

The background of the cover is a photograph of several surgeons in an operating room. They are wearing blue scrubs, surgical masks, and caps. They are focused on a patient lying on the operating table, illuminated by bright overhead surgical lights. The scene is a typical clinical setting.

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Heparin

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Heparin: An Important Drug Enters Its Seventh Decade

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Despite its structural complexity and multiple activities, heparin enjoys widespread clinical use and interest in its potential is growing

Heparin is the drug most widely used to prevent blood from clotting. Approximately 33 metric tons, representing 500 million doses, of heparin are used worldwide each year. Its anticoagulant activity is exploited in circumstances where the normal propensity for blood to clot must be overcome. Surgical procedures often require heparinisation, as do extra-corporeal therapies, such as heart-lung oxygenation and kidney dialysis. In these procedures, the blood removed from the body comes into contact with blood-incompatible polymer surface.

Heparin also controls the clotting of blood in diseased vessels. In diseases, including deep vein thrombosis and stroke, its use can effectively reