail. PF4 binds with high affinity to a particularly large HS motif of approximately 9 kDa in size, protecting it from heparinase digestion; this motif consists of two S domains with an NA-domain linking sequence between them (the SAS motif), able to wrap around the PF4 tetramer so that each S domain can reach one of the heparin binding sites (Stringer and Gallagher, 1997). The unsulfated linking domain does not interact with PF4. The antibodies that cause HIT are raised to closely packed complexes of multiple PF4 tetramers bound together with heparin (Rauova et al., 2005; Greinacher et al., 2006); large, highly sulfated heparin molecules are likely to offer more opportunities for multivalent complexes to form than LMWH or fondaparinux (Greinacher et al., 2006). It may also be the case that heparin binding induces conformational change, leading to partial unfolding of PF4 (Mikhailov et al., 1999).

The heparin binding sites of both MIP-1α/CCL3 and MIP-1b/CCL4 have been identified (Koopmann and Krangel, 1997; Koopmann et al., 1999) and the MIP-1α/CCL3 binding motif HS identified (Stringer et al., 2002) as another SAS motif, in which long S domains (12–14 monosaccharides) are separated by a short NA domain linker.

The chemokine RANTES/CCL5 has been crystallized in complex with heparin oligosaccharides (Shaw et al., 2004). The structure was used to engineer nonheparin binding RANTES/CCL5 variants that act as inhibitors of endogenous RANTES/CCL5 (Johnson et al., 2004; Shaw et al., 2004). Other modified RANTES/CCL5 variants, lacking the ability to dimerize, also lose their GAG binding capacity and act as inhibitors of wild-type RANTES/CCL5 (Rek et al., 2009).

The interactions of multiple chemokines in the presence of heparin or HS are not independent. It has recently been suggested that the phenomenon of chemokine cooperativity, in which one chemokine can enhance the activity of a second, is due to competition for matrix GAGs, rather than downstream signaling pathway convergence or heterodimerization (Verkaar et al., 2014).

3. Bone Morphogenetic Proteins and Their Antagonists. The bone morphogenetic proteins (BMPs) and growth and differentiation factors are members of the transforming growth factor (TGF)-β structural superfamily, as are most of the group of proteins known as BMP antagonists. Many members of this family, including TGFβ itself (Lyon et al., 1997), bind heparin. Together they form complex systems involved in developmental morphogenesis that is not by any means restricted to bone generation (Rider and Mulloy, 2010; Brazil et al., 2015). HS is critical for the functionality of some, but not all, of these morphogenetic systems, and manipulation of their heparin binding properties can be used in drug design and in controlled-release systems for therapeutic proteins (Reguera-Nuñez et al., 2014; Lee et al., 2015). With the exception of BMP-1, all of the BMPs have a cystine knot structure and can form homo- or heterodimers. Their heparin binding sites are generally thought to be in the flexible N-terminal tail, outside the structured cystine knot, and not seen in crystal structures (Rider and Mulloy, 2010; Gandhi and Mancera, 2012).

Crystal structures of follistatin in complex with sucrose octasulfate and with inositol hexasulfate have indicated the likely location of its heparin binding site (Innis and Hyvönen, 2003). Follistatin antagonizes myostatin and activin, and the follistatin-activin and follistatin-myostatin complexes have higher affinity for heparin than follistatin alone (Zhang et al., 2012a). An engineered construct of follistatin, developed for therapeutic use in muscle regeneration, lacks heparin-binding capacity and thus has improved pharmacokinetics (Datta-Mannan et al., 2013; Yaden et al., 2014).

The BMP antagonist noggin binds with high affinity to BMP-2, BMP-4, and BMP-7 (Zimmerman et al., 1996). Noggin binds heparin and cell-surface HS through a contiguous sequence rich in basic residues (Paine-Saunders et al., 2002), which, in the crystal structure of the noggin/BMP-7 complex, is located distant from the BMP binding site (Groppe et al., 2002). A mutation in the heparin binding site of noggin has recently been associated with hearing loss (Masuda et al., 2014).

The structures of two heparin-binding members of the closely related Cerberus and Dan (CAN) family of BMP antagonists have been solved: sclerostin by NMR spectroscopy (2K8P.pdb and 2KD3.pdb, respectively) (Veverka et al., 2009; Weidauer et al., 2009), and protein related to DAN and Cerberus (PRDC; also known as...
The heparin binding site of VEGF forms a belt-like region in the C-terminal domain of the HS-binding isoform of the protein (Robinson et al., 2006; Krilleke et al., 2007; Jeong et al., 2013). Angiogenesis is guided by VEGF held in the ECM by HS proteoglycans (Zoeller et al., 2009); this interaction, and its disruption by heparanase, is important for development, wound healing, and cancer (Vlodavsky et al., 2011). Some, but not all, snake venom VEGFs also bind heparin (Nakamura et al., 2014), probably via their unstructured C-terminal domains (Suto et al., 2005).

Midkine and pleiotrophin are di-domain growth factors with limited expression in normal tissues, but they are upregulated in malignancy (Kadomatsu et al., 2013). The interaction between the multifunctional growth factor midkine and heparin is unusual in that the hinge region between the two domains is important for heparin binding, as determined by an NMR study using the pentasaccharide fondaparinux (Lim et al., 2013). Pleiotrophin is associated with chondroitin sulfate–mediated neurite outgrowth (Li et al., 2010) and is important in bone repair (Lamprou et al., 2014).

The multidomain growth factor HGF/SF has a heparin binding site in the NK1 domain (Zhou et al., 1998, 1999) and has been crystallized in complex with a heparin oligosaccharide (Lietha et al., 2001), demonstrating interactions with both the N and K1 domains. HS is required for HGF/SF signaling (Sakata et al., 1997) and the smallest even-numbered heparin oligosaccharide capable of supporting signaling is a tetrasaccharide (Delehedde et al., 2002), but a smaller trisaccharide with only two sulfate substituents is also effective (Deakin et al., 2009), regardless of the positions of sulfation. It is therefore not surprising that effective small molecule heparin mimetics have been designed that act as inhibitors of HGF signaling (Raiber et al., 2007).

5. Fibroblast Growth Factors. In addition to restriction of diffusion through the matrix, HS can act at the cell surface as a coreceptor for some, although not all, FGFs. The FGF-19 group of endocrine growth factors (FGF-19, FGF-21, and FGF-23) has low affinity for heparin and thus can diffuse freely through the matrix and circulation (Harmer et al., 2004b; Beenken and Mohammadi, 2012). However, sulfated GAGs are necessary for signaling, probably through interaction with the receptor complex (Nakamura et al., 2011). FGF-1 and FGF-2 have been the subjects of particularly intense structural biology studies with apparently contradictory and controversial results. The coreceptor activity of HS involves the formation of a ternary complex of FGF, fibroblast growth factor receptor (FGFR), and HS, so that both HS-FGF and HS-FGFR interactions may in principle be involved in addition to FGF/FGFR complex formation.

The crystal structures of FGF-1 and FGF-2 in complex with heparin oligosaccharides have different stoichiometry. The crystal structures of FGF-2 with heparin (1BFΒ and 1BFC.pdb) (Faham et al., 1996) have 1:1 stoichiometry. By contrast, FGF-1 forms a dimer with no direct contact between protein monomers, around a single heparin oligosaccharide (1AXM, 2AXM.pdb) (DiGabriele et al., 1998). The sulfate clusters on one side of the heparin chain interact with one monomer, and the sulfates on the other side interact with the other. The two monomers therefore interact with opposite polarities of the heparin chain, and thus interact with different sequence motifs; superficial analysis of the binding epitope could mean that many heparin sequences might display one copy of either motif and thus bind 1:1 with FGF-1.

Crystallized ternary structures of heparin decasaccharide and FGF-2 with the ligand-binding region of FGFR1 (1FQ9.pdb) (Schlessinger et al., 2000) and heparin dodecasaccharide with FGF-1 and FGFR2 (1E00.pdb) (Pellegrini et al., 2000) are not the same (Pellegrini, 2001), either in structure or stoichiometry. The 2:2:1 FGF-1/FGFR2/heparin complex is reminiscent of the FGF-1/heparin crystal structure and contains the same asymmetry, and two receptor monomers bind each to a monomer of FGF-1; the single heparin molecule forms a bridge between the two FGF-1/FGFR2 complexes. By contrast, the FGF-2/FGFR1/heparin complex has a 2:2:2 stoichiometry: the two receptor monomers interact directly, and a deep linear cleft accommodates the nonreducing ends of two heparin chains. The physiologic significance of these two types of complex is not clear, but neither is an artifact, both can be prepared in solution, and it has been suggested that both complex types could be involved in signaling through larger focal complexes (Harmer et al., 2004a).

Numerous studies have attempted to define clear HS sequence preferences for different FGFs, in terms of direct interaction with the growth factor and of functional interaction in the FGFR complex. The size and sequence requirements for heparin potentiation of FGF signaling seem to vary depending on the exact
FGF-FGFR pairing concerned (Ostrovsky et al., 2002; Rudd et al., 2010b). It has been proposed that individual FGF-FGFR complexes form a canyon with HS selectivity defined by the combination, rather than simply the sum of FGF-HS and FGFR-HS requirements (Mohammadi et al., 2005). The use of synthetic heparin-like oligosaccharides has been particularly informative, particularly a set of hexasaccharides with sulfate substitution arranged so that only one side of the polysaccharide chain displays the sulfate clusters characteristic of heparin, as illustrated in Fig. 3 (Muñoz-García et al., 2014). Remarkably, one of these oligosaccharides binds to FGF-1 and supports signaling (Fig. 3B), whereas the other has lower affinity for FGF-1 and does not support signaling (Fig. 3C) (Angulo et al., 2004). The ability to support formation of the FGF-1 dimer as seen in the crystal structure of the complex (DiGabriele et al., 1998) is therefore not always necessary for the HS coreceptor. It is also worth noting that although the active hexasaccharide may be a minimal sequence for FGF-1 activity, it is not uniquely active, because the opposite side of the chain can bear either a full set of sulfates or none at all and probably any pattern between the two extremes. In addition, it has also been determined that the minimum length of fully sulfated heparin oligosaccharide that can induce dimerization of FGF-1 is an octasaccharide (Brown et al., 2013). A minimal sequence for binding to FGF-2 was determined as -UA-GlcNS-UA-GlcNS-IdoA2S- (Maccarana et al., 1993). The ability of HS to FGF-2 was determined as -UA-GlcNS-UA-GlcNS- can induce dimerization of FGF-1 is an octasaccharide length of fully sulfated heparin oligosaccharide that and probably any pattern between the two extremes. In chain can bear either a full set of sulfates or none at all and is not uniquely active, because the opposite side of the chain can bear either a full set of sulfates or none at all and probably any pattern between the two extremes. In addition, it has also been determined that the minimum length of fully sulfated heparin oligosaccharide that can induce dimerization of FGF-1 is an octasaccharide (Brown et al., 2013). A minimal sequence for binding to FGF-2 was determined as -UA-GlcNS-UA-GlcNS-IdoA2S- (Maccarana et al., 1993). The ability of HS fragments and chemoenzymatically generated HS-like oligosaccharides to form ternary complexes with several FGF-FGFR combinations have not demonstrated any dependence on a particular sequence of 2-O- and 6-O-sulfation, but on the length and overall degree of sulfation (Jastrebova et al., 2006). A similar charge dependence was also shown for the capacity of oligosaccharides to support FGF-2 signaling in Chinese hamster ovary cells (Jastrebova et al., 2010); however, in all of these cases, a minimal active sulfation pattern could be embedded into numerous more highly sulfated sequences, thus escaping detection in these experiments (Lindahl and Kjellén, 2013).

A systematic survey of binding of FGF-2, VEGF, and SDF-1 with carefully characterized preparations of partially desulfated LMWH fractions using surface plasmon resonance (SPR) has been reported (Roy et al., 2011). Although all the compounds tested remained heterogeneous, it was possible to show that 6-O-sulfation had more of an effect on binding to VEGF and SDF-1 and 2-O-sulfation on FGF-2.

D. Heparin, the Complement System, and Innate Immunity

The complement pathway acts to facilitate the combat of infection in after activation of both the innate and adaptive immune systems. It is initiated through three distinct pathways: the classic pathway, through the adaptive immune response; the alternative pathway, through exposure to anionic surfaces; and the lectin pathway, through the recognition of foreign carbohydrate structures. Via a complex protease cascade mechanism, all of the pathways lead to activation of the complement protein C3, ultimately resulting in the formation of the membrane attack complex that directly attacks foreign cells leading to cell lysis, and the so-called anaphylotoxins by products, C5a and C3a, which are known to act as chemoattractants for neutrophils (Jose et al., 1990). C3a and C5a can also activate and degranulate mast cells. It is therefore of interest in the regulation of the inflammatory response that many complement proteins are capable of binding to heparin, and a survey of the relative affinities of such proteins for a heparin-coated surface has been carried out, leading to an estimate that complement proteins in the circulation may well be bound to heparin to a significant extent at therapeutic heparin concentrations (Yu et al., 2005a). The ability of heparin (exogenous and presumably endogenous) and its mimetics to influence the complement system may also, in time, lead to new therapies for diseases involving complement activation, such as in the treatment of renal failure (Zaferani et al., 2014).

Heparin acts to modulate the complement system at more than one stage. In the early stages of the complement cascade, linked into the contact pathway of coagulation initiation, heparin acts in its role as a positive modulator of serpin activity, by interaction with the C1 esterase inhibitor (C1inh). A detailed study of C1inh, heparin, and complement component 1 subcomponent s (C1s) carried out using consecutive double-capture SPR (Rajabi et al., 2012) demonstrated a significant potentiation of C1inh inhibition of C1s. Heparin also potentiates the activity of C1inh against coagulation factor XIa, but has no effect on C1inh inhibition of kKIIa (Wuillemin et al., 1996).

C1inh consists of a serine protease inhibitor (serpin) domain at its C terminus, together with a highly glycosylated N-terminal domain, apparently not directly involved in serpin activity (Zeerleder, 2011). Mutants with altered glycosylation in the serpin domain have been studied (Rossi et al., 2010) and are active. C1inh with an N-terminal truncation has been crystallized (2OAY.pdb), and a potential heparin binding site was identified, not at the helix D location (which is obscured by the N-terminal domain) as for AT, but at the enzyme-inhibitor interface (Beinrohr et al., 2007). This has allowed a rationalization of the differential effects of heparin on inhibition by C1inh of different proteases, in terms of the surface charges of the protease; the face of kKIIa that interacts with C1inh is acidic, rather than basic as is the case for Xa and C1s (Beinrohr et al., 2007).

The direct interaction of sulfated polysaccharides such as fucoidan with the C1 complement protein
complex, specifically with C1q, has been proposed as a mechanism for inhibition of the complement cascade (Tissot et al., 2003). Crystallographic studies have identified a heparin binding site on the globular region of C1q (Garlatti et al., 2010). In addition, fucoidan also exerts anticomplement activity through interacting with C4; structural studies of the interaction between C4 and a fucoidan oligosaccharide have shown that branching enhances affinity (Clément et al., 2010).

The alternative pathway of complement activation is both positively and negatively influenced by proteins bound to cell surface HS. The protein properdin is a positive regulator of the alternative pathway and has been observed to bind to the HS proteoglycan syndecan 1 in kidney proximal tubule epithelial cells of proteinuric rats, promoting the deposition of C3 on the cells (Zaferani et al., 2011). Another heparin-binding protein, complement factor H (CFH), also interacts with HS at the host cell surface, in this case protecting host cells from the complement system by interacting with C3b. CFH has also been shown to bind to the HS of kidney proximal tubule epithelial cells of proteinuric rats, but to a distinct epitope (Zaferani et al., 2012), raising the possibility that the fine structure of HS can influence the balance of activation and inhibition of complement at the cell surface.

CFH is a multidomain protein, consisting of a chain of 20 complement control protein (CCP) repeats, also referred to as the short consensus repeat. CFH has at least two distinct heparin binding sites, one in CCP 6–8 and one in CCP 19–20. Disruption of HS-CFH interactions may lead to loss of CFH from the host cell surface, and thus loss of protection against the complement system (Perkins et al., 2010).

It is known that mutations at or near the heparin binding site in CFH CCP 6–8 can predispose to age-related macular degeneration (Prosser et al., 2007), and that mutations at or near the CCP 19–20 site are associated with atypical hemolytic uremic syndrome (Józsi et al., 2006). There is also evidence that the two heparin binding sites have differential affinities for HS fine structure motifs, so that the 6–8 site might recognize an HS motif prevalent in the Bruch’s membrane of the eye and the 19–20 site might prefer a motif found in the kidney glomerular basement membrane (Clark et al., 2013); this leads to the concept of the HS “zip code,” a correlation between specific HS structures and their location within organs and tissues (Langford-Smith et al., 2014). An alternative approach to this question considers the two heparin binding sites together, rather than independently, and is based on studies that show bivalent CFH-heparin binding in vitro (Khan et al., 2012). If cooperative binding at both sites is necessary for secure anchoring of CFH to the cell surface, loss of affinity at one site may considerably weaken affinity of the cell surface for CFH (Perkins et al., 2014). This point of view does not require selectivity for specific local HS motifs, but it is not incompatible with the concept. However, the interaction between CFH 19–20 and sialic acid host glycans was characterized recently, raising the possibility that HS may not be the only, or even the major, glycan involved in CFH interaction in the kidney glomerular basement membrane (Blaum et al., 2015).

Peptidoglycan recognition proteins (PRGPs) form part of the mammalian innate immune system. One of them, PRGP-S isolated from camel milk, has been crystallized with heparin disaccharide in the binding pocket (3OGX.pdb) (Sharma et al., 2012). The interaction has an estimated $K_d$ (from SPR) of $3.3 \times 10^{-7}$ M. The functional significance of this interaction is not clear, because PRGPs bind to pathogenic carbohydrates. Although Sharma et al. point out that the E. coli K5 polysaccharide is closely related to structures in heparin, the disaccharide in their crystal structure is trisulfated, and the sulfates play a major part in the interaction.

### E. Other Heparin Binding Proteins

1. **Adhesion Molecules**. Heparin has been shown to bind to several adhesion proteins, including the leukocyte integrin αMβ2 or macrophage-1 (Mac-1) (Peter et al., 1999), the platelet integrin α(IIb)β3 (Sobel et al., 2001), and platelet endothelial cell adhesion molecule (PECAM)-1 (Coombe et al., 2008; Gandhi et al., 2008). Heparin inhibits several selectin-mediated processes involved in metastasis and inflammatory cell infiltration (see below), and the heparin-selectin interaction may well be a major mechanism by which heparin attenuates cancer metastasis (Laubli and Borsig, 2009). The selectins bind to several sialylated, fucosylated, and sulfated glycans; heparin, and HS, bind to L- and P-selectin, but not to E-selectin (Koenig et al., 1998). Although no atomic resolution structure for the selectin/heparin complex has been solved to date, the kinetics of heparin and LMWH binding to L- and P-selectin have been measured using the quartz microbalance (Simonis et al., 2007a,b).

Several anionic polysaccharides other than heparin have been studied for their ability to interact with selectins. Examples come from marine invertebrates and plants (Bachelet et al., 2009; Fitton, 2011; Kozlowski et al., 2011; Pomin, 2012; Gomes et al., 2015). An example is fucosylated chondroitin sulfate (Borsig et al., 2007), for which there is strong evidence of a conformational similarity between the branched GAG and the Lewis-X glycan motif, that has high affinity for L- and P-selectin (Panagos et al., 2014). Several anticoagulant properties of heparin have been studied in the context of cancer, as a drug for treating cancer patients (Sun and Walker, 1986). Heparin and its derivatives are used as anticoagulants in medical practice, and they are also used as antithrombotic agents for the treatment of cardiovascular diseases. Heparin has been shown to inhibit the activity of platelet-activating factor (PAF) receptors, which play a key role in the regulation of platelet aggregation and the formation of blood clots. In addition, heparin has been shown to inhibit the activation of coagulation factor Xa, which is an important step in the coagulation cascade.

2. **Snake Venom Cardiotoxins**. In 1986, heparin was noted to ameliorate the toxicity of a cobra cardiotoxin toward rat cardiac tissue in vivo (Sun and Walker, 1986). Cobra cardiotoxins are basic proteins, to which heparin binds at a cationic belt of conserved residues (Vyas et al., 1997). NMR spectroscopy and molecular...
modeling has confirmed the heparin binding site of cardiotoxins A1 and A3 from *Naja atra* (Sue et al., 2001; Tjong et al., 2007), and a crystal structure of *Naja atra* A3 cardiotoxin bound to a heparin hexasaccharide demonstrated the conformational change and dimerization of the toxin in the presence of heparin and citrate, which helps to explain the interaction of cardiotoxins with the cell surface (Lee et al., 2005). Although synthetic oligosaccharides were designed to explore the detailed molecular structure of the cardiotoxin binding motif in heparin or HS (Ke et al., 2005), a later study concluded that binding was more dependent on charge cluster organization in heparin rather than fine structure (Tjong et al., 2007).

3. Eosinophil-Granule Major Basic Protein and Eosinophil Cationic Protein. Eosinophil-granule major basic protein (EMBP) has a structure resembling the C-type lectins that bind carbohydrates in a calcium-dependent manner (Swaminathan et al., 2001). A crystal structure has been determined for EMBP in complex with a heparin disaccharide (2BRS), identifying a heparin binding site at an exposed loop area and no calcium coordination is involved in the binding (Swaminathan et al., 2005). In the same study, it was shown by SPR that both heparin and HS bind to immobilized EMBP. The authors speculate that binding to the cell surface HS allows EMBP to aggregate at the cell membrane, inducing cell lysis. Eosinophils are part of mammalian host defense against parasitic infections and eosinophil-derived granule proteins such as EMBP are thought to contribute to host defense through their ability to induce cell lysis. Another eosinophil-granule protein, eosinophil cationic protein (ECP), also binds heparin (Fredens et al., 1991; Torrent et al., 2011) and the structure of a complex of ECP with a synthetic heparin trisaccharide has been determined by NMR spectroscopy and molecular dynamics (2LVZ) (García-Mayoral et al., 2013). ECP is a member of the RNase A superfamily, but its cytotoxicity is not a function of its weak RNase activity. However, the catalytic triad of the RNase active site is involved in the interaction with heparin. As has been proposed for MBP, ECP may be concentrated at the cell surface by binding to HS, and the interaction may orient the protein in such a way as to aid destabilization of the cell membrane (García-Mayoral et al., 2013). Inappropriate release of such cytotoxic proteins during eosinophilic diseases such as asthma and rhinitis has been implicated in the damage to the respiratory epithelial cells characterizing these conditions. It is of interest therefore that the cytotoxicity of respiratory epithelial cells induced by cationic protein (Motojima et al., 1989) is inhibited by heparin (Fredens et al., 1991).

4. Annexin. The annexins are multifunctional proteins found both inside and outside the cell. The crystal structures of annexin-A2 bound to heparin tetrasaccharide (1HYU.pdb) and hexasaccharide (1HYV.pdb) (Shao et al., 2006) show an unusual degree of calcium dependence in their interaction, which indicates that a minimum of four to five monosaccharide units are necessary for the interaction. SPR gave a $K_d$ for polymeric heparin of 17 nM, at neutral pH in the presence of calcium.

5. Thrombospondin. The thrombospondins are extracellular proteins with numerous ligands, including heparin/HS. The N-terminal domain of thrombospondin (TSPN)-1 is involved in GAG binding and has anti-adhesive activity, as well as mediating the uptake and clearance of thrombospondin-1. A crystal structure has been determined for a complex between TSPN-1 and the synthetic compound fondaparinux, based on the pentasaccharide with high affinity for AT (Fig. 1A) (Tan et al., 2006) and subsequently for heparin oligosaccharides of dp 8 and dp 10 (Tan et al., 2008). All three structures agree on the location of the heparin binding site, and the special features of the fondaparinux pentasaccharide do not appear to be necessary for binding to thrombospondin. The longer oligosaccharides appear to elicit dimerization in TSPN-1 in a fashion dependent on oligosaccharide length; TSPN-1 complexed with the octamer forms a *trans*-dimer (one protein monomer of either side of the heparin chain), but with the decamer forms a *cis*-dimer (both protein monomers on the same side of the heparin chain). In this way, it is possible to see how a full-length heparin chain could aggregate several thrombospondin-1 molecules.

F. Protein Aggregation in the Nervous System

The intimate involvement of GAGs in the processes by which protein aggregates form and spread in nervous tissue was recently summarized (Dudas and Semenik, 2012). The amyloid precursor protein (APP) gives rise to the amyloid β-peptide (Aβ) implicated in Alzheimer’s disease. Although the physiologic role of APP is not known, it has been suggested that the secreted ectodomain of APP acts as a growth factor and utilizes cell surface HS as a low-affinity coreceptor (Reinhard et al., 2013). The roles of GAGs and proteoglycans in Aβ aggregation, and as potential antiamyloidogenesis agents, are the subject of an interesting review (Ariga et al., 2010), in which a number of potential therapeutic applications of GAGs are discussed.

APP and its mammalian homologs, the APP-like proteins, can form intracellular homo- and heterodimers; although APP is monomeric in solution in vitro, it dimerizes in the presence of heparin (Gralle et al., 2006), thus possibly reducing Aβ generation (Eggert et al., 2009). The E2 domain of APP-like protein 1 crystallizes as a dimer, and a heparin hexasaccharide soaked into the crystal is bound asymmetrically within the dimer, making different contacts with the two protein monomers; one heparin oligosaccharide is bound to each dimer (3QMK) (Xue et al., 2011). The stoichiometry holds also in solution, as determined by isothermal titration calorimetry.
The enzyme that cleaves APP, β-secretase (BACE)-1, is formed by cleavage of the zymogen pro–BACE-1, a process that is potentiated by heparin. A model of the proenzyme suggests a specific heparin binding site and a possible mechanism for this effect in which heparin-induced conformational change in the protein promotes easier contact between the substrate and active site (Klaver et al., 2010). However, heparin, HS, and heparin mimetics are observed to inhibit the production of Aβ both in vitro (Patey et al., 2006) and in cortical cells (Cui et al., 2012). The relationship between HS fine structure, molecular weight, and anti–BACE-1 activity has been actively pursued (Patey et al., 2008; Arungundram et al., 2009; Cui et al., 2012) and has led to the development of promising lead compounds in the octasaccharide to decasaccharide size range; the repeating non-natural disaccharide sequences GlcNAc6S-IDoA2S and GlcNAc6S-IDoA2S are found to be particularly effective, with minimal anticoagulant side effects (Schwörer et al., 2013).

Also associated with Alzheimer’s disease, as a major component of neurofibrillary tangles, is the microtubule-stabilizing protein τ (Avila et al., 2004). Heparin interacts with τ at several sites on the protein, inducing changes in secondary structure and becoming incorporated into paired helical filaments (Sibille et al., 2006). The propagation of τ aggregates through neural networks, involving release of τ into the extracellular space and uptake by adjacent cells, has been recognized as a major mechanism for the spread of neurofibrillary pathology (Medina and Avila, 2014), which may involve HS as a cell surface receptor (Holmes et al., 2013). HS is also found to act as a receptor for synuclein aggregates, but not for huntingtin; although heparin is known to induce synuclein aggregation (Cohlberg et al., 2002), there are no reports that this is the case for huntingtin.

Heparin and its mimetics, particularly the sulfated xylan known as pentosan polysulfate, have also attracted attention as possible anti–transmissible spongiform encephalopathy agents (Panegyres and Armari, 2013). In common with other disease states in which fibrillation is involved, the effect of heparin on prion protein aggregates has proved to be concentration dependent. At low heparin/prion ratios, induction of β-sheet structure and fibril formation in prion protein PrP(106–206) were enhanced by heparin; however, at high heparin/prion ratios, the opposite was the case, and fibril formation was inhibited (Bazar and Jelinek, 2010).

**G. Interactions between Heparin and Pathogens**

1. **Viruses.** Heparin affinity chromatography can be used to purify several viruses from complex biologic media, including hepatitis B and C (Zahn and Allain, 2005), human papilloma virus (HPV) (Kim et al., 2012), porcine reproductive and respiratory syndrome virus (Hu et al., 2010), and lentiviruses (Segura et al., 2010), including human immunodeficiency virus HIV-1 (Nassar et al., 2012), as recent examples.

Heparin and other GAGs bind to the HIV-1 envelope glycoprotein gp120, involving heparin binding sites at the V3 loop and elsewhere (Crublet et al., 2008). SPR of GAGs on immobilized gp120 has been used to determine that binding is dependent on both length and degree of sulfation (Matos et al., 2014). Remarkably, sequence similarities have been noted between the heparin-binding V3 loop of HIV-1 and the heparin-binding Duffy binding protein of *Plasmodium vivax* (Bolton and Garry, 2011a,b).

HS is involved in cell attachment and entry of the foot-and-mouth disease virus subtype O1. The crystal structure of the viral capsid with heparin (1IQP) (Fry et al., 1999) shows a heparin binding domain made up from elements of three separate peptides, the capsid proteins V1, V2, and V3. There is evidence that heparin/HS binding is acquired or enhanced in cell culture, as opposed to field strains, and the capsid of the A10(61) cell culture adapted strain has also been crystallized with heparin (IZBE) (Fry et al., 2005).

HPV 18 and HPV 16 are both capable of causing cervical cancer, and use cell surface HS as a receptor. Crystal structures of HPV18 (3OFL) and of HPV 16 (3OAE) with bound heparin oligosaccharides show numerous heparin binding sites, in contrast with foot-and-mouth disease virus (Dasgupta et al., 2011).

Adeno-associated virus serotype 2 is used as a vector in experimental gene therapy. It binds heparin and can be isolated using heparin affinity chromatography (McClure et al., 2011). Electron microscopy has been used to study the capsid-heparin complex in two studies published almost simultaneously (Levy et al., 2009; O’Donnell et al., 2009). In one study (O’Donnell et al., 2009), the heterogeneity and conformational complexity of the UH used as ligand made it difficult to produce more than a rough model of the binding site. The second study (Levy et al., 2009), using a partially depolymerized heparin, suffered the same problems, perhaps to a lesser extent; however, both studies agreed on the location of the major heparin binding site in the valleys between protrusions at the 3-fold axis of the icosahedral capsid. The studies differ on the subject of conformational change to the capsid on heparin binding; if such changes occur, they are likely to be fairly subtle.

HSV-1, but not HSV-2, can use cell surface HS as a coreceptor (Akhtar and Shukla, 2009). HSV-1 has an unusual interaction with HS, because it requires 3-O-sulfation to interact with the viral capsid protein gD and to trigger membrane fusion. Moreover, the AT-binding sequence formed by the 3-OST-1 isoform (Fig. 1A) has no affinity for gD; the sequences formed by 3-OST-2, 3-OST-3, 3-OST-4, and 3-OST-6 are necessary, because in addition to a 3-O-sulfated glucosamine, an upstream 2-O-sulfated iduronic acid is also required. A gD binding octasaccharide containing the characteristic sequence

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*Mulloy et al.*
IdoA2S-GlcNH23S6S- has been isolated from bovine kidney HS (Liu et al., 2002).

HS is also implicated as a receptor for dengue virus. Its binding site on the DENV envelope protein has been identified by site-directed mutagenesis (Watterson et al., 2012), and comparison of DENV from several different strains has provided a rationalization of the differences in heparin binding between them, in terms of charge distribution on the envelope surface (Kostyuchenko et al., 2014).

2. Malaria. The apicomplexan parasite *Plasmodium falciparum* causes severe malaria in humans. Heparin inhibits merozoite invasion of erythrocytes, binding to numerous apical proteins (Kobayashi et al., 2013). Infected erythrocytes bind to each other (“rosetting”) and to the vascular endothelium, leading to occlusion of the microvasculature. The *P. falciparum* erythrocyte membrane protein PfEMP1 is present at the surface of infected erythrocytes, and this protein binds to endothelial and cell surface HS in a manner that can be competed out by intravenous injection of modified heparin with minimal anticoagulant activity (Vogt et al., 2006). Low-anticoagulant heparin also disrupts rosetting in fresh clinical isolates (Leitgeb et al., 2011). The interaction between heparin and infected erythrocytes has, unusually, been investigated by single-molecule force microscopy (Valle-Delgado et al., 2013); a binding force in the range 28–46 pN was observed for infected cells, with no binding to noninfected cells.

The heparin binding site of PfEMP1 lies near the N terminus of the multidomain protein and has been located on the Duffy-binding–like DBLa module of VarO strain PfEMP by cocystallography of the N-terminal sequence–DBLa fragment with heparin dodecamer, with additional experimental evidence from site-directed mutagenesis and molecular modeling (Juillerat et al., 2011). Homology modeling of DBLa modules from a number of *P. falciparum* strains has also been used, along with docking calculations, to locate the heparin binding site (Agrawal et al., 2014), but it is difficult to compare the two studies, because the N-terminal sequence was not included in the homology modeling study.

3. Bacterial Heparin Lyases. Heparinases I, II, and III are bacterial heparin lyases from *Pedobacter hepaticus*, with distinct specificities for particular linkages in heparin and HS (Jandik et al., 1994). For some of them, crystal structures in complex with heparin oligosaccharides have been solved (Table 1). They are useful in the detailed study of heparins from different sources, for example (Zhang et al., 2011).

The specificities of the bacterial heparin and heparan lyases have been studied by enzymic cleavage of well-defined oligosaccharides (Linhardt et al., 1990; Rhomberg et al., 1998a,b). Heparinase I cleaves the linkage GlcNS6S-IdoA2S, leaving an unsaturated uronic acid at the nonreducing end of the product oligosaccharides. This enzyme has been described as acting in a random endolytic fashion (Jandik et al., 1994) and also in a processive, exolytic mode (Ernst et al., 1998). Heparinases I and II can cleave the AT-binding motif (Shriver et al., 2000b). Heparinase II, the enzyme that breaks down both S and NA domains, can be mutated at a single active site residue so that it can cleave at this important site (Zhao et al., 2011). Heparinase III, also known as heparitinase I, cleaves the unsulfated NA domains of heparin and HS and thus is a valuable reagent for determination of the S-domain size and spacing in HS (see Barne et al., 2000).

**H. Heparin in Its Native Environment**

1. Interactions with Granule Contents. Heparin proteoglycan (serglycin) is packed into mast cell secretory granules, along with histamine and abundant serine proteases (in humans, principally chymase 1, β-tryptase, and carboxypeptidase A3) (Hellman and Thorpe, 2014; Wernersson and Pejler, 2014). In this context, heparin, and sometimes chondroitin or dermatan sulfate, is assumed to act simply as a polyelectrolyte, allowing efficient packing of the basic proteins and histamine (Rönnberg et al., 2012). Serglycin is necessary to preserve the morphology of mast cell granules and storage of mast cell proteases (Rönnberg and Pejler, 2012), and the absence of the NDST-2 isoform that initiates the sulfation of heparin has similar, but less marked, effects as the absence of serglycin (Forsberg et al., 1999; Humphries et al., 1999).

On degranulation, heparin is released from the mast cell into surrounding tissues (Green et al., 1993; Wang and Kovanen, 1999) along with the proteases. In sharp contrast with the net effect of heparin on serine proteases and their inhibitors in the coagulation system (see below), heparin protects and enhances the activity of mast cell serine proteases (Pejler and Sadler, 1999; Hallgren et al., 2005). Once degranulation has taken place, the net effect of the high concentration of heparin, proteases and histamine on local tissue is not clear cut. The released proteases have both pro- and anti-inflammatory activities; heparin activates the proteases and can also bind to and protect their cytokine substrates. The exact course of events during an inflammatory episode must be unique to each set of circumstances. The contribution of the potent anticoagulant activity of heparin to the mix is still unknown.

Human β-tryptase II crystallizes as a square-shaped tetramer (Pereira et al., 1998) with the active sites of the four monomers facing inward. The periphery of the tetramer displays a long linear area of positive charge, presumably the binding site through which heparin stabilizes the active tetramer; in the absence of heparin, tryptase dissociates into an inactive, monomeric form. The heparin binding site contains histidine residues, protonated only at the low pH of the mast cell granule,
so that on release into neutral pH, the protease should rapidly dissociate from heparin and presumably lose activity.

There is no evidence that chymase adopts a heparin-stabilized oligomer; this protease has an acidic face at and around its active site and an opposite basic face that will inevitably interact with heparin (McGrath et al., 1997). Approximation of the enzyme with positively charged peptide substrates may explain heparin’s activation of chymase (Pejler and Sadler, 1999).

Other hematopoietic cell types also contain protease-filled granules (of a wider variety than mast cells) and serglycin (Wernersson and Pejler, 2014). Some of these are basic and will bind heparin; some are not. The classification of mast cells into connective tissue and mucosa, with distinct heparin or chondroitin sulphate contents, is based on rodent examples and may not be generalizable to all species and tissues. More recently, confirmed human mast cell proteases include granzyme H (Rönnberg et al., 2014).

2. Enzymes of Heparin Breakdown. Mammalian heparanase is a glycosyl hydrolase. No crystal structure is available, but its three-dimensional structure has been modeled (Ilan et al., 2006). The active enzyme is a heterodimer of two peptides, resulting from cleavage of a single precursor peptide, and contains two predicted heparin binding domains. The structures of HS and heparan can be significantly modified by heparanase when it is upregulated in inflammation (Li and Vlodavsky, 2009), by endoglycolytic activity of the enzyme, cleaving at the reducing side of glucuronic acid residues. Heparanase partially depolymerizes mast cell heparin (Gong et al., 2003) to give chain-length distribution in the same range as commercially purified heparin (although the manufacturing process also plays a part) (Mulloy et al., 2014). Heparin (including LMWH) is also an inhibitor of heparanase activity, when measured using ECM as a substrate (Naggi et al., 2005). The inhibitory activity of heparin is much increased by chemical modification to its structure, such as glycol splitting and N-acetylation, resulting in a product that is not such a good substrate (Naggi et al., 2005). Because heparanase is strongly implicated in the breakdown of matrix in tumor metastasis, these modified heparin compounds have potential as antimetastatic agents (Casu et al., 2008). The detailed substrate specificity of heparanase was studied recently (Mao et al., 2014) using a mass spectrometric approach. Heparanase was found to cleave at the nonreducing side of highly sulfated domains, exposing potential FGF-2 binding motifs and thereby increasing the FGF-2 potentiating capacity of treated HS.

Both UH and LMWHs are cleared in the liver, initially by binding to the hyaluronic acid receptor for endocytosis, also known as stabilin-2, then by endocytosis into lysosomes (Harris et al., 2008). The enzymes of lysosomal breakdown of HS, when dysfunctional, give rise to some of the group of conditions known as mucopolysaccharidoses (MPSs). As a result, the structures of the enzymes have been intensively studied. The best known of these is Hurler syndrome MPS-1, in which α-L-iduronidase is dysfunctional. The human enzyme was described by Freeman and Hopwood (1992). Recent structural studies have revealed a remarkable involvement of the N-glycan in the catalytic action of this enzyme (Maita et al., 2013). A recombinant form of this enzyme is available for treatment of MPS-1. Hunter syndrome, or MPS-2, is caused by a deficiency in iduronate sulfatase (Tylki-Szymańska, 2014), and again a recombinant enzyme is used in treatment. Heparin acetyl-coenzyme A/N-acetylglucomaminide N-acetyltransferase is an integral lysosomal membrane protein containing 11 transmembrane domains. Deficiencies of this enzyme lead to mucopolysaccharidosis IIIC (Fan et al., 2011a).

V. Analysis

Heparin as a drug was traditionally characterized principally in terms of its anticoagulant activity, applied as a test for both identity and quantity. The development of the LMWHs, a series of related products differing in their molecular weight distributions and in the structural consequences of their depolymerization, made the development of new analytical tools necessary. These new methods were only sporadically applied to UH until the events of 2007 to 2008, when contamination of heparin with OSCS caused adverse events in a number of patients in several countries, including a number of fatalities in the United States (Blossom et al., 2008; Chess et al., 2012). In addition, the possibility of the reintroduction of bovine heparin for clinical use has spurred the application of analytical strategies to compare heparin from different species (Santos et al., 2014). Recent reviews summarize methods for the characterization of heparin (Jones et al., 2011; Shriver et al., 2012).

A. Physicochemical Identification

The definitive tests for the identity of heparin must be those that rely on its specific enhancement of the inhibitory activity of AT toward thrombin and FXa. However, the physicochemical properties of heparin—its overall high negative charge, its molecular weight, and its sugar composition—can all be used to design chromatographic and spectroscopic tests for the identity of heparin. For UH, the purpose of an identification test would be to distinguish heparin from other sulfated polysaccharides, and it might also be used as an assurance of the absence of contaminating compounds such as OSCS. For LMWHs, identification tests also must distinguish between individual products.

Identity tests for heparin can be both spectroscopic and chromatographic, and a secure identification is best
based on two or more tests that respond to different distinguishing properties of heparin; this strategy is referred to as “orthogonal” testing. For example, the pharmacopeias of both Europe and the United States contain both NMR spectroscopy and ion exchange chromatography tests for UH. In addition, the U.S. Pharmacopeia monograph requires UH to comply with molecular weight criteria (see below).

B. Molecular Weight Measurements for Heparin

Mast cell heparin proteoglycan (serglycin) is partially degraded by heparanase (Gong et al., 2003) and heparin prepared from pig intestinal mucosa is a polydisperse polysaccharide with a mean molecular weight in the range of 14,000–20,000, the exact figure depending on the manufacturing process in addition to the starting material (Mulloy et al., 2014). It is likely that some low molecular weight fragments are left behind; a highly sulfated, low molecular weight HS isolated from heparin byproducts (Casu et al., 1983) may in fact be of mast cell origin. Many of the biologic activities of heparin are molecular weight dependent, and analytic techniques have been developed based largely on size exclusion chromatography (SEC), although electrophoretic (Edens et al., 1992) and spectroscopic (Desai and Linhardt, 1995) methods have also been devised. For UH, the use of SEC calibrated using light scattering detection is the most common method in use (Kristensen et al., 1991; Bertini et al., 2005; Sommers et al., 2011). However, interlaboratory variation in results from this method is not insignificant; for comendial use, a broad-standard calibrant material has been established for heparin sodium (Mulloy et al., 2014). It has been understood for many years that the hydrodynamic properties of heparin are such that protein or neutral polysaccharide standards do not provide accurate results for SEC of heparin (Harenberg and de Vries, 1983; Volpi and Bolognani, 1993; Khan et al., 2010).

In the case of LMWH, molecular weight distribution is a defining parameter distinguishing one product from another (Gray et al., 2008). The same techniques that are applied to UH can be used for molecular weight determinations (Desai and Linhardt, 1995; Knobloch and Shaklee, 1997). In addition, a calibration technique can be based on the UV absorbance of terminal unsaturated uronic acids of LMWH produced by the β-elimination mechanism (Nielsen, 1992). If it is assumed that the UV label is evenly distributed over all molecular weights, the UV absorbance of the sample is proportional to molar concentration. The refractive index (RI) increment of a heparin solution is proportional to weight concentration. A chromatographic system equipped with both UV and RI detectors can therefore be used to provide the ratio of weight concentration to molar concentration and thus derive the molecular weight at any given retention time, for a calibrant of known mean molecular weight (Van Dedem and Nielsen, 1991). In another calibration method, a single polydisperse LMWH can be used as a broad standard, requiring only RI detection (Mulloy et al., 1997; Mulloy and Hogwood, 2015).

C. Spectroscopy of Heparin

NMR spectroscopy of heparin started almost 50 years ago (Jaques et al., 1966; Perlin et al., 1970) and has been crucial in research to establish the composition and sequence of heparin as well as its conformational properties in solution (see above). The use of NMR for analytical purposes grew as equipment of the necessary sensitivity and resolving power became generally available (Neville et al., 1989; Tachibana et al., 1990), and $^{13}$C NMR was incorporated into the European Pharmacopeial monographs for LMWH products. NMR has been recognized as a particularly powerful analytical tool, allowing the simultaneous determination of the major and minor structural motifs that characterize LMWHs (Casu and Torri, 1999; Guerrini and Bisio, 2012).

The subjective test of “similarity” between the spectrum of a heparin sample and of a heparin reference material is insufficient to guarantee the absence of a contaminating substance. Pharmacopeial monographs now provide objective criteria for the positions and intensity of certain $^1$H NMR signals and require the identification of unrecognized signals; this has led to the elucidation of several minor structures in heparin (Kellenbach et al., 2011; Mourier et al., 2011, 2012; Beccati et al., 2012).

The increased emphasis on well defined physicochemical quality criteria for heparin has made gross contamination of heparin exceptionally difficult, but the introduction of a trace contaminant is harder to discourage in this way. In addition, the stipulation that heparin must be derived solely from porcine mucosa, common to both the U.S. and European pharmacopeial monographs for heparin, is not easy to monitor by chromatographic or spectroscopic methods. However, it is not impossible; recently developed systematic methods of mathematical analysis of NMR spectra, using database comparisons, are able to detect samples containing only 1% ovine or bovine heparin in porcine heparin (Rudd et al., 2012).

D. Physicochemical Quantification

Physicochemical quantification of heparin in biologic fluids is, perversely, much less straightforward than quantification of anti-Xa or anti-IIa activity using chromogenic substrates (see the relevant sections). Interaction with small molecule dyes can be used for heparin in aqueous solutions of low ionic strength (Templeton, 1988), but the dyes Mallard blue and Heparin Red, capable of binding heparin quantitatively in serum, were only recently developed (described above; Bromfield et al., 2013a). Earlier methods have
relied on prior removal of heparin from the biologic medium using precipitation, cation exchange chromatography (Jaques et al., 1990), or immobilized polylysine (Mohammad et al., 1980).

Heparin sensors have recently been reviewed (Bromfield et al., 2013b). A sensor capable of quantitative response to heparin in therapeutic concentrations in plasma has been a goal of many research groups in the past few decades. Problems of ionic strength and autofluorescence have hampered the achievement of sufficiently high sensitivity (Bromfield et al., 2013b). Researchers devised a poliyon-sensitive membrane electrode that is capable of estimating heparin concentration in blood by potentiometric titration with protamine (Ramamurthy et al., 1998, 1999).

Graphene oxide (GO) is a water-soluble single carbon layer capable of interacting with many biomolecules (Cai et al., 2011). A heparin sensor system has been designed in which the fluorescence of a pyrene-based oligoelectrolyte is quenched by interaction with GO; the fluorescence is rescued by heparin, which competes with GO for the oligoelectrolyte (Cai et al., 2011). Gold nanorods aggregated on a GO surface by protamine disaggregate in the presence of heparin, changing the SPR response and color, with a lower limit of quantitation of 3.0 ng/ml in aqueous solution (Fu et al., 2012a,b).

E. Sequence Analysis

In spite of the problem of heterogeneity, it is possible to apply some sequence determination techniques to the analysis of heparin. Two main approaches can be distinguished: 1) oligosaccharide fingerprinting, including disaccharide composition, applicable to any heparin/HS regardless of molecular weight distribution and sequence heterogeneity; and 2) full sequence analysis, only applicable to homogeneous, short oligosaccharides.

1. Disaccharide Analysis. The action of heparinases I, II, and III in combination yields a mixture of disaccharides, and these may be separated by HPLC (Zhang et al., 2011) or capillary electrophoresis (Shriver and Sasisekharan, 2015) in the analysis of heparin preparations. Heparin lyases work by a β-elimination mechanism, leaving an unsaturated uronic acid at the nonreducing end. The UV absorbance of this residue can be used for detection, or a fluorescent label can be used to increase sensitivity (Skidmore et al., 2010). This method of depolymerization has the great disadvantage that both iduronate and glucuronate yield the same unsaturated residue at the nonreducing end; information on the nonreducing terminal uronic acid is therefore lost. Techniques for separation and mass spectrometric characterization of nitrous acid depolymerized oligosaccharides, with intact uronic acids at the nonreducing ends, have been devised (Gill et al., 2012). Combined techniques, including a separation step and a mass spectrometry (MS) step, are powerful (Zaia, 2009). The separation step allows for quantitation of the fragments and the MS step allows for their identification. For example, ion-pairing reverse-phase ultra-performance liquid chromatography (LC) with electrospray time-of-flight (TOF) MS, for disaccharide analysis of heterogeneous heparin samples, can distinguish between bovine and porcine mucosal heparins (Korir et al., 2008). Zhang et al. (2009) describe a quantitative disaccharide composition approach, using 13C-, 15N-labeled disaccharides as internal standards in ion-pairing reverse LC-MS.

The use of cetyltrimethylammonium-coated columns has been noted to improve the performance of strong anion exchange chromatography in the separation of heparin-derived oligosaccharides (Mourier and Viskov, 2004). The resulting column was used in a sequencing strategy without the use of MS, merely specific fragmentation and chromatography; minor disaccharides were resolved and identified by NMR. The method has been used in an investigation of the effects on activity of non-native minor structural components of LMWH introduced by chemical depolymerization (Guerrini et al., 2010).

Volpi and Linhardt (2010) provided a useful protocol for the recovery of GAGs from tissue samples, or GAG capsules from E. coli, as well as enzyme digestion, SAX chromatography, or reverse-phase ion-pair HPLC with UV or MS detection. Direct electrospray ionization MS on intact GAGs, inducing fragmentation to monosaccharides, can be used as an analytical tool and can detect less than 1% chondroitin sulphate/dermatan sulphate in heparin (Hu et al., 2009, 2011).

2. Sequence Determination. Full sequence determination of oligosaccharides cannot be used as an analytical technique for heparin in any kind of routine application. However, some interesting research techniques based on MS have been developed for heparin as for other carbohydrates (Kailemia et al., 2014). Labeling techniques can be used to mark reducing termini as well as to aid sensitive detection. Rhomberg et al. (1998a) used matrix-assisted laser desorption ionization MS and capillary electrophoresis of defined oligosaccharide substrates to follow the time course of heparinase I and heparinase II digestion. They use a semicarbazone “mass tag” to distinguish the original reducing ends from those generated during digestion, and so derive more detailed sequence information than from disaccharide analysis alone. Other digestion procedures, using both chemical and enzymatic depolymerization methods with fluorescent labeling, can also give improved sequence data (Turnbull et al., 1999). PAGE can be used to separate oligosaccharides longer than a disaccharide. Matrix-assisted laser desorption ionization MS, plus enzymatic and chemical degradation, has been used to sequence decasaccharides from heparin with reducing-end GlcNS3S6S (Shriver et al., 2000a). An optimized method involving the use of a crystallinonorharmane matrix and ionic liquid,
1-methylimidazolium-α-cyano-4-hydroxycinnamate was introduced for the matrix-assisted laser desorption ionization TOF/TOF analysis of heparin oligosaccharides (Oguma et al., 2007), and a bioinformatics tool was devised for the semiautomated analysis of the resulting mass spectra (Lawrence et al., 2008).

A particular problem for mass spectrometric studies of heparin is a strong tendency to sulfate loss on ionization, although there are methods that can ameliorate this problem at least for shorter oligosaccharides (Kailemia et al., 2012). The practical limit of useful MS data is reached for oligosaccharides approximately 22 monosaccharides in length; longer chains can be detected, but MS is insufficiently sensitive for quantitative use (Doneanu et al., 2009). An LC-MS method with additives in the buffer system could identify molecular ions with no sulfate loss up to a 22 mer for oligosaccharides from the LMWH tinzaparin. In a different approach, hydrophilic interaction chromatography and Fourier transform MS has been used to compare two intact LMWH products (Li et al., 2012). It remains the case that LC-MS cannot be used to determine the molecular weight of LMWH, because MS rapidly loses sensitivity at high molecular weight.

MS can also play a different part in the stable isotope analysis of heparin from different species and areas of origin (Jasper et al., 2015).

**F. Detection of Contaminants and Impurities**

Heparin is a natural product, isolated from animal tissues using a series of chemical treatments. The finished drug substance product is likely to contain impurities from the tissue of origin and from the reagents used in its manufacture. Pharmacopeial monographs have specified limits for protein and nucleic acid impurities for many years, until recently, using simple, low-technology methods. In addition, when NMR spectroscopy was used to survey heparin products on the market, it was found that heparin contained a sizeable percentage of other GAGs, particularly dermatan sulfate (Fig. 1D), which copurifies with heparin in several protocols (Liverani et al., 2009). The production and regulation of UH manufacture was uneventful for several decades until 2007 to 2008, when it came to light that some heparin lots were associated with serious adverse effects, including fatalities (Sasisekharan and Shriver, 2009). The narrative of this series of events, from the point of view of a heparin manufacturer involved, was presented elsewhere (Chess et al., 2012).

The reaction to these events by regulators was prompt, and a group of experts was rapidly convened. Identification of the structure of the contaminant by Guerrini et al. (2008) was performed and published in a matter of a few weeks in February and March 2008. The heparin lots associated with adverse events contained varying proportions of OSCS (Fig. 1F), a semisynthetic modified GAG made by per-O-sulfonation of naturally occurring chondroitin sulfate (Fig. 1E) (Maruyama et al., 1998). Two-dimensional NMR established the identity of the contaminant by comparison with a synthetic standard; digestion with chondroitinase ABC followed by disaccharide analysis confirmed the chondroitin backbone. The biologic basis for the adverse effects was ascribed to activation of the contact system (Kishimoto et al., 2008), and epidemiologic studies were able to trace the link between specific batches of contaminated heparin and the adverse reactions of specific patients (Blossom et al., 2008). In addition to activating the contact system, OSCS (and OSCS-contaminated heparin) also inhibits the complement cascade through its interaction with C1inh (Zhou et al., 2012b), more strongly than heparin alone; this is consistent with earlier observations that dextran sulfate is an effective potentiator of C1inh (Wuillemin et al., 1997).

The initial analytical response from the U.S. Food and Drug Administration was to introduce tests for the presence of OSCS in heparin samples using two methods: $^1$H NMR and a capillary electrophoresis separation. Since then, numerous alternative methods have been proposed, some of which have been incorporated into pharmacopeial monographs. The use of $^1$H NMR spectroscopy has been widely adopted for compendial purposes (McEwen et al., 2008, 2010). The contaminant OSCS has a distinctive $^1$H NMR spectrum (Maruyama et al., 1998) with an intense signal arising from its N-acetyl methyl, well resolved and clearly visible when present in the $^1$H NMR spectrum of heparin (Guerrini et al., 2008). This allowed the rapid screening of heparin samples for the presence of the contaminant that could be interpreted quantitatively (McEwen et al., 2008). Both qualitative and quantitative estimations of the presence of OSCS can be devised. NMR spectroscopy for compendial identity tests is frequently performed by subjective comparison of a spectrum with that of a reference material; detection of OSCS or other yet-unknown sulfated polysaccharides demands objective criteria, listing features of the spectrum that must be present as well as those that should not be present, with some semiquantitative measures of relative peak heights (VSP Monograph on Heparin, 2014). A potential problem for detailed analysis of the heparin spectrum is the effect of divalent cations, such as calcium or manganese (from potassium permanganate bleaching processes). The addition of EDTA corrects the chemical shift and linewidth variations induced by divalent cations (McEwen, 2010), and the calcium effects can be utilized to aid the identification and quantification of OSCS (McEwen et al., 2009).

Anion exchange chromatography can separate untreated GAGs by charge and has sufficient resolving power to separate heparin from GAGs with lower charge (dermatan and chondroitin sulfates and artificially oversulfated GAGs such as OSCS). Both SAX (Trehy et al., 2009; Keire et al., 2010) and weak anion
exchange (Hashii et al., 2010) can be used. An extra dimension of specificity can be added to chromatographic separations of heparin and OSCS by means of nitrous acid digestion that depolymerizes heparin while leaving NA chondroitins untouched (Viskov et al., 2009a).

The presence of OSCS in heparin products raises the concern that other “side-stream” components of crude heparin may be persulfonated as well. Guerrini et al. (2009b) used two-dimensional heteronuclear single quantum coherence to resolve signals from other GAGs. Waste products at various stages of heparin production were analyzed, persulfonated, and then compared with OSCS-contaminated heparin. It was clear that the OSCS contamination is not oversulfated GAG waste because no sign of oversulfated heparin or HS was found.

Overviews of the contamination problem and the rapid analytical response to it were provided by Beni et al. (2009a) and Sasissekharan and Shriver (2009). The development of new analytical methods for heparin was reviewed by Beni et al. (2011).

McKee et al. (2010) report on the acute response to contaminated heparin, with the mean onset time being about 5 minutes in dialysis patients. Using a sensitive enzyme immunoassay, Bairstow et al. (2009) examined the effects in vivo of OSCS-contaminated heparin. The maximum no observed effects level for OSCS is approximately 1 mg/kg in rats and pigs; 2 mg/kg produces significant hypotension. The potential possibility of OSCS-induced HIT has also been discussed (Warkentin and Greinacher, 2009).

Methods for quantification and detection of OSCS include a Taq polymerase inhibition assay (Tami et al., 2008), enzyme immunoassay (Bairstow et al., 2009), and potentiometric methods (Wang and Meyerhoff, 2010) that can be developed into sensor technology (Gemene and Meyerhoff, 2010; Kang et al., 2011). Other ingenious methods for detection and quantification of OSCS in heparin include a heparinase inhibition assay (Aich et al., 2011), a convenient disposable strip-type electrochemical polyanion sensor (Kang et al., 2011), pyrolysis MS (Nemes et al., 2013), and spectropolarimetry (Chmielewski et al., 2011; Stanley et al., 2011).

Although the OSCS contamination problem chiefly affected UH, contamination of LMWH was also possible. OSCS was found to be depolymerized by both base-catalyzed eliminative cleavage of the heparin benzyl ester and by hydrogen peroxide treatment, although not by nitrous acid or heparinase I (Wang and Meyerhoff, 2010).

In addition to OSCS, the possibility of contamination with other sulfated polysaccharides is real and these may be more difficult to detect. Nonetheless, some ingenious methods have been devised to screen heparin products for oversulfated polysaccharides in general. The structural feature of OSCS used in the standard one-dimensional $^1$H NMR tests for its presence is the N-acetyl methyl; a similar compound in which the N-sulfate replaces N-acetyl has been prepared and a detection method based on radical depolymerization and LC-MS has been developed (Li et al., 2014a,b). Diffusion-ordered NMR spectroscopy can also be used to assess whether a heparin sample has been mixed with another component of different molecular weight (Bednarek et al., 2010).

VI. Mechanism of Action of Anticoagulant Activity

Heparin exerts its anticoagulant action through interaction with coagulation factors and inhibitors. Coagulation is a complex process involving soluble proteins, platelets and cellular components such as endothelial cells and monocytes (Versteeg et al., 2013), and hemostasis is maintained when actions of procoagulant clotting factors are counteracted by coagulation inhibitors. The major plasma coagulation inhibitor is AT, which targets activated coagulation factors such as FxIIa, FxIa, Fxa, FxVa, FxVIIa, and FIIa. Since these activated coagulation factors are serine proteases, AT is classified as a serine protease inhibitor (Serpin). Heparin, acting as a catalyst, exerts its anticoagulant action through potentiation of the inhibitory activity of AT and other serpins (Fig. 4). Although the interaction with AT requires a specific pentasaccharide sequence, no specific structural requirements have been identified for binding to other serpins (Huntington, 2011). Another important heparin binding inhibitor in the extrinsic system is tissue factor pathway inhibitor (TFPI) (Ellery and Adams, 2014). Figure 5 illustrates the inhibitory action of the various heparin binding coagulation inhibitors on the coagulation cascade and the mechanism of actions of heparin and inhibitor complexes are described in more detail by Gray et al. (2012). The essential aspects of the effects of heparin on coagulation, with some more recent advances, are summarized below.

Although the mode of interaction of heparin and AT was partially elucidated by 1982 (Björk and Lindahl, 1982), a full description of the way in which heparin affects the activity of AT had to wait for advances in the 1990s and 2000s ( Olson et al., 2010; Huntington, 2011). The crucial discovery was the identification of the specific sequences in heparin and HS with high affinity for AT (Fig. 1C). Within the cardiovascular system, AT interacts with HS in the glycoalyx of vascular endothelial cells (Chappell et al., 2009); this interaction may play a more prominent role in anti-inflammatory protection of the endothelium than in anticoagulation (Shworak et al., 2010).

In its nonheparin-bound state, AT is a relatively slow-acting serpin, because its reactive center loop is partially folded into the center of a $\beta$-sheet structure. When bound to heparin, AT shows a considerable increase in inhibitory action, which is a hallmark of serpins (Huntington, 2003). The high-affinity pentasaccharide sequence in heparin binds to antithrombin in a two-stage
process; first, an initial binding event involves three of the monosaccharide units and initiates conformational change in AT, and then the interaction is completed, stabilizing the heparin-activated conformation of AT (Desai et al., 1998). Conformational changes are transmitted through the AT structure, leading to expulsion of the reactive center loop region and an increase in the exposure of an AT exosite that binds directly to FXa (Fig. 4A).

The reactive center is a substrate-like structure, present in all serpins, to which the active sites of several serine proteases involved in coagulation can bind to, but not cleave, and thus form a stochiometric and inactive complex. The interaction of the protease active site will cleave the reactive center loop and cause further conformational change in AT, which draws in the protease causing inactivation by deformation as well as directly by blocking the enzyme’s active site. Once the inhibitor and protease are bound, heparin is released from the complex and can act to catalyze another reaction. Unlike AT, heparin is not consumed in the inhibitory reaction (Huntington, 2006).

A. Antithrombin-Mediated Inhibition of Factor IIa (Thrombin) and Factor Xa

The activated conformation of AT induced by the pentasaccharide sequence is sufficient to enhance the inhibitory action on FXa, the protease that converts prothrombin to thrombin (FIIa) (Fig. 5). FXa interacts directly with AT at a specific exosite exposed on binding to heparin (Izaguirre et al., 2014). In addition, in the presence of calcium, a single heparin chain can bind to both AT and FXa directly, enhancing the AT-FXa interaction further (Rezaie and Olson, 2000; Lin et al., 2001), but it is not an absolute requirement. This calcium-dependent effect explains earlier reports of a calcium-induced molecular weight dependence of the inhibition by AT of FXa (Barrowcliffe and Le Shirley, 1989).

In contrast with FXa, potentiation of thrombin inhibition by AT requires an additional 13-saccharide chain attached to the nonreducing end of the pentasaccharide sequence, so that thrombin and AT both bind to the same heparin molecule. Thrombin interacts with heparin at its exosite II, in a fundamentally different mode than AT (Mosier et al., 2012). No particular heparin sequence is required for this interaction; thrombin has been crystallized with a regular heparin fragment (Carter et al., 2005), and a complex of AT and thrombin with a heparin mimetic has been crystallized in which the thrombin binding moiety is a sulfated oligoglucose (1TB6.pdb) (Li et al., 2004).

B. Antithrombin Inhibition of Other Coagulation Factors

AT is also able to inhibit a number of other coagulation proteases (Fig. 5). The mode of inhibition of FIXa has been found to be similar to FXa, with FIXa binding to the same exosite on AT. A high-resolution crystal structure of a pentasaccharide-FIXa-AT complex (3KCG.pdb) has shown that one pentasaccharide binds to AT and a second binds to an exosite on FIXa, in such a way as to suggest the possibility of association of both proteins with a single heparin molecule (Johnson et al., 2010). As with heparin-mediated FXa binding to AT, it has been shown that longer heparin chains will bind with good affinity in the presence of Ca2+, further enhancing inhibition (Wiebe et al., 2003).

In the intrinsic arm of the coagulation pathway, the ability of heparin to enhance AT inhibition of FXIa (Olson et al., 2004) and kallikrein (Gozzo et al., 2006) is relatively limited compared with FXa and thrombin. It is interesting to note that the anti-FXIa activity of a mutant AT lacking its heparin binding site is still potentiated by heparin, indicating that direct interaction between heparin and FXIa is involved (Yang et al., 2010); a potential heparin binding site has been identified in the catalytic domain of FXIa (Jin et al., 2005).

AT inhibits the FVIIa complex in the extrinsic pathway, and this inhibition is enhanced by fondaparinux, LMWH and UH. A direct, calcium-dependent interaction has been shown between FVIIa and heparin (Martínez-Martínez et al., 2011).

C. Other Heparin-Activated Serpins

Heparin cofactor II (HCII), another serpin that is potentiated by heparin, is a coagulation inhibitor that inhibits only thrombin; HCII is reported to also inhibit
chymotrypsin and neutrophil cathepsin G (Huntington, 2013). HCII is present in plasma at similar levels to AT (Tollefsen, 1997), but HCII cannot substitute for AT in AT deficiency. HCII deficiency has no effect on plasma coagulation, but it does give rise to an increase in the formation of occlusive arterial thrombi after damage to the endothelium (He et al., 2002). In vivo HCII is potentiated by dermatan sulfate, which is present in blood vessel walls (Tovar et al., 2005). Dermatan sulfate–activated HCII may therefore play a part in the prevention of excessive thrombosis in vascular injury (Tollefsen, 2010).

Heparin and dermatan sulfate both potentiate the inhibition of thrombin by HCII by several orders of magnitude (Tollefsen, 1997), but HCII cannot substitute for AT in AT deficiency. HCII deficiency has no effect on plasma coagulation, but it does give rise to an increase in the formation of occlusive arterial thrombi after damage to the endothelium (He et al., 2002). In vivo HCII is potentiated by dermatan sulfate, which is present in blood vessel walls (Tovar et al., 2005). Dermatan sulfate–activated HCII may therefore play a part in the prevention of excessive thrombosis in vascular injury (Tollefsen, 2010).

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Protein C inhibitor (PCI) regulates the activity of activated protein C (APC), which is the active form of the zymogen protein C. Protein C is converted to APC by thrombin (Comp et al., 1982), and in this active form acts as an anticoagulant by inactivating FVa and FVIIIa, in the presence of a cofactor protein S. PCI therefore regulates an inhibitor of coagulation, acting in a manner that can promote coagulation, rather than inhibit it. The binding of heparin to PCI accelerates the inhibition of APC and also of FXa in the presence of calcium (Sun et al., 2009). Long heparin chains are needed to enhance APC inhibition (Van Walderveen

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**Fig. 5.** Serpin interactions with heparin. Interaction of heparins with four serpins that inhibit coagulation serine proteases, from crystal structures. Serpins are shown as ribbon diagrams coded according to secondary structure (β-strands in blue, helices in red, and turns in green). Bound heparin molecules are shown as stick diagrams, and atoms are colored by element (carbon in gray, nitrogen blue, oxygen in red, and sulfur in yellow). (A) AT in its native (1TIF.pdb, on the left) and heparin-activated (from 3KCG.pdb, on the right) crystal structures. The bound heparin pentasaccharide (1) lengthens helix D (2), thus expelling a strand segment (3) and extending the reactive center loop, colored orange (4). The exosite that binds to FXa is exposed (5). (B) Complexes of thrombin (colored magenta) with AT and a synthetic heparin mimetic (1TB6.pdb) on the left and HCII (1JMO.pdb) on the right. Thrombin engages with the reactive center loop of the two serpins in different geometries; the orientation of thrombin with respect to the serpin is maintained by the heparin-thrombin interaction in the case of AT, and by a long, hirudin-like N-terminal sequence in HCII. (C) PCI (2HI9.pdb) with a heparin oligosaccharide placed close to the predicted heparin binding site on helix H. Two orientations are shown: one (on the left) corresponds to the other diagrams in this figure, and the other (on the right) is rotated 90°. The crystal structure of a PCI-heparin-thrombin complex (3B9F.pdb; not shown) supports the idea that heparin forms a bridge between the heparin binding sites of PCI and thrombin. (D) Protease nexin 1 in complex with thrombin (colored magenta) (4DYT.pdb). Two different orientations of PN-1 and thrombin are seen in the crystal structure: one (on the left) in which the reactive center loop is engaged with the active site of thrombin, and another (on the right) in which the engagement is not productive, but the heparin binding residues (yellow ball and stick) of the two proteins are aligned. These two complexes may represent two stages of the interaction (expanded with permission from Gray et al., 2012).
et al., 2010), which suggests that both PCI and the protease need to bind to heparin simultaneously. The structure of native PCI complexed with heparin and thrombin has been solved (3B9F.pdb) (Li et al., 2008), revealing a binding site for heparin involving helix H, which is close to the reactive loop (Fig. 5C).

The importance of protease nexin (PN)-1 in hemostasis and vascular biology was recently recognized (Bouton et al., 2012). This serpin has long been known to inhibit thrombin approximately 100-fold faster than AT in vitro (Wallace et al., 1989), and heparin enhances this rate by about three orders of magnitude (Evans et al., 1991). However, PN-1 does not contribute to the anticoagulant activity of heparin in vivo because its concentration in plasma is very low. Rather, PN-1 is found bound to cell surfaces in numerous organs and tissues (Bouton et al., 2012), including blood vessel walls. PN-1 has been detected in platelet granules on the platelet surface and is secreted during platelet activation (Boulaftali et al., 2010). In this context, its contribution to the antithrombotic activity of heparin in vivo may have been underestimated.

The crystal structure of PN-1 has been solved in complex with heparin (4DY0.pdb) and with thrombin (4DV7.pdb) (Li and Huntington, 2012) (Fig. 5D). The protein has a typical serpin fold with a heparin binding site on helix D. In contrast with AT, however, the heparin binding sites of PN-1 and thrombin are not aligned when the reactive centre loop of PN-1 is productively engaged with the active site of thrombin. Initial formation of a ternary complex of PN-1 with both thrombin and heparin may therefore be the first stage of a two-phase interaction, with the heparin-thrombin interaction lost when the covalent PN-1–thrombin complex forms. Heparin is not released on the formation of the PN-1–thrombin complex (Evans et al., 1991), indicating that the PN-1/thrombin complex is likely to remain bound to the cell surface by HS.

The protein Z–dependent protease inhibitor is a serpin known to inhibit both FXa and FXIa (Yang et al., 2012). Heparin accelerates these interactions 20- to 100-fold (Huang et al., 2011). The heparin binding site on protein Z–dependent protease inhibitor involves basic residues on helix D (like AT) and helix C (unlike AT), and the presence of an unstructured N-terminal tail may indicate some similarity with HCII (Yang et al., 2012).

Another serpin, C1inh, inhibits both the complement cascade and the contact intrinsic pathway, where the coagulation and the innate immune systems interact. C1inh deficiency leads to hereditary angioedema through continuous overactivity of the contact system (Konings et al., 2013). The properties of C1inh are not limited to the complement and contact systems (Zeerleder, 2011). Heparin potentiates the activity of C1inh (Gozzo et al., 2003), and the crystal structure of C1inh (Beinrohr et al., 2007) indicates a different mode of action toward the protease and a wholly different heparin binding site to AT.

It has been noted that adverse reactions to OSCS-contaminated heparin are inversely correlated with C1inh plasma levels (Zhou et al., 2012a). On one hand, OSCS appears to activate the contact system through prekallikrein activation; on the other hand, OCSC seems to inhibit the contact system through potentiation of C1inh.

D. A Non-Serpin Inhibitor: Tissue Factor Pathway Inhibitor

TFPI is a serine protease inhibitor but it is not, structurally speaking, a serpin. It is the major inhibitor of the extrinsic pathway (Vadivel and Bajaj, 2012; Ellery and Adams, 2014) and is the subject of recent reviews (Adams, 2012; Ellery and Adams, 2014). The heparin-binding isofrom TFPIα consists of an acidic N-terminal region, three Kunitz tandem domains, and a basic C-terminal end (Bajaj et al., 2001). The Kunitz domains are involved in anticoagulant activity, with the first domain inhibiting FVIIa/tissue factor complexes and the second domain inhibiting FXa (Broze et al., 1988). The C-terminal sequence in TFPI has high affinity for heparin (Ye et al., 1998). Injection of heparin releases endothelium-bound TFPI (Sandset et al., 1988) into the circulation. Heparin bound to TFPI potentiates its inhibitory activity on free FXa and on FXa in FVIIa/tissue factor/FXa complex (Xu et al., 2002).

E. Measurement of Anticoagulant Activity of Heparin

Accurate measurement of the anticoagulant activity of heparin is important to labeling of therapeutic products and clinical monitoring. UH and LMWH are extracted from animal sources and are complex polydisperse molecules; as such, gravimetric mass units obtained using physicochemical methods (see the section on analysis) do not provide adequate information on their anticoagulant action. Similar to other biologics, the measurement of anticoagulant activity requires comparison with a reference standard, in a bioassay; and results are expressed as relative potency or relative activity to the standard. World Health Organization international and pharmacopoeial reference standards are available for potency assignment of heparin products. The history and development of heparin and LMWH units, standardization landmarks, and statistical considerations for bioassays are detailed by Gray (2012). Bioassays using citrated plasma or purified reagents are designed based on the ability of heparin to potentiate the inhibitory action of plasma coagulation factor inhibitors such as AT and HCII.

1. Plasma-Based Assays. The plasma-based assays are global assays and measure the potentiation of the inhibitory effect of coagulation factor inhibitors by heparin on activated coagulation factor inhibitors such as FXa, FIXa, FXa, and FIIa (thrombin). The end point of these
assays is clot formation; with an increasing amount of heparin, there is an increase in the prolongation of clotting times. A number of assays, including activated partial thromboplastin time (APTT) and protamine sulfate titration, have been used for measurement of heparin especially in clinical settings, and the final readouts from these assays are influenced by the quality of the plasma (e.g., high concentrations of PF4 and the presence of other anticoagulants). Although these plasma-based assays are commonly used for the measurement of UH, they are seldom used for LMWHs.

APTT is a screening test for detection of clotting factor deficiency. It is highly sensitive to heparin and is currently the method of choice for clinical monitoring of UH treatment. This method involves activation of plasma via the intrinsic pathway with a negatively charged activator (for example ellagic acid), in the presence of phospholipid and the clotting time is recorded after the addition of calcium. Although the APTT is easily adapted to run on automated instruments, results are variable and highly dependent on the APTT reagent used. It is recommended that therapeutic APTT ranges should be determined locally against therapeutic heparin levels obtained using anti-Xa assay or protamine titration (Hirsh and Raschke, 2004; Baglin et al., 2006). Until recently, variations on this test, using sheep plasma instead of human plasma, were used by the European Pharmacopeia and the U.S. Pharmacopeia as the pharmacopeial monograph methods for potency labeling of therapeutic UH. These methods were revised after the contamination of heparin with OCS and the current European Pharmacopeia and U.S. Pharmacopeia methods are based on the potentiation of the inhibitory action of AT on FXa and thrombin. The establishment of these new monograph assays may prevent future adulteration with contaminants that may increase overall anticoagulant activity in plasma-based systems.

The “protamine sulfate” titration assay has also been used for measurement of heparin in patient plasma samples and is based on the ability of protamine sulfate, a highly positively charged protein, to neutralize the anticoagulant activity of heparin (Refn and Vestergaard, 1954; Newall, 2013). The principle of the assay is based on the normalization of the heparin-prolonged thrombin clotting times by protamine sulfate. However, this assay is not easily automated and since protamine can also act as an anticoagulant (Kresowik et al., 1988), addition of excess protamine can lead to an incorrect estimation of heparin. This assay is therefore not recommended for potency labeling of heparin products.

2. Purified System Assays. The purified reagent methods are the methods of choice for potency labeling of therapeutic heparins. The current European Pharmacopeia and U.S. Pharmacopeia potency assays for both UH and LMWHs are based on the ability of heparin to potentiate the inhibition of thrombin (FIIa) or FXa by AT (U.S. Pharmacopeial Convention, 2014; European Pharmacopeia, 2015) and are known as anti-Xa or anti-IIa assays. These AT-dependent assays are highly specific for heparins because only heparin, LMWHs, and HS (and the synthetic pentasaccharide) are known to possess the essential pentasaccharide sequence that binds to AT (see the section on mechanism of action). These assays utilize purified proteins (AT, FXa, and FIIa) and are carried out by incubation of the heparin/AT mixture with either FXa or FIIa for a specified length of time. The residual FIIs or FXa cleave a chromogen from chromogenic substrates that are specific for FIIa or FXa. Color development is inversely proportional to the concentration of heparin.

Anti-Xa assays are also commercially available for monitoring LMWH treatment and the source of AT may come from the patient’s own plasma or exogenous AT may be included in the kit to avoid low level or depletion of AT in the patient’s plasma, which may lead to an underestimation of heparin concentration.

VII. Clinical Use as an Anticoagulant/Antithrombotic

The major clinical indications for heparins, both UH and LMWH, are in the prophylaxis and the treatment of venous thromboembolism (VTE), which is the focus of this section. However, other important uses are in acute coronary syndromes (Howard et al., 2014), extracorporeal circuitry (Lim et al., 2004; Sonawane et al., 2006; Cronin and Reilly, 2010; Davenport, 2011; Pon et al., 2014), and maintenance of the patency of indwelling catheters (Wang et al., 2013; Kordzadeh et al., 2014). It should be noted that LMWH preparations differ in their characteristics (Hovanessian 1999; Hirsh et al., 2001; Gray et al., 2008; Garcia et al., 2012; Guerrini and Bisio, 2012); hence, their clinical effects cannot be expected to be identical or interchangeable. However, an individual analysis of these agents is outside the scope of this review; thus, they are generally referred to here as a collective. Furthermore, a recent study assessing the quality of clinical trials that support the use of LMWHs in VTE prophylaxis found that there was a wide variation between individual studies, in particular among those performed before 1990 (Agnelli et al., 2015), which makes indirect comparison of these agents even more difficult.

Key risk factors for VTE are advanced age, obesity, cancer and its treatment, prolonged immobility, genetic factors such as known thrombophilia or family history, surgery (especially major orthopedic), and hormonal therapies such as hormone replacement therapy or combined oral contraception. All risks arise from the principles of Virchow’s triad, such that disturbance of the normal physiologic state of the blood itself, the flow of blood or the vessel wall will predispose toward
thrombosis, and a combination thereof will further increase the overall risk (Reitsma et al., 2012).

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are currently considered as the single clinical entity VTE and, as such, the prevention and treatment of DVT and PE follow a common approach. Indeed, associated and often asymptomatic PE can be demonstrated by appropriate diagnostic methodology in a significant proportion of patients with DVT (Huisman et al., 1989; Moser et al., 1994; Hughes et al., 2014) and, reciprocally, evidence of lower limb DVT is detectable in the majority of patients with symptomatic PE (Dalen, 2002a,b). Furthermore, it has been found that in the absence of treatment, approximately one-quarter of lower leg DVT cases extend to become proximal, with approximately one-half of those embolizing to the pulmonary circulation (Dalen, 2002a). VTE presents a major healthcare burden worldwide, with an incidence that increases with advancing age (ISTH Steering Committee for World Thrombosis Day, 2014); as such, VTE is unlikely to decline in significance as general life expectancy increases.

A. Venous Thromboembolism

1. Treatment of Venous Thromboembolism. Use of heparins in the treatment of PE and DVT is standard practice and has been subject to refinement over the last half-century, since the key report that UH (albeit in combination with a vitamin K antagonist), compared with no anticoagulant treatment, reduced death and recurrence of thrombosis in a small trial of patients with symptomatic PE (Barritt and Jordan, 1960). Once it had been demonstrated that fixed-dose regimens using LMWH, without the same need for routine monitoring, were as safe as treatment with UH with dose adjustment (based on results of plasma coagulation assays) for DVT (Holm et al., 1986; Prandoni et al., 1992) and PE (Bratt et al., 1985; Hull et al., 1992; Théry et al., 1992), the way was paved for the replacement of UH with LMWH to a large extent. After confirmation of efficacy and safety in larger clinical studies (Dalen, 2002b), LMWH treatment is now the preferred approach in the initial management of VTE (Garcia et al., 2012; Kearon et al., 2012; Cohen et al., 2014) and is used routinely in cases of PE or proximal DVT. However, although LMWHs are now standard therapy for VTE treatment and prophylaxis (Dalen, 2002b; Gray et al., 2008; Garcia et al., 2012; Kearon et al., 2012), UH may be more suitable in patients considered to be at a high risk of bleeding (Garcia et al., 2012; Cohen et al., 2014), owing to the relatively rapid cessation of anticoagulant effects upon termination of the infusion and, where necessary, greater susceptibility to reversal by protamine administration (Garcia et al., 2012; Pai and Crowther, 2012). Furthermore, UH with dosing guided by APTT monitoring is the agent of choice in patients with significant impairment of renal function (Cohen et al., 2014), in whom the rate of LMWH clearance tends to be reduced, thus predisposing these patients to bleeding, although not all LMWH preparations are equally affected in this respect (Rabbat et al., 2005; Lim et al., 2006; Cook et al., 2008; Siguret et al., 2011; Johansen and Balchen, 2013) and it may be appropriate in selected patients to use LMWH with dose adjustment based on anti-Xa measurements (Garcia et al., 2012). In general terms, however, the convenience of fixed-dosage regimens with once- or twice-daily administration—a afforded to LMWHs by their more predictable pharmacokinetic profile, as well as the lack of requirement for routine laboratory monitoring (Gray et al., 2008; Weitz and Weitz, 2010)—makes LMWHs the more attractive agents.

Treatment with anticoagulants is sufficient in the vast majority of cases of VTE, notwithstanding those involving severe hemodynamic instability owing to PE, which may necessitate the use of thrombolytic therapy or surgical embolectomy (Kearon et al., 2012; Konstantinides et al., 2014). In treatment of VTE, LMWH or UH is given by subcutaneous injection or, alternatively, a loading dose of UH is given by intravenous bolus followed by continuous intravenous infusion. Regular monitoring of the effects of UH, usually by daily APTT measurement, is required, whereas monitoring of the effects of LMWH therapy, usually achieved by indirect assays of FXa activity, is less commonly necessary (Gray et al., 2008; Weitz and Weitz, 2010; Garcia et al., 2012). Early treatment of PE tends to be extremely effective and fixed-dose LMWH regimens have been shown to be as effective as dose-adjusted UH in treatment of PE, but with a reduced rate of recurrence of thrombosis and a reduced rate of significant bleeding events (Quinlan et al., 2004). Typically, heparin-based treatment would be continued for a minimum of 5 days before transfer of the patient to an oral anticoagulant, with continuation of heparin until stabilization of the international normalized ratio within therapeutic range in the case of a vitamin K antagonist. However, invariably in patients who are pregnant, and most commonly in those with cancer, anticoagulation in the outpatient setting would be maintained with LMWH for the remainder of the treatment period. The optimum length of treatment with anticoagulants after a first VTE event has been the subject of some debate. In patients who are pregnant, LMWH therapy would continue throughout the remainder of pregnancy and the puerperium (6 weeks postdelivery). In patients with cancer, LMWH therapy would continue for 6 months before reassessment of the risk versus benefit of further continuation. However, in other patients, the risk of recurrent VTE needs to be weighed against the risk of side effects, most notably bleeding events, on an individual basis. Recurrence of thrombosis after unprovoked VTE, which would be defined as that which occurs in a patient without known significant risk factors such as major surgery, trauma, or
pregnancy in the prior 3 months, has an annual incidence of 5%–10% after the cessation of 3–6 months of anticoagulant therapy (Marcucci et al., 2015). However, a recent analysis concluded that prolonged treatment after VTE in patients with defined and transient risk factors that led to the episode exposes these patients unnecessarily to bleeding risks (Ageno et al., 2015). Current suggestions are that anticoagulant therapy should be for 3 months or indefinitely, given that the active treatment period is complete by 3 months (Agnelli and Becattini, 2008; Kearon and Akl, 2014). The decision whether to continue indefinitely after a single event depends on the patient's individual bleeding risk offset against the likelihood of recurrence, which is higher if, for example, the initial thrombosis event was PE or if the patient is male (Kearon et al., 2012; Kearon and Akl, 2014).

2. Venous Thromboembolism Prophylaxis in Medical Patients. In the prophylaxis of VTE in hospitalized patients considered to be at risk, because of the presence of one or more risk factors, heparin is likely to be given unless the risk of hemorrhage outweighs the thrombotic risk. Both UH and LMWH significantly reduce the incidence of DVT in acutely ill medical patients, although they also increase the risk of serious bleeding without a clear effect on all-cause or PE-related mortality. However, LMWH has been found to cause less bleeding than UH in this context, and LMWH is also shown to have a greater effect on preventing DVT (Alikhan et al., 2014), although an earlier meta-analysis did not detect significant differences in DVT prevention or bleeding outcomes between UH- and LMWH-treated medical patients (Kanaan et al., 2007). UH and LMWH regimens are safe and effective in acutely ill medical patients, with the consensus being that LMWHs have an improved safety profile in this setting (Cohen et al., 2010). In addition to mechanical prophylaxis, unless contraindicated (e.g., due to peripheral arterial disease, cardiac failure, sensory impairment or certain dermatoses), heparin would be offered to patients assessed to be at risk, from as soon as possible until the risk is considered to be removed, after regular reassessment. A significant risk of thrombosis might be assessed in cases in which mobility is likely to be considerably impaired for several days or longer, especially in the presence of any known additional risk factor, or if significant surgery under general anesthesia is planned. However, if regional anesthesia is likely, then the timing of anticoagulant therapy should be considered in relation to this, such that dosing is avoided for 12 hours beforehand to reduce the risk of epidural hematoma. Similarly, spinal anesthesia or an epidural within the previous 4 hours would contraindicate the use of heparin. Other risk factors for bleeding that would preclude the use of pharmacologic anticoagulation include active bleeding, thrombocytopenia, acquired bleeding tendency (e.g., hepatic failure), acute stroke, and uncontrolled systolic hypertension. Low-dose LMWH appears to be the safest option in patients with ischemic stroke outside the acute phase who require VTE prophylaxis (Kamphuisen and Agnelli, 2007).

Critically ill patients present an additional challenge for a number of reasons. Renal impairment is common in such patients, and it is associated with an increased risk of VTE but also with an increased risk of developing bleeding complications (Cook et al., 2008). In addition, risk of VTE in critically ill patients shows significant individual variability dependent on underlying pathology, and treatments and the consequences of even relatively minor PE in these patients could be severe on account of reduced cardiopulmonary function (McLeod and Geerts, 2011). A large, multicenter trial found a LMWH (dalteparin) to be nonsuperior to UH in prevention of proximal DVT (the primary outcome) in the critical care setting, although the PE rate was reduced in the LMWH group (Cook et al., 2011). No significant differences in bleeding events were observed between the two treatment groups (Cook et al., 2011; Arnold et al., 2013) and the LMWH was found to be relatively cost-effective (Fowler et al., 2014).

3. Venous Thromboembolism Prophylaxis in Surgical Patients. Major orthopedic surgery, of the hips or legs, is generally considered to present a high risk for the development of VTE, owing to extensive activation of coagulation mechanisms as a result of the procedure, relatively prolonged anesthesia, and extended immobility postoperatively, and a significant proportion of patients undergoing such procedures could be expected to develop VTE without prophylaxis. Furthermore, risk of VTE persists for weeks after surgery and commonly presents after discharge from the hospital (Leclerc et al., 1998; White et al., 1998, 2003). However, it is currently acknowledged that the baseline risk of thrombosis associated with major orthopedic surgery has fallen over time, as a result of refinements to surgical and anesthesiological approaches and perioperative management, independently of the use of thromboprophylaxis (Falck-Ytter et al., 2012; Francis, 2013; Chan et al., 2015). Nonetheless, risk of death from VTE was found to be very low in patients managed with modern anticoagulant regimens, including enoxaparin, in the trials analyzed in a recent systematic review (Chan et al., 2015), and routine pharmacological thromboprophylaxis is recommended in the majority of patients undergoing major orthopedic procedures (Falck-Ytter et al., 2012). While presenting a considerable risk in itself, the presence of additional risk factors in patients undergoing major surgery may further increase the likelihood of significant thrombosis; a large retrospective cohort study of patients who had undergone major orthopedic surgery found that female sex and a previous history of VTE were the major determinants of thrombosis risk in this setting (Donath et al., 2012).
In the majority of orthopedic patients, LMWH prophylaxis is preferred to UH on the basis of at least equivalent and generally greater efficacy and safety, along with more convenient dosing schedules (Hirsh et al., 2001; Gray et al., 2008). Indeed, LMWH regimens are well established to be effective and safe for VTE prevention in major surgical (hip and knee) and trauma cases (Hovanessian, 1999; Hirsh et al., 2001; Falck-Ytter et al., 2012; Francis, 2013), although UH may be used in the case of significant renal impairment. VTE prophylaxis (mechanical and pharmacological, unless there are specific contraindications) should be used in all such patients (Falck-Ytter et al., 2012; Francis, 2013). Mechanical prophylaxis should be used while mobility is significantly reduced and pharmacological prophylaxis with LMWH is continued for several weeks postoperatively, including into the outpatient setting. The recommended timing of heparin therapy in relation to the surgical procedure varies, although initiation 6–12 hours after surgical closure appears to suitably address the balance between effective thromboprophylaxis and increased risk of blood loss. A recent prospective study in patients undergoing total knee replacement was, however, carried out to investigate the safety and efficacy of delayed initiation of DVT prophylaxis with LMWH. The incidence of symptomatic DVT was not significantly different when LMWH therapy was initiated 24 hours after surgical closure, compared with a standard regimen (12 hours), whereas a significant reduction in total blood loss associated with the procedure was observed with the delayed initiation protocol (Liu et al., 2014). If VTE prophylaxis is required preoperatively (e.g., due to immobility in hip fracture), then a suitable heparin-free period is usually required before surgery, avoiding administration within 4 hours on either side of the procedure (Falck-Ytter et al., 2012).

In the case of other surgery, including orthopedic surgery to the upper limbs or to the lower limbs that is nonmajor (e.g., arthroscopy), the patient’s individual risk would be assessed and prophylaxis only initiated if risk of thrombosis clearly outweighed risk of bleeding. If required, LMWH would be used as the first-line treatment and would be used postsurgically until a level of mobility is restored sufficient to negate the risk. It is not universally agreed whether patients with immobilization of the lower legs require routine thromboprophylaxis and it is generally not recommended as a routine approach (Falck-Ytter et al., 2012), although the use of LMWH in these patients has been reported to be safe and effective where necessary (Ettema et al., 2008; Testroote et al., 2008). LMWH prophylaxis has also been found to be effective and safe in patients with short-term reductions in mobility (e.g., owing to leg fracture or arthroscopy); a meta-analysis of trials in such patients found a significant reduction in major VTE events on the order of 70%, at the expense of a nonsignificant 35% increase in the risk of major bleeding (Chapelle et al., 2014). Other types of surgery (e.g., gynecologic, gastrointestinal, cardiac, or vascular, as well as general surgery including day cases) would be similarly treated; prophylaxis with a combination of mechanical means and heparin (LMWH preferred) would be provided throughout any period of significantly reduced mobility (typically no more than 1 week). An exception would be in the case of major abdominal or pelvic surgery for cancer, whereby pharmacological prophylaxis would be extended further into the postoperative period because of the high underlying thrombosis risk in patients with cancer.

B. Pregnancy

Normal pregnancy presents as a hypercoagulable state and pregnancy-associated VTE remains a significant cause of maternal morbidity and mortality in developed countries (Kher et al., 2007; Lussana et al., 2012; Berresheim et al., 2014; Guimicheva et al., 2015), accounting for approximately 10% of maternal deaths (Marshall, 2014). Pregnancy places an estimated 4- to 5-fold increase in risk of VTE compared with the nonpregnant state, which rises further during the puerperium and can be compounded by underlying maternal risk factors (James, 2007; Lussana et al., 2012; Marshall, 2014). Such risk factors may include those that are not specifically related to pregnancy, such as thrombophilias or history of VTE, or factors such as preeclampsia, multiple pregnancy, or hyperemesis gravidarum.

Normal pregnancy is associated with increased thrombin generation and reduced protein S levels (de Boer et al., 1989; Eichinger et al., 1999; Hoke et al., 2004), as well as increased plasma concentrations of coagulation factors VII and VIII and fibrinogen (Stirling et al., 1984; Chunilal et al., 2002), which extends to additional reductions in AT and protein C levels in the presence of preeclampsia (de Boer et al., 1989). Moreover, changes in the APTT in response to UH therapy can be altered in pregnancy as a reflection of these changes and perhaps of increases in general in levels of heparin binding proteins (Chunilal et al., 2002).

UH and LMWHs do not cross the placenta (Flessa et al., 1965; Forestier et al., 1984, 1987). Both are considered safe for use in pregnancy and can be used in this setting for the treatment and prophylaxis of VTE, in contrast with other available anticoagulant agents that cannot be used in pregnancy either due to known teratogenicity, such as vitamin K antagonists, or lack of established safety information, such as novel oral anticoagulants (NOACs) (Marshall, 2014). In most cases, LMWHs are the preferred agent in VTE treatment and/or prophylaxis in pregnancy (Hirsh et al., 2001; James, 2007; Regitz-Zagrosek et al., 2011; Bates et al., 2012; Konstantinides et al., 2014) and have been shown to be safe and effective in this population (Lepercq et al.,
2001; Rodie et al., 2002; Greer and Nelson-Piercy, 2005; Kher et al., 2007). The reduced propensity of LMWH to cause the side effects of osteoporosis and thrombocytopenia, compared with UH (Bates et al., 2012; Lussana et al., 2012), may be particularly important in terms of the relatively protracted period of exposure that is indicated in the case of VTE prophylaxis throughout pregnancy and the puerperium. Upward adjustment of normal weight-based regimens tends to be required, especially in the case of LMWHs, in large part because of the increased rate of renal clearance of these agents (secondary to increased volume of distribution) that is associated with pregnancy (Kher et al., 2007). Although routine monitoring of anti-Xa activity tends not to be required with LMWH therapy in pregnancy, it has been suggested that in high-risk patients, peak and trough levels should be considered for optimal dose selection. VTE prophylaxis should be considered in patients who are admitted to the hospital during pregnancy or within 6 weeks of delivery, if risk factors in addition to pregnancy itself are present or if surgery (including caesarean section) is planned. However, the likelihood of the need for regional anesthesia would need to be considered in terms of the timing of heparin therapy and mechanical means of prophylaxis used until the risk of epidural hematoma is considered to be low, in line with local guidelines.

C. Cancer

Monotherapy with LMWH is currently the recommended option for the treatment and prophylaxis of VTE in patients with cancer (Lee, 2010; Carrier et al., 2014; Elyamany et al., 2014; Kuderer and Lyman, 2014). A recent analysis suggested that LMWH may be slightly superior to UH in terms of mortality outcomes in the initial treatment of PE in patients with cancer, although differences in the quality of studies comparing the different treatments makes it difficult to draw a firm conclusion (Akl et al., 2014a). However, the relative convenience associated with a LMWH-based approach should be considered along with its established efficacy.

VTE risk among patients with cancer varies significantly and is dependent on the site of primary tumor, biochemical and hematologic parameters, therapeutic approach, and underlying patient characteristics, such that patients should be individually assessed for risk at the start of their treatment regimen and periodically throughout (Khorana, 2010; Khorana and McCrae, 2014). Malignancy itself is a hypercoagulable state (Cohen et al., 2010, 2014), but chemotherapeutic regimens add to this risk, such that VTE prophylaxis is often warranted (Lee et al., 2003; Falanga and Zacharski, 2005; Di Nisio et al., 2014), including in the outpatient setting (Khorana, 2012), and almost invariably in the inpatient setting (Kamphuisen and Beyer-Westendorf, 2014). The generalized risk of VTE in patients with cancer is reported to be increased 7-fold over those without (Khorana, 2012; Noble, 2012; Franchini and Mannucci, 2015); the rate of occurrence has been estimated at between 1% and 30% (Lee et al., 2003) and is up to 50% in the palliative care setting (Noble, 2012). Indeed, the link between cancer and thrombosis is sufficiently strong that the development of unprovoked VTE in a patient not known to have cancer may be seen as an indication for at least basic investigations for the presence of undiagnosed malignant disease. However, a recent meta-analysis concluded that it is not currently clear whether routine testing for cancer in patients with unprovoked DVT leads to overall benefit to patients, although testing does lead to a diagnosis of cancer earlier in the disease if present (Robertson et al., 2015).

The risk of thrombosis in patients with cancer may also be compounded by the need for central venous catheterization (Ryan et al., 2012; Debourdeau et al., 2013; Elyamany et al., 2014), which in itself is reported to contribute an average risk of 1.7 VTE events per 1000 catheter-days, and which may be treated with, but is not reliably prevented by, anticoagulant therapy (Jasti and Streiff, 2014). Furthermore, patients with cancer are at increased risk of developing complications during anticoagulant therapy, which may include an increased risk of major bleeding, as well as treatment failure manifesting as recurrent thrombosis, compared with other patient groups (Prandoni et al., 2002, 2015; Lee et al., 2003; Kamphuisen and Beyer-Westendorf, 2014; Kyrie, 2014). However, therapy with LMWH has been found to be more effective than a vitamin K antagonist in reducing the rate of recurrent thrombosis in patients with cancer who initially present with proximal DVT, PE, or both (Lee et al., 2003); treatment with LMWH is also shown to provide better survival outcomes in primary thromboprophylaxis (Di Nisio et al., 2014) and over long-term treatment after symptomatic PE (Zhang et al., 2015). A recent meta-analysis of trials assessing primary prophylaxis of VTE with LMWH in ambulatory patients undergoing chemotherapy found that although survival at 1 year was unaffected (where this outcome was reported), the rate of symptomatic DVT and PE was significantly reduced, with no significant increase in major bleeding events (Ben-Aharon et al., 2014). Positive effects were particularly apparent in patients being treated for tumors of the lung or pancreas, which tend to have a greater association with VTE than most other types of cancer (Noble, 2012). It should be noted that the fact that heparins have effects on tumor progression and survival that are not directly related to the prevention of thrombotic events confounds analysis of the effects of VTE prophylaxis on survival outcomes (Lee, 2010; Noble, 2012; Franchini and Mannucci, 2015). However, a systematic review of randomized controlled trials assessing the benefits and harms of UH or LMWH in ambulatory patients with...
cancer suggested a modest improvement in survival rates after 1 and 2 years, accompanied by a significant reduction in the risk of VTE and a small increase in the risk of minor bleeding events (Akl et al., 2014b). Moreover, given that VTE is the second-leading cause of death in patients with cancer after cancer progression (Khorana, 2010), reducing thrombosis risk should inevitably have some effect on survival rates.

D. Acute Coronary Syndromes

Antithrombotic therapy with UH or LMWH is given in the general management of patients with ST elevation myocardial infarction and also as an adjunct to thrombolytic therapy with tPA-based agents and/or during angioplasty (percutaneous coronary intervention) (De Luca and Suryapranata, 2015). The usefulness of UH and LMWH in patients with non–ST-segment elevation myocardial infarction (STEMI) was found to be equivocal in a recent meta-analysis (Andrade-Castellanos et al., 2014), in that risk of myocardial infarction in heparin-treated patients was reduced, but overall mortality and rate of recurrent angina was unaffected versus placebo. LMWH have been suggested to be more effective in this context (Magee et al., 2003), as well as in acute myocardial infarction (Puyimurat et al., 2012), and when used for anticoagulant cover during percutaneous coronary intervention (Silvain et al., 2012), in terms of both survival and bleeding outcomes. However, UH is often preferred because its effects are more readily reversed. A lack of data exists to suggest the optimal duration of anticoagulation in non-STEMI, with longer durations tending to present an increased risk of bleeding without clear improvements in outcome (Riaz et al., 2014).

E. Fondaparinux and Other Alternatives

Fondaparinux is a synthetic AT-binding pentasaccharide that is identical to the corresponding pentasaccharide sequence found in natural heparin. The anticoagulant activity of fondaparinux is achieved through specific inhibition of FXa via AT, without any effect on FIIa; in this regard, fondaparinux can be viewed as an extreme example of a LMWH. Fondaparinux can be used as an alternative to UH or LMWH in the majority of indications for antithrombotic treatment or prophylaxis, in patients with competent renal function, by once-daily subcutaneous delivery without the need for laboratory monitoring (Garcia et al., 2012). For example, efficacy and safety of fondaparinux have been found to be comparable with intravenous UH in the treatment of PE (Büller et al., 2003), LMWH in VTE prophylaxis after major orthopedic surgery (Donath et al., 2012), and LMWH in non-STEMI, whereby bleeding risk was found to be lower with fondaparinux (Szummer et al., 2015). Fondaparinux is reported to have been used successfully during pregnancy in the case of hypersensitivity to heparin (Elsaigh et al., 2015), but it is not currently recommended because of the lack of safety data in this setting (Konstantinides, 2014).

Because of its defined size, fondaparinux has the advantage that it should not provoke side effects of heparin that are attributable to protein-binding interactions requiring larger heparin structures, key among these being a lack of propensity to cause HIT (Konstantinides, 2014). Moreover, reports suggest that fondaparinux is frequently used in patients who cannot receive UH or LMWH because of HIT (Schindewolf et al., 2014), and there are reports both of fondaparinux being used successfully in patients with existing HIT (Lobo et al., 2008; Kang et al., 2015) and of a lack of cross-reaction with HIT antibodies (Savi et al., 2005; Weitz and Weitz, 2010). However, there are also reports that fondaparinux can, in rare cases, cause problems in this setting (Warkentin, 2010; Warkentin et al., 2007, 2014; Bhatt et al., 2013). Looking forward, it seems likely that use of the orally active direct FIIa and FXa inhibitors (NOACs) will become the established treatment approach in patients with HIT, because reports of their successful use in this context exist (Hantson et al., 2015; Sharif et al., 2015). Indeed, whether NOACs will eclipse heparins (and indeed vitamin K agonists; Rollins et al., 2014) in the long term for at least some of the established indications of heparins discussed here (Gómez-Outes et al., 2012, 2014; Neumann et al., 2012; Yoshida et al., 2013; Lazo-Langner et al., 2014), especially if agents for their specific or at least more effective reversal become available (Greinacher et al., 2015), remains to be seen.

F. Adverse Reactions

The adverse effects of heparin can be related to the vast array of biologic activities that heparin has. The most important complication that can arise from heparin treatment is bleeding, which results directly from the dose of heparin given. The risk of bleeding is difficult to determine as a result of the multiple factors that can be involved, including the amount and duration of heparin treatment, patient indication, procedure undertaken, and comedication. The nonbleeding risks are associated with heparin binding to other proteins not involved in maintaining blood fluidity. These risks are very low, with the most well known being HIT. Other risks include osteoporosis (observation needed especially in pregnant or elderly patients) and skin lesions, with some reported incidence of alopecia and elevation of liver enzymes.

1. Bleeding. All antithrombotics carry the risk of bleeding as a complication. Heparin shifts the hemostatic balance by inhibiting normal coagulation, limiting clotting and repair at sites of vascular injury. Although the risk of bleeding is increased by heparin, the baseline status of the patient and the procedure (if any) that he or she undergoes will all contribute. This topic was comprehensively reviewed recently by Alban...
subsequently stored in the through antibodies that recognize complexes of PF4 and (Greinacher et al., 2009). The pathogenesis of HIT is platelet activation and aggregation as a result of HIT bocytopenia has given rise to an enhanced risk of venous LMWH. The condition is paradoxical, whereby throm-

a prothrombotic condition, in which there is a drop in LMWH (Martel et al., 2005). HIT is characterized as with a risk of 2.6% when using UH and 0.2% with (International Stroke Trial Collaborative Group, 1997).

2. Heparin-Induced Thrombocytopenia. The most common nonbleeding side effect of heparin therapy is the immunologic response, HIT. There are two types of HIT: type 1 and type 2. Type 1 is more common, with mild thrombocytopenia that occurs near the onset of treatment and normalizes with continued treatment (Warkentin et al., 2008). This type of HIT occurs in 10%–30% patients and does not require cessation of heparin therapy (Shantsila et al., 2009). The mechan-

ism for type 1 is not autoimmune, is due to heparin directly effecting platelet activation (Chong and Castaldi, 1986), and is referred to as heparin-associated thrombocytopenia. Type 2 is a more serious, immune-related reaction and occurs after longer (>4 days) exposure to heparin (Martel et al., 2005). Nonspecific reference to HIT most commonly refers to this type.

The general incidence of HIT is clinically very low, with a risk of 2.6% when using UH and 0.2% with LMWH (Martel et al., 2005). HIT is characterized as a prothrombotic condition, in which there is a drop in platelet count in patients who have been given UH or LMWH. The condition is paradoxical, whereby throm-

bocytopenia has given rise to an enhanced risk of venous and/or arterial thrombosis. The thrombotic events are platelet activation and aggregation as a result of HIT (Greinacher et al., 2009). The pathogenesis of HIT is through antibodies that recognize complexes of PF4 and heparin. PF4 is produced by megakaryocytes and is subsequently stored in the α-granules of platelets. Upon activation, platelets release PF4, which binds to negatively charged GAGs such as HS on endothelial cell surfaces. This binding displaces AT from HS, switching the endothelial surface to be more prothrombotic, a de-

sirable feature at sites of vascular injury. Heparin will disrupt this interaction due to PF4 having a higher affinity for heparin than cell surface HS (Zucker and Katz, 1991). This leads to endothelial cell–bound PF4 being released into the circulation and gives rise to the PF4-heparin complex, which can become immunogenic (Greinacher et al., 2008).

The interaction between PF4 and heparin is principally charge dependent with some influence from sulfation patterns (Rauova et al., 2005). This correlates well with LWMH and the pentasaccharide fondaparinux having lower immunogenicity (incidence of HIT) relative to UH (Greinacher et al., 2008). The formation of PF4/heparin complexes requires roughly equimolar concentrations, with higher or lower concentrations affecting the relative size of the complexes formed (Greinacher et al., 2006; Suvarna et al., 2007). The formation of large complexes (>670 kDa) is crucial for the pathogenesis of HIT (Rauova et al., 2005), with immunogenicity driven by the size and stability of the complex.

The antibodies formed against PF4/heparin are mainly of the IgG isotype, which are able to interact with cells through their Fc receptors. When the large immune complexes form, the antibodies bound to the complexes can bind to the FcγIIa receptors on platelets, which crosslink platelets together (Kelton et al., 1988) and will cause platelet aggregation and activation. These two aspects give rise to the clearance of platelets (thrombocytopenia) and release of procoagulant elements that drive a thrombotic response (Hughes et al., 2000; Tardy-Poncet et al., 2009). HIT antibodies can also cause activation of endothelial cells (Cines et al., 1987), monocytes (Pouplard et al., 2001), and neutro-

phils (Xiao et al., 2008). The activation of these cells can all contribute to the prothrombotic state that HIT triggers.

HIT cannot be resolved simply by cessation of treat-

ment with heparin because the prothrombotic state will in itself require therapy using alternative anticoagulant approaches such as argatroban. The risk of a thrombotic event requires treatment with the alternative anticoagulant for several weeks (Warkentin and Kelton, 1996), during which platelet count will rebound.

3. Skin Lesions. The induction of skin lesions is a second immunologic response to heparin, which occurs at the site of subcutaneous injection several days after administration (Hirsh et al., 2001). Such reactions are often related to the use of LMWH, which is adminis-
ter and can be characterized as allergic reactions or related to HIT. The incidence of skin lesions was recently assessed in a clinical study and was found to be much higher at 8% (Schindewolf et al., 2009) than the previously accepted incidence of 2% (Bircher et al., 1990). There are three types of skin lesions, with the most frequent being delayed hypersensitivity, then immediate hypersensitivity, and the least common being skin necrosis (Warkentin, 1996).

Delayed hypersensitivity reactions occur after sensiti-

zation to heparin of approximately 2 weeks, but they have been observed after long periods of treatment (Bircher et al., 1995; Boehncke et al., 1996). Most reactions present with erythema at the sites of admin-

istration (Ludwig et al., 2006) and can range from mild to papulovesicular erythematous plaques (Schindewolf et al., 2010a). Histologic analysis has shown infiltration by neutrophils and eosinophils, which is characteristic
of delayed type hypersensitivity reactions (Grassegger et al., 2001; Schindewolf et al., 2010a). There is some evidence that the hypersensitivity reaction is molecular weight dependent (Ludwig et al., 2005), with the pentasaccharide having a much lower incidence compared with other heparins (Schindewolf et al., 2010b).

Immediate hypersensitivity reactions have been attributed to contaminants or preservatives in heparin preparations (Hirsh et al., 1998; Bottio et al., 2003). However, there has been a description of a direct immediate reaction to heparin that caused pruritic skin lesions, with the patient showing cross-reactivity to other LMWH preparations (Harr et al., 2006). This type of reaction is, however, extremely rare.

Skin necrosis induced by heparin is also a very rare response, but it is considered the most serious type of reaction (Kelly et al., 1981). The route of heparin administration is not related to the occurrence, with the reaction developing around ten days into treatment, manifesting on the extremities (Levine et al., 1983; Drew et al., 1999). The exact mechanism for lesion formation is not clear, with several possible pathways suggested: allergic or hypersensitivity reactions, local trauma, and allergic vasculitis (Drew et al., 1999; Jappe, 2006). However, most frequently necrosis occurs as part of HIT, with HIT antibodies found in many patients (Handschin et al., 2005), although only about a quarter will actually present with HIT (Warkentin et al., 2005).

4. Osteoporosis. Long-term use of heparin is well known to increase the risk of osteoporosis (Jaffe and Willis, 1965), with an incidence of approximately 5% (Le Templier and Rodger, 2008). Clinically, daily doses of UH >15,000 IU for longer than 6 months will typically give rise to fractures (Griffith et al., 1965; Hirsh et al., 1995). Studies that incorporate prolonged use of heparin generally involve pregnant women, who are naturally at a higher risk of osteoporosis (Rajgopal et al., 2008). In trials involving VTE prophylaxis with UH during pregnancy, an incidence of 5% was reported for vertebra fractures (Howell et al., 1983). In a trial using UH to treat VTE, there was a higher incidence of 10% (Monreal et al., 1994), albeit in a much older age group (average 68 years). In this latter study, no difference in bone density was observed between the group who developed fractures and those without fractures, which contrasts with an observation made in pregnant women who had low bone density several years after heparin treatment (Pettilä et al., 2002). The risk associated with LMWH is much lower, with a large data review (N = 2777 patients), again in pregnant women, finding only one reported instance (0.04%) of osteoporotic fracture (Greer and Hunt, 2005; Greer and Nelson-Piercy, 2005). The mechanism by which heparin can impair bone formation is complex. A study in rats found that UH decreases the rate of bone formation and increases the rate of bone resorption (Muir et al., 1996). A subsequent study using LMWH found that it is able to affect bone formation but has no effect on bone resorption (Muir et al., 1997). Examination using a calcium release assay with osteoblasts has shown that the size and sulfation of heparin are factors that promote bone resorption (Shaughnessy et al., 1995), which corresponds with LMWH being associated with a lower risk of osteoporosis. Osteoprotegerin (OPG), involved in the development of osteoblast lineage cells, has been found to bind to heparin (Irie et al., 2007), which prevents OPG inhibitory activity on bone resorption and corresponds with elevated OPG levels in patients administered heparin (Vik et al., 2007).

G. Other Side Effects

1. Alopecia. The occurrence of alopecia has been known as a side effect of long-term UH therapy for some time (Hirschboeck et al., 1954). LMWHs have also been noted to cause alopecia (Barnes et al., 2000; Sarris et al., 2003; Wang and Po, 2006); however, the frequency of this reaction, as with UH, is considered very low, although the occurrence has not been precisely defined. The mechanism involved is unclear, and there are suggestions that heparin influences the growth cycle of epithelial cells (Paus, 1991) and may interfere with heparanase, which also possesses a role in hair growth (Zcharia et al., 2005).

2. Elevation of Liver Enzymes. A number of studies have reported the elevation of the liver enzymes alanine aminotransferase and aspartate aminotransferase with UH and LMWH treatment (Monreal et al., 1989; Carlson et al., 2001), with an incidence estimated at approximately 15% (Arora and Goldhaber, 2006). The mechanism for this effect has not been identified, but the enzyme levels return to normal after treatment has finished or, in some cases, during therapy (Dukes et al., 1984).

3. Hyperkalemia. Heparin causes a reversible inhibition of aldosterone synthesis, which has the effect of promoting natriuresis and inhibiting kaliuresis. The effect appears to be independent of anticoagulant activity, occurs at relatively low doses and is specific to aldosterone, mediated at least in part by reduced angiotensin II receptor expression in the zona glomerulosa (Oster et al., 1995). In the majority of patients receiving heparin, physiologic compensation prevents hyperkalemia from occurring; however, monitoring of serum potassium levels is prudent in individuals with impaired renal function or diabetes or in the presence of additional medications that promote potassium retention (e.g., aldosterone receptor antagonists, angiotensin-converting enzyme inhibitors) (Preston et al., 1998; Ben Salem et al., 2014).

VIII. Non-Anticoagulant Effects of Heparin

Beyond its well recognized anticoagulant effects, it has long been appreciated that heparin possesses a wide range of other biologic actions (Jaques, 1979). In particular, heparin exhibits a broad range of activities...
that are of potential relevance to control of the inflammatory response (Lever and Page, 2012) and progression of cancer, particularly metastasis (Vlodavsky et al., 1992), which shares many similarities with leukocyte diapedesis in inflammation. Furthermore, there is growing interest in heparin and related drugs in controlling infectious diseases, ranging from effects on prions (Vieira et al., 2014) and viruses (de Boer et al., 2012) to effects on bacteria (McCrea et al., 2014). Many of the activities of heparin are now considered to be independent of anticoagulant activities and as such are ripe for exploitation as novel approaches to a wide range of diseases (see section X and Table 2). Heparin is thought to exert many of its non-anticoagulant actions of relevance to inflammation through binding of proteins such as chemokines and growth factors that are functionally dependent upon binding to HS (reviewed in Turnbull et al., 2001; Powell et al., 2004; Table 1). However, the exact structural characteristics that mediate the anti-inflammatory effects of heparin are, in the majority of cases, not fully known. Interactions between heparin and proteins can vary from highly sequence specific, such as the binding of AT, to relatively nonspecific (Mulloy et al., 1996) (see section III); hence, a wide array of physiologically relevant molecules are bound by this highly polyanionic molecule (Tyrrell et al., 1999; Table 1). A significant number of proteins that can be classed as heparin binding are fundamentally associated with the inflammatory response, including, but by no means limited to, cytokines (Muramatsu and Muramatsu, 2008), chemokines (Miller and Krangel, 1992; Handel et al., 2005; Shute, 2012), growth factors (Skinner et al., 1991; Watt et al., 1993; Diamond et al., 1995; Bono et al., 1997; Koenig et al., 1998), adhesion molecules (Lever et al., 2000), cytotoxic peptides (Fredens et al., 1991), and tissue-degrading enzymes (Redini et al., 1988; Walsh et al., 1991).

As discussed previously, the heparin-binding domains of many proinflammatory proteins are either known or can be predicted by modern techniques. However, there is a clear rationale for characterization of the corresponding sequences within the heparin molecule required for interaction with these sites, which, if isolated, may possess useful properties as drugs for the treatment of inflammatory diseases or other conditions aside from thrombosis. An analogous approach has recently been taken in identifying oligosaccharide samples isolated from polysaccharides obtained from a marine organism, *Holothuria forskali*, which can recognize the adhesion molecule P-selectin (Panagos et al., 2014). Despite the ability of heparin to inhibit a range of inflammatory diseases (see below), the use of standard heparin for the treatment of these conditions is, theoretically at least, limited by its anticoagulant effects. However, a greater understanding of the interactions between heparin and specific mediators of the inflammatory response could facilitate the discovery and development of novel anti-inflammatory drugs lacking anticoagulant activity.

### A. Effects of Heparin on Inflammation and Inflammatory Mediators

Heparin has been reported to inhibit the activation of a number of inflammatory cell types (Slungaard et al., 1990; Ahmed et al., 1992; Rohrer et al., 1992; Bazzoni et al., 1993; Inase et al., 1993; Piccardoni et al., 1996; Teixeira et al., 1996; Brown et al., 2003; Lever et al., 2007). These effects can at least partially be attributed to the binding and neutralization of mediators and enzymes released during the inflammatory response (Tyrrell et al., 1999) that would otherwise lead to inflammatory cell activation, although inhibition of the release of inflammatory mediators from cells may also contribute to the net effect (Rao et al., 1991; Ahmed et al., 1994; Brown et al., 2003; Ji et al., 2004; Zeng et al., 2004) (see below). Furthermore, certain inflammatory cell-derived enzymes and cytotoxic mediators, involved in promotion of the inflammatory response and subsequent tissue damage and remodeling, have also been shown to be inhibited by heparin, including elastase (Redini et al., 1988; Walsh et al., 1991), cathepsin G (Redini et al., 1988), eosinophil peroxidase (Pégiorier et al., 2006), ECP (Fredens et al., 1991), and EMPB (Swaminathan et al., 2005).

Many growth factors, including basic FGF (FGF-2) (Bono et al., 1997) and transforming growth factor-β (McCaffrey et al., 1989; Okona-Mensah et al., 1998) are bound by heparin, which results in an inhibition of their effects on smooth muscle cell proliferation, a feature of the tissue remodeling seen in diseases that include asthma, atherosclerosis, and coronary stenosis. Indeed, inhibition of vascular smooth muscle proliferation is a long established property of heparin (Clowes and Karnowsky, 1977), unrelated to anticoagulant activity (Guyton et al., 1980), that has since been described in airway smooth muscle as well (Kilfeather et al., 1995; Okona-Mensah et al., 1998; Kanabar et al., 2005). Heparin acts as a potent competitive inhibitor of inositol 1,4,5-triphosphate–dependent calcium release from the endoplasmic reticulum (Ghosh et al., 1988), an effect that is considered to underlie the inhibition of human mast cells that has been described in vitro (Inase et al., 1993) and found to be independent of anticoagulant activity (Ahmed et al., 1997). LMWH has also been demonstrated to inhibit the release of cytokines from mast cells (Baram et al., 1997), and oligosaccharides derived from heparin have been found to inhibit the release of IL-4 and IL-5 from blood T lymphocytes (Ji et al., 2004). Heparin also inhibits the cytotoxic effects of tumor necrosis factor-α–activated eosinophils on endothelial cells (Slungaard et al., 1990), as well as the homotypic aggregation and chemotaxis of eosinophils in response to complement factor C5a, which is bound by heparin (Matzner et al., 1984; Teixeira et al., 1996).
Heparin has been shown to bind to the surface of neutrophils (Leculier et al., 1992) and to inhibit their degranulation (Brown et al., 2003; Lever et al., 2007) and homotypic aggregation (Laghi Pasini et al., 1984; Freischlag et al., 1992; Bazzoni et al., 1993; Brown et al., 2003), as well as the production of superoxide anions, the activity of lysosomal enzymes (Bazzoni et al., 1993), the release of elastase (Brown et al., 2003), and the ability of neutrophils to activate platelets (Bazzoni et al., 1993; Piccardoni et al., 1996). Furthermore, neutrophil activation in response to thrombin-stimulated platelet products is also inhibited, as is thrombin-induced platelet aggregation under certain conditions (Piccardoni et al., 1996). In endothelial cells, UH has been demonstrated to inhibit lipopolysaccharide-induced inflammatory responses via blocking p38 mitogen activated protein kinase (MAPK), phosphoinositide-3-kinase and nuclear factor-κB activation of endothelial cells (Li et al., 2009). The same group has also extended these studies to show that UH suppresses lipopolysaccharide-induced MCP-1 (CCL2) expression in human microvascular endothelial cells by blocking Krüppel-like factor 5 and the nuclear factor-κB pathway (Li et al., 2009). Heparin has also recently been shown to inhibit melanosome uptake and phagocytosis into human epidermal keratinocytes via an effect on blocking P13k/Akt and ME/ERK signaling pathways (Makino-Okamura et al., 2014), extending the evidence supporting the ability of heparin to interfere with various intracellular signaling mechanisms.

B. Inflammatory Cellular Adhesion and Trafficking

In addition to inhibiting the release of inflammatory cell-derived mediators, heparin is also effective in limiting the recruitment of many inflammatory cell types into tissues, through modulation of interactions between leukocytes and vascular endothelial cells at a number of levels. Heparin has been shown to inhibit the adhesion of leukocytes to endothelium both in vitro (Bazzoni et al., 1993; Silvestro et al., 1994; Lever et al., 2000; Smailbegovic et al., 2001) and in vivo (Ley et al., 1991; Tangelder and Arfors, 1991; Nelson et al., 1993; Xie et al., 1997; Salas et al., 2000; Johnson et al., 2004; Lever et al., 2010). Furthermore, the ultimate accumulation of cells in inflamed tissue sites, in response to both allergic (Sasaki et al., 1993; Seeds et al., 1995; Seeds and Page, 2001; Vancheri et al., 2001) and nonallergic (Nelson et al., 1993; Teixeira and Hellewell, 1993; Yanaka and Nose, 1996; Johnson et al., 2004; Lever et al., 2010) stimuli, is inhibited by heparin.

As discussed previously, heparin is known to bind to several adhesion molecules expressed during inflammatory responses, and the structural requirements for these interactions are becoming increasingly well characterized (Fritzsche et al., 2006) (see section III above). L-Selectin, a molecule expressed by leukocytes that is involved in early interactions between inflammatory cells and the endothelium, is bound by heparin (Koenig et al., 1998) and endothelial HS is able to act as an endothelial ligand for this molecule during cell rolling (Giuffrè et al., 1997). The β2-integrin adhesion molecule Mac-1 (CD11b/CD18), essential for the firm adhesion of leukocytes to endothelial cells, is also bound by heparin (Diamond et al., 1995; Peter et al., 1999) to the extent that surface immobilized heparin is able to support Mac-1–dependent neutrophil adhesion under flow conditions in vitro (Diamond et al., 1995). In vivo, inhibition of Mac-1–dependent interactions have been shown to underlie the inhibitory effect of heparin on interactions between leukocytes and the endothelium (Salas et al., 2000). On endothelial cells, heparin binds to P-selectin (Skinner et al., 1991), a selectin adhesion molecule involved in the early sequestration of neutrophils during inflammation, now recognized as being secondary to P-selectin–dependent leukocyte/platelet interactions (Pitchford et al., 2005) and interaction with P-selectin glycoprotein ligand-1 (PSGL1) (Sreevarkumar et al., 2014). The selectins are a family of glycoprotein adhesion molecules comprising an epidermal growth factor-like moiety, repeating sequences mimicking those found on complement binding proteins and an NH₂-terminal lectin domain and it is via the lectin domain that these molecules Ca²⁺-dependently bind to carbohydrate counterligands. Selectins are predominantly involved in the early, rolling stages of leukocyte adhesion, without which firm adhesion and transmigration cannot proceed (Lawrence and Springer, 1991). However, despite structural similarities between the selectins, it is established that heparin is unable to bind to E-selectin (Koenig et al., 1998). This difference is known to rely upon two specific amino acid residues in the EGF-like domain of the selectins, in that if these residues are altered, E-selectin can be made to bind heparin, and the ability of P-selectin to bind heparin can be reduced (Revelle et al., 1996). This differential effect may possess physiologic significance with respect to the role of endogenous heparin and/or HS in the inflammatory process, and a similar selectivity of binding can be observed among members of the immunoglobulin superfamily (IgSF) of adhesion molecules. Heparin has been shown to bind PECAM-1 (Watt et al., 1993), an IgSF member thought to be involved in leukocyte transmigration because of its location at intercellular junctions on the endothelium. The homotypic aggregation of PECAM-1–transfected fibroblasts was also found to be inhibited by heparin in a manner dependent on interaction with the second immunoglobulin domain (DeLisser et al., 1993). Heparin is also able to bind to the neuronal cell adhesion molecule through a heparin-binding region on the second immunoglobulin domain (Cole et al., 1986), as well as through a further heparin-binding region on the first immunoglobulin domain (Kiselyov et al., 1997); such interactions with HS are important for the physiologic functioning of this protein.
in neuronal development (Kallapur and Akeson, 1992). However, the IgSF adhesion molecules intercellular adhesion molecule (ICAM)-1 and ICAM-2, expressed on vascular endothelium and important ligands for the leukocyte β2-integrins, do not appear to bind heparin. It is possible that the cell trafficking associated with physiologic immune surveillance, such as interactions between lymphocyte function-related antigen-1 (CD11a/CD18) and ICAM-1/ICAM-2, may be spared while those associated with excessive cell recruitment during inflammation may be inhibited, given that heparin can affect the functioning of ICAM-1 indirectly, through binding of Mac-1. Heparin can therefore interfere with each step of the events involved in inflammatory cell recruitment, including rolling, inflammatory cell activation, adhesion, and transmigration. We also recently demonstrated that a non-anticoagulant fraction of heparin, O-desulfated heparin, can inhibit leukocyte infiltration in vivo via an effect on platelet/leukocyte interactions (Riffo-Vasquez et al., 2015). Furthermore, recent data in mice demonstrated that bis-deoxycholic acid conjugated to a 6-O-desulfated LMWH was internalized into endothelial cells and that this intracellular modified heparin was able to inhibit the transendothelial recruitment of T cells via inhibition of a Rho-A-dependent mechanism (Kang et al., 2014).

C. Effects on Heparanase in Inflammation

The ubiquitous distribution of heparan sulfate proteoglycans (HSPGs) in mammalian systems provides a clear indication of the physiologic importance of these molecules. HSPGs possess roles in growth and development, are key structural components of extracellular matrices, and are involved in the localization and bioactivity of a wide array of mediators, including enzymes, growth factors, cytokines, and chemokines (Turnbull et al., 2001; Powell et al., 2004). Heparanase is responsible for the site-selective cleavage of HS chains, thus regulating the activity of the wide range of proteins that is functionally dependent upon HSPG.

Heparanase activity has been demonstrated in immune tissue, including the spleen, lymph nodes, leukocytes, and platelets, as well as in endothelial and smooth muscle cells. With respect to the accepted role of heparanase in cancer (McKenzie, 2007), heparanase mRNA is significantly increased in human tumors, compared with corresponding normal tissues, whereby heparanase activity in tumor cells correlates positively with their metastatic potential (McKenzie et al., 2000).

In the context of both inflammatory diseases and cancer, release of heparanase by tumor or inflammatory cells facilitates their diapedesis and migration to tissue sites and promotes angiogenesis and tissue remodeling through release or activation of growth factors. We recently reported that human recombinant heparanase is also able to promote leukocyte infiltration in vivo (Lever et al., 2014) and the pathophysiological importance of heparanase activity is further illustrated by the fact that in tissue sections from patients with inflammatory bowel disease, compared with healthy tissues, areas of extensive GAG disruption are visible on the vascular endothelium and basement membrane, associated with localized areas of inflammation (Murch et al., 1993). Furthermore, increased levels of GAG degradation products have been found in the urine of patients with asthma, which is thought to reflect the breakdown of ECMs as a result of the inflammatory process in the airway (Shute et al., 1997), possibly reflecting the degradation of matrix as inflammatory leukocytes migrate through tissue.

Heparin has long been known to be an inhibitor of heparanase activity (Bar-Ner et al., 1987), and it is also well established that heparin-degrading enzymes are released by certain inflammatory cells during the process of diapedesis (Lider et al., 1990; Matzner et al., 1992). In particular, when heparin is used at low doses in models of inflammatory disease that can be considered to be lymphocyte driven, such as allergic encephalomyelitis (Willenborg and Parish, 1988; Lider et al., 1990), delayed type hypersensitivity (Sy et al., 1983), and graft versus host reactions (Gorski et al., 1991; Naparstek et al., 1993), infiltration of cells into tissues is markedly inhibited. It has further been demonstrated that exposure of vascular endothelial cells to proinflammatory cytokines leads to the secretion of heparanase (Chen et al., 2004; Edovitsky et al., 2006), further suggesting an important role for this enzyme in the inflammatory response, strengthened by the observation that development of delayed hypersensitivity reactions in mice correlates with endothelial heparanase expression (Edovitsky et al., 2006).

Inhibition of heparanase (Uno et al., 2001; Edovitsky et al., 2004; Joyce et al., 2005; Basche et al., 2006), including with heparin (Nakajima et al., 1988; Sciumbata et al., 1996), reduces tumor cell adhesion, migration, and subsequent colonization of tissues, making this enzyme a valid therapeutic target in cancer (McKenzie, 2007). Given the clear similarities that exist between the processes of tumor cell metastasis and inflammatory cell diapedesis, along with the role of heparan-degrading enzymes demonstrated in models of inflammation, heparanase inhibition is likely to contribute to the overall anti-inflammatory effects of heparin and related molecules. These observations suggest heparanase itself to also be a potential target for novel anti-inflammatory drugs (McKenzie, 2007). In support of this, we recently showed that low molecular weight heparanase inhibitors are able to inhibit allergen-induced inflammatory responses in the lung (Morris et al., 2015). However, the role of heparanase in lymphocyte and neutrophil diapedesis was recently challenged, at least in mice (Stoler-Barak et al., 2015). Furthermore, recent work suggests that heparanase overexpression in mice actually impairs the inflammatory response and macrophage
clearance of amyloid-β in a model of neuroinflammation (Zhang et al., 2012). Such results suggest that heparanase may well be anti-inflammatory under certain circumstances or in certain tissues. Nonetheless, heparanase was also recently demonstrated to contribute to the renal dysfunction during sepsis in mice (Lygizos et al., 2013). Furthermore, heparanase inhibitors have been demonstrated to prevent the endotoxemia associated loss of the glycocalyx on the vascular endothelium and neutrophil attachment (Schmidt et al., 2012). These effects of the heparanase inhibitors led to an attenuation of sepsis-induced acute lung injury and mortality in mice (Schmidt et al., 2012) and reinforce the important regulatory role played by the endothelial glycocalyx in leukocyte trafficking, first suggested many years earlier after the seminal experiments of Görög and Born (1982), whereby removal of sialic acid residues from the endothelial surface with neuraminidase led to massive leukocyte adhesion to the endothelium.

IX. Non-Anticoagulant Effects of Heparin: Preclinical and Clinical Studies

A. Acute Inflammatory Reactions

Pretreatment with heparin in animal models of inflammation has been shown to inhibit eosinophil infiltration into the lung (Sasaki et al., 1993; Seeds et al., 1993, 1995) and skin in response to a range of inflammatory insults (Teixeira and Hellewell, 1993), neutrophil accumulation in the inflamed peritoneal cavity (Nelson et al., 1993; Lever et al., 2010), independently of anticoagulant activity (Lever et al., 2010), and vascular permeability in the skin (Carr, 1979; Jones et al., 2002). Furthermore, heparin has been shown to inhibit bronchial hyper-responsiveness in rabbits in response to platelet-activating factor (Sasaki et al., 1993); in sheep in response to inhaled allergen (Ahmed et al., 1992), an effect that was shared by very low molecular weight and non-anticoagulant heparins (Ahmed et al., 1997); and in guinea-pigs, in which the protective effect of heparin against airway hyper-responsiveness to methacholine was found to be attributable to preservation of nitric oxide signaling (Maarsingh et al., 2004). Inhaled heparin was recently demonstrated to reduce the expression of IL-17A, IL-4, and IL-13 in the lungs of house dust–sensitized mice (Huang et al., 2014).

Heparin has also been found to protect against ischemia-reperfusion injury in a variety of experimental models, including the hamster dorsal skin chamber (Becker et al., 1994), cardiac muscle ischemia (Kilgore et al., 1999), and transient focal cerebral ischemia in rats, whereby treatment with heparin was found to reduce the degree of brain injury by inhibiting reperfusion-induced leukocyte accumulation (Yanaka and Nose, 1996). Interestingly, heparin has been suggested as a potential treatment of limitation of the delayed neurologic injury that follows subarachnoid hemorrhage (Simard et al., 2010), on account of its broad anti-inflammatory effects, whereby it has been suggested that subanticoagulant doses may be sufficient to provide benefit in this setting (Simard et al., 2010). Intracisternal administration of heparin was previously found to be protective after experimental subarachnoid hemorrhage in rats (Tekkök et al., 1994). However, given that many of the anti-inflammatory properties of heparin appear to be broadly separable from its anticoagulant effects, related molecules that lack anticoagulant activity could prove to be more useful and acceptable in this situation. Furthermore, recent studies in mice demonstrated that heparin can reduce both the increased plasma protein levels and leukocyte infiltration into the lung elicited by exposure to chlorine gas (Zarogiannis et al., 2014) and can reduce the lung injury after smoke inhalation or burns (Miller et al., 2009, 2014).

B. Human Inflammatory Diseases

In addition to having beneficial effects in experimental models of inflammation, heparin has long been considered to be of potential use in a range of human inflammatory diseases and was first assessed for this purpose in the 1960s (Dolowitz and Dougherty, 1960, 1965), in small, subjectively assessed trials in people with allergic disease. More recently, in controlled studies, heparin has shown clinical benefit in patients with asthma (Ahmed et al., 1993; Bowler et al., 1993; Antczak and Kuna, 1995; Diamant et al., 1996) or COPD (Brown et al., 2006). In patients with COPD, early studies suggested that heparin may be of benefit on account of its ability to neutralize the induction of emphysema by elastase (Rao et al., 1990; Laufma et al., 1991). Treatment of patients with allergic rhinitis with topical heparin reduces eosinophil recruitment into the nose after allergen exposure (Vancheri et al., 2001). Furthermore, administration of heparin exhibits clinical benefits in the treatment of patients with inflammatory bowel disease, even in individuals known to be resistant to treatment with glucocorticosteroids (Gaffney et al., 1991, 1995; Evans et al., 1997; Michell et al., 2001), although meta-analyses of these trials concluded that there is insufficient evidence to support the use of heparin for the treatment of active ulcerative colitis (Shen et al., 2007; Chandé et al., 2008).

A very important point is that heparin treatment was not found to elicit significant hemorrhagic side effects in any of these clinical studies, either when administered systemically or locally. In a study that was carried out specifically to address the effects of inhaled heparin on blood coagulation, it was found that almost 40% of a single inhaled dose of heparin is still detectable in the lung 24 hours after administration, with no significant anticoagulant effects detectable (Bendstrup et al., 2002). Markart et al. (2010) investigated inhalation of
150,000 IU of heparin for 28 days in healthy volunteers and in patients with idiopathic pulmonary fibrosis, and they concluded that heparin administered by this route was safe and showed a local alveolar anticoagulant effect up to 7 hours after inhalation. Again, it seems likely that novel drugs that retain the anti-inflammatory effects of heparin, but lack the anticoagulant effects, will be useful in the management of a range of inflammatory diseases that have been found to respond positively to the administration of heparin.

C. Cancer

Because of the common use of heparins for the prophylaxis of VTE in patients with cancer, significant evidence exists to suggest that heparin confers benefit in the treatment of cancer that extends beyond the prevention of thrombosis (Zacharski and Ornstein, 1998; Engelberg, 1999; Hettiarachchi et al., 1999; Zacharski et al., 2000; Smorenburg and Van Noorden, 2001; Niers et al., 2007; Borsig, 2010; Mousa, 2010). Analysis of trials of heparin treatment in patients with cancer indicate an improved rate of survival (Hettiarachchi et al., 1999; Smorenburg et al., 1999), and meta-analyses performed specifically to assess the effects of UH and LMWH treatment on survival in patients with cancer have indicated positive effects (Akl et al., 2007; Kuderer et al., 2007).

As discussed previously, the accumulation of metastatic tumor cells in tissue sites shares considerable similarities with inflammatory cell recruitment that requires adhesion to the vascular endothelium and subsequent diapedesis across the vessel wall (Vlodavsky et al., 1999), including a dependence on P-selectin and platelet activation (Borsig et al., 2001; Pitchford et al., 2003). However, the extent to which the anticoagulant actions of heparin contribute to these effects is not as clear as is perhaps the case for many of the anti-inflammatory properties of heparin. Heparin has repeatedly been demonstrated to reduce metastasis of carcinoma cells in animal models (Nakajima et al., 1988; Alonso et al., 1996; Sciumbata et al., 1996; Parish et al., 2001; Mousa et al., 2006) and the basis of the antimetastatic effects of UH are thought to depend at least partly on inhibition of fibrin deposition around tumor cells and subsequent protection from attack by the immune system (Alonso et al., 1996). Nonetheless, many studies have found that fractions of heparin with reduced or absent anticoagulant activity can also inhibit metastasis (Sciumbata et al., 1996; Mousa et al., 2006). Specific mechanisms thought to be involved in this effect include inhibition of heparanase activity (Engelberg, 1999), inhibition of selectins (Borsig et al., 2001), and inhibition of the TFPI (Amirkhosravi et al., 2007), the latter having been found to promote angiogenesis and metastasis through a combination of anticoagulant and non-anticoagulant mechanisms (Mousa et al., 2004). Inhibition of selectin function appears to be important and separable from anticoagulant effects, in that clinically relevant anticoagulant levels of LMWH have been shown to inhibit experimental metastasis in a manner that correlates with the ability to inhibit P- and L-selectin function, whereas the pentasaccharide fondaparinux, which lacks this ability, was without effect in the same assays, at levels normalized for anticoagulant activity (Stevenson et al., 2005). Furthermore, mice deficient in both P- and L-selectin are protected against experimental metastasis and, importantly, treatment of these mice with heparin was found to provide no additional protection (Stevenson et al., 2007), in contrast with the marked effects seen in wild-type animals in a range of studies.

Protective effects of heparin in cancer models extend beyond the inhibition of metastasis to include modulation of tumor growth and angiogenesis. Heparin has long been known to inhibit angiogenesis, and its inhibitory effects on heparanase are well established to be involved in this effect (Vlodavsky et al., 1992). Growth factor–induced endothelial cell proliferation is inhibited by both UH and LMWHs (Khorana et al., 2003; Takahashi et al., 2005; Marchetti et al., 2008). Although LMWHs in this respect were found to be more potent than UH (Khorana et al., 2003; Marchetti et al., 2008), ultra-LMWH species, including fondaparinux, were without effect (Marchetti et al., 2008). Moreover, inhibitory effects on the chemokine system may also mediate some of the antiangiogenic and antimetastatic effects of heparin (Mehrad et al., 2007).

D. Would Healing and Tissue Repair

Administration of heparin by inhalation has also been suggested as a treatment option in the management of smoke inhalation injury (Cancio, 2009; Toon et al., 2010). Heparin, when administered alone or in combination with N-acetylcysteine, reduces the acute lung injury resulting from smoke inhalation that contributes significantly to the morbidity and mortality observed in these patients (Miller et al., 2009).

A number of reports exist of heparin also being used, topically or systemically, to treat burns, although there is currently a lack of controlled studies in this area for clear conclusions to be drawn on its efficacy in this setting (Oremus et al., 2007). However, isolated case reports continue to emerge that suggest heparin is able to promote tissue repair and inhibit inflammation in patients with burns (Ferreira Chacon et al., 2010). In animal models, application of heparin-binding epidermal growth factor–like growth factor (HB-EGF)—which is known to be upregulated both in human burn tissue and during healing of partial-thickness burn injuries specifically through potentiation of the expression of transforming growth factor-α, another member of the EGF family of growth factors involved in wound repair (Cribbs et al., 1998)—can promote healing of ileal tissue.
after experimental reanastomosis surgery (Radulescu et al., 2011). Localization of HB-EGF through binding to HSPG facilitates juxtacrine inhibition of cell proliferation by this growth factor, and disruption of this binding allows the released HB-EGF to function as an autocrine mitogen (Prince et al., 2010). Therefore, it is possible that soluble heparin at the site of injury could act competitively to release this growth factor from HS binding sites and promote participation in tissue repair. Furthermore, heparin has been found to promote tissue repair in rabbit trachea after topical application in experimental airway surgery (Sen et al., 2009), further suggesting that the ability of heparin to bind a range of growth factors may be useful in the specific situation of tissue repair after localized tissue injury (Table 1).

E. Protraction of Labor

Some clinicians have noted that pregnant women administered LMWHs for the prevention of thrombosis appeared to have a shorter induction time during labor (Ekman-Ordeberg et al., 2010). Results from experimental models using uterine biopsies suggest that LMWH may have an influence on the inflammatory cytokine IL-8 (Ekman-Ordeberg et al., 2009) and increase contractility. A non-anticoagulant LMWH, tafosiparin, was prepared to assess this observation seen in labor. In the phase 2 study, tafosiparin (http://www.dilafor.com) was found to be effective in reducing the incidence of protracted labor. This suggests that the anticoagulant action of heparin is not essential for this clinical effect of heparin.

Labor is considered an inflammatory process, and heparin may have a role in this setting by modulating inflammatory markers such as IL-8 and proinflammatory cytokine expression in the myometrium and cervix during labor (Osman et al., 2003). In the context of inflammation, heparin has been shown to help transport cytokines through cell matrix layers, preventing sequestration, and this may be part of how heparin inhibits protracted labor (Tanino et al., 2010). Alternatively, it is plausible that heparin is able to influence the ability of contractile agents acting on uterine smooth muscle, such as oxytocin.

F. Endogenous Heparin

Mast cells are the only cell type in mammalian species to contain heparin, but the physiologic role of mast cell–derived heparin is not clearly understood. Mast cells are classically associated with allergic and inflammatory responses; although heparin is known to be important in the storage of histamine and certain proinflammatory granule proteins within the mast cell (Forsberg et al., 1999; Humphries et al., 1999), it would seem unlikely that a potent anticoagulant should be biosynthesized solely for the purpose of storing inflammatory mediators. However, the situation of mast cells close to blood vessels of the microcirculation—and, indeed, their more recent observation in pathologic sites such as atheromas and tumors—suggests that endogenous heparin may play an important role in regulation of both physiologic and pathophysiologic responses. Despite the fact that cancer tends to be a prothrombotic state, it has been observed in malignant melanoma that blood specifically within tumors fails to clot. It was shown in both murine and human melanoma that mast cells infiltrate these tumors in large numbers. Moreover, inhibition of endogenous heparin activity in mouse models, with protamine, heparinase, or genetic NDST-2 deficiency, was found both to restore thrombosis within tumors and lead to increased tumor size, suggesting that mast cell–derived heparin plays an inhibitory role in melanoma progression (Samoszuk et al., 2003). The same investigators also demonstrated that mast cell–derived heparin was able to inhibit the growth of a human breast cancer cell line when in coculture with fibroblasts, but not in their absence, the potential significance of which is illustrated by histologic observations of degranulating mast cells in fibrous tissues in a range of human tumor types (Samoszuk et al., 2005).

Heparin released from degranulating rat mast cells was found to inhibit the proliferation of aortic smooth muscle cells more potently than commercial heparins of lower molecular weights than the mast cell species isolated. In particular, high molecular weight proteoglycans released from the mast cell granules were found to provide the majority of the antiproliferative effect, although GAGs subsequently purified from these proteoglycans were also found to possess inhibitory activity (Wang and Kovanen, 1999). The results of this study and others (Kovanen, 2009) suggest that mast cells in general, and mast cell–derived heparin in particular, may possess a protective role in the process of atherogenesis. Deficiency of endogenous heparin has in fact been suggested to be a potential contributing factor in the progression of atherosclerosis, through loss of heparin-derived anti-inflammatory and anticoagulant mechanisms relevant to this process (Engelberg, 2001). Furthermore, a small study compared serum levels of immunoglobulin E, endogenous heparin-like material, and thrombin-AT complexes between healthy controls and patients with a recent history of myocardial infarction. Heparin-like material was demonstrated in the blood of patients with peripheral arterial occlusive disease, all of whom showed an increased coagulation tendency compared with healthy controls, in a manner that correlated with disease severity (Shankar et al., 2008). By contrast, patients with allergic disease have an increased level of circulating “heparin-like material” and these patients have been suggested to have less calcification of the major arteries, suggestive of a role for endogenous heparin in preventing cardiovascular problems (Lasser et al., 1987). Human mast cells have been reported to release heparin after allergen stimulation (Green et al., 1993).
In the case of allergic inflammation, suggestions as to the role of mast cell–derived heparin have been mixed. On the one hand, given the known anti-inflammatory effects of heparin, it has been proposed that heparin released from degranulating mast cells during the allergic asthmatic response functions to limit the extent of subsequent inflammation in response to the initial stimulus, and that inhibition of mast cell degranulation by drugs such as β-adrenoceptor agonists may thus impair this protective function (Page, 1991). By contrast, others have suggested that heparin from mast cells may contribute to allergic inflammation, because heparin proteoglycans, isolated from a murine mastocytoma line (Brunnée et al., 1997) and from human lung (Noga et al., 1999), were found to initiate the contact system via FXII activation. This group (Brunnée et al. 1997; Nogi et al. 1999) suggested that in allergic reactions mast cell–derived heparin can act as a suitable surface to promote this cascade and can contribute to the generation of kinins to participate in the allergic response. However, a small clinical study compared plasma levels of endogenous heparin-like material between a group of patients with controlled asthma and healthy controls and found these levels to be reduced in the patient group; the authors conclude that the lack of heparin-associated anti-inflammatory activity in these individuals may be a significant factor in the disease (Davids et al., 2010).

X. Heparin in Biomaterials and Regenerative Medicine

A. Immobilized Heparin in Medical Devices and Regenerative Medicine

A protocol for immobilization of heparin onto agarose beads (Sepharose) was published in 1971 (Iverius, 1971), and the resulting affinity chromatography medium was used to purify lipoprotein lipase from milk (Egelrud and Olivecrona, 1972; Iverius et al., 1972). This technique has been widely adopted, as its use has consistently been found to simplify and improve protein purification for heparin-binding proteins. These include plasma proteins such as AT (Thaler and Schmer, 1975; Martínez-Martínez et al., 2012), viral proteins (McClure et al., 2011; Adamson et al., 2012), whole viruses (WuDunn and Spear, 1989), and growth factors (Lobb and Fett, 1984; Fan et al., 2011b). The properties of the resulting purified growth factors may, however, be dependent on the purification method. FGF-2 prepared by heparin affinity chromatography has different oligomerization properties than FGF-2 purified by ion exchange chromatography (Platonova et al., 2014), possibly owing to trace amounts of heparin fragments from the affinity medium (a popular, commercially available prepacked column).

B. Heparin-Treated Medical Devices

Many biocompatible medical devices intended for contact with the circulation are treated with heparin so as to diminish their prothrombotic properties. The technologies that have been developed for coating blood-contacting devices (e.g., stents, vascular grafts, and extracorporeal circulation components) with heparin have been clearly summarized (Tanzi, 2005). Heparin-coated stents have been in use for many years (Kocsis et al., 2000) with some controversy as to the efficacy of the heparin coating in preventing thrombosis and restenosis (Wöhrl et al., 2001). Heparin is also the most common coating for extracorporeal life support circuit components (Sievert et al., 2009), and a survey of the literature has concluded that heparin-coated components justify their extra cost in terms of clinical benefit to patients undergoing cardiac surgery. Recently, silk-based heparin-containing grafts have been designed (Liu et al., 2013; Zhu et al., 2014).

Bridging the gap between heparin-treated prostheses and tissue engineering are developments such as SDF-1α/heparin–coated vascular grafts for the recruitment of progenitor cells (Yu et al., 2012).

C. Regenerative Medicine: Stem Cells and Heparan Sulfate

HS is essential to normal embryonic development (Holley et al., 2014). Embryonic stem cells lacking HS do not differentiate, but they can be induced to do so in the presence of heparin (Tamm et al., 2012). Response to the combination of multiple signaling pathways determines the developmental fate of each cell, and HS is a key element in this process (Holley et al., 2014). Pending development of specific HS-based reagents to steer cell proliferation and differentiation in regenerative medicine, wound healing, and tissue engineering strategies have been described based on the more readily available heparin. There is a considerable recent literature on the subject of tissue engineering ‘scaffolds’, biocompatible constructs which imitate the structural and functional aspects of extracellular matrix, to be populated either by the body’s own endogenous cells or by cultured cells such as stem cells. These scaffolds are often designed to incorporate covalently attached heparin, HS (Meade et al., 2013), or HS mimetic molecules, giving rise to the concept of the neoproteoglycan (Weyers and Linhardt, 2013).

Much work in the past decade has been undertaken designing heparin-incorporating scaffold chemistries for specific functions, aimed at the replacement of missing tissue or the strengthening of debilitated tissues; this field was recently reviewed (Liang and Kiick, 2014). A few examples provide an idea of the possible therapeutic scope: they include induction of blood vessel formation by low concentrations of growth factors in nanostructures consisting of peptides and heparin (Rajangam et al., 2006), heparin crosslinked gel used as a slow-release matrix for OPG (McGonigle et al., 2008), and slow release of FGF-2 from a heparin-containing poly(caprolactine)-gelatin matrix (Kim
et al., 2010). The heparin mimetic alginate-sulfate in an alginate hydrogel gives controlled release of HGF (Ruvinov et al., 2010) and improved tissue vascularization, intended for cardiovascular repair. Injectable gelatin/heparin gels loaded with growth factors have been devised to encourage urethral muscle development in urinary incontinence (Park et al., 2014).

In bone tissue engineering, a heparinized scaffold may be used to carry BMP-2 (Noh et al., 2015) and to improve mineralization of mesenchymal stem cells (MSCs) (Honda et al., 2013). Heparin crosslinked to a chitosan scaffold retains FGF-2 and thus sustains survival and growth of neural stem cells for treatment of central nervous system injury (Skop et al., 2013); heparin-gelatin scaffolds have been developed for eye surgery as replacements for cadaveric corneas (Niu et al., 2014). The delivery of lentiviral vectors for gene therapy in spinal cord injury can also be aided using heparinized scaffolds (Thomas and Shea, 2013).

Complex scaffolds containing heparin and growth factors can attract endogenous cells for wound-healing applications as well as maintaining stem cells and directing their growth and differentiation. Recruitment of endogenous cells to a heparin-containing collagen scaffold can be achieved by loading with SDF-1α (CXCL12) (Bladergroen et al., 2009) and incorporation of heparin into electrospun scaffolds has been shown to guide differentiation of MSC toward an endothelial phenotype (Centola et al., 2010). Slow release of platelet-derived growth factor BB from an electrospun heparinized scaffold enhanced smooth muscle cell infiltration and proliferation (Lee et al., 2012).

Within a scaffold, a uniform concentration of heparin and growth factors may not be optimal. Introduction of spatial patterning can be achieved in several ways, such as gradient formation of glial cell–derived neurotrophic factor in scaffolds for peripheral nerve regeneration (Roam et al., 2014). In the future, differentially micropatterned scaffold or membrane surfaces could be used to guide fine structure and cell growth and phenotype in tissue regeneration. Heparin can be immobilized in micropatterns on a surface, as was demonstrated by Park and Ito (2000) using a polystyrene plate; in the presence of FGF-2, mouse fibroblast growth was enhanced on the heparinized regions. Similar techniques can be used to heparin coat the surfaces of microchannels on biodegradable composite membranes, with effects on the morphology of fibroblasts grown on the membrane (Baran et al., 2011). Heparin hydrogel microwells containing collagen 1 can be used to maintain the phenotype of hepatocytes (You et al., 2014).

X. Novel Drugs Based on the Non-Anticoagulant Actions of Heparin

It is clear from the large number of studies that have been published over recent decades, from basic science investigations to clinical observations in human diseases, that heparin and related molecules possess considerable promise for the treatment of a range of conditions that are not specifically associated with disorders of blood coagulation. However, the potential utility of heparin as it stands, for many of these indications, may be limited by two key factors: lack of selectivity of action, including the need to dissociate specific non-anticoagulant effects from anticoagulant activity, and inconvenience of the parenteral route of administration.

As described above, many of the actions of heparin are independent of its anticoagulant activity, raising the possibility of developing derivatives of heparin and/or heparin mimetics lacking this property. One such derivative is O-desulfated heparin, which as been shown to have a range of anti-inflammatory (Fryer et al., 1997; Seeds and Page, 2001) and antifibrotic (Fryer et al., 1997) actions. It has also been investigated in humans for the treatment of COPD (Decraner et al., 2013).

Others have attempted to identify non-anticoagulant oligosaccharides by depolymerizing UH or LMWH (Turnbull et al., 1992) or by ion exchange chromatography (Shastri et al., 2013). Some of these oligosaccharides possess anti-inflammatory activity (Lever et al., 2007; Shastri et al., 2013). Interestingly, one tetrasaccharide has been shown to be anti-inflammatory after oral dosing in allergic sheep (Ahmed et al., 2012, 2013), although the same tetrasaccharide failed to show any consistent effect in patients with allergic asthma undergoing an allergen challenge (Duong et al., 2008).

It seems likely that novel heparin-based treatments will emerge that isolate specific activities of the parent molecule. Aside from the archetypal example of the AT-binding pentasaccharide, it is established that selectivity of protein binding exists within GAG chains (Taylor and Gallo, 2006) and structural requirements within heparin for the binding of certain growth factors, enzymes, and adhesion molecules have been identified (Karnovsky et al., 1989; Casu et al., 2008). Moreover, the knowledge that minimum GAG chain lengths exist for the effective binding of specific proteins, including thrombin and PF4, has already been exploited in the development and use of anticoagulant heparins with more predictable effects (Gray et al., 2008; Petitou et al., 2009). We previously described that very-LWMH preparations, of defined and homogenous chain length, have differential and size-dependent effects on neutrophil degranulation (Lever et al., 2007), and a simple approach such as molecular weight limitation could alone go some way toward removing activities that are unwanted for a specific indication.

In terms of the physiologic roles of heparin, the fact that commercially available heparin has been standardized based on its anticoagulant activity, and given the heterogeneity of naturally occurring heparin chains, it is not necessarily the case that alternative and unrelated activities will be present in a manner that
correlates with the former. Furthermore, it is possible that certain activities may be lost or selected out in the processing of raw heparin for clinical use as an anticoagulant. Full characterization of the structural features within heparin that account for specific non-anticoagulant effects could, in the future, allow standardization of native heparin for these activities.

Regarding the route of administration of heparin, there has long been interest in developing heparin-based anticoagulants that do not require parenteral administration; for example, with respect to the management of chronic inflammatory diseases, the need for convenient and palatable methods of drug delivery is arguably an even greater issue. In some circumstances, such as inflammatory diseases of the lung, local administration of heparin by inhalation is an option; however, where systemic effects are required, an efficient and predictable drug absorption profile becomes necessary. Clearly, it would be an advantage to developing oral formulations of heparin; indeed, Jaques (1979) cited work that heparin did demonstrate some biologic activity when administered orally but suffered from very poor oral bioavailability. Since these observations, there have been various attempts to improve the bioavailability of heparin, such as via the use of sodium N-[8(-2-hydroxybenzoyl)amino]caprylate (Baughman et al., 1998). Other approaches include the use of chitosan nanoconstructs (Paliwal et al., 2012) and the use of polyaniminomethacrylate coacervates (Viehof and Lamprecht, 2013) to improve the oral delivery of heparin and LMWHs. Attempts have also been made to create solid formulations of heparin for oral delivery by use of heparin conjugated with deoxycholic acid, formulated with the polymer Poloxamer 407 (Park et al., 2010). A recent article reviewed the current status of the different approaches to deliver oral formulations of heparin (Schlüter and Lamprecht, 2014).

Others have developed transdermal approaches to deliver heparin (Lanke et al., 2009), and there have also been various attempts to develop inhaled formulations of heparin. Thus, as discussed above, heparin has been administered safely to humans by inhalation for up to 28 days (Markart et al., 2010), and in most studies in patients, inhaled delivery of heparin is not associated with adverse effects and indeed does not cause systemic effects when administered by alternative means, is promising with respect to the potential delivery of heparin species with alternative pharmacological profiles.

Furthermore, heparin was administered successfully in validation studies of needle-free injection devices (Baer et al., 1996; Hollingsworth et al., 2000; Wagner et al., 2004), presenting a possible alternative to conventional subcutaneous injection of heparins. In these studies, measurement of coagulation parameters was used to assess the efficacy of heparin delivery; the fact that a robust and dose-related effect of heparin can be measured, after the administration of standard heparins by alternative means, is promising with respect to the potential delivery of heparin species with alternative pharmacological profiles.

**XII. Summary**

Over the past quarter-century, heparin has proved to be a consistently useful drug in all of its forms, in spite of its nature as a complex and variable natural product with multiple biologic effects. It is hard to imagine that UH would be taken seriously as a new drug in the 21st century, so we have to count ourselves fortunate (as patients, physicians, or researchers) that it is an old drug with decades of use and development behind it. The elucidation of heparin’s AT-dependent anticoagulant ability, the development of LMWH products, and the use of heparin as a model compound in the advancement of our understanding of proteoglycan function, are high points; and the appalling OSCS contamination incident of 2007 to 2008 is a low point in the recent history of heparin.

In the future, the prospects are more complex, perhaps it will become possible to disentangle the relationships between the structure of heparin/HIS and its numerous functions and interactions, so that more conventional synthetic compounds can be developed for therapeutic use. Heparin mimetics from natural sources, lacking anticoagulant activity, might prove useful for non-anticoagulant indications. As the demand for heparin increases, the sources from which it is derived may well widen; this in itself may uncover more new structures and functions. It is certain that in the long run, heparin studies will pave the way toward advances in regenerative medicine as well as in the treatment of inflammatory disease and cancer. Heparin is indeed an anionic polyelectrolyte; the past 25 years have shown that it is a great deal more as well.

**Authorship Contributions**

Wrote or contributed to the writing of the manuscript: Mulloy, Hogwood, Gray, Lever, Page.

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Pharmacology of Heparin and Related Drugs


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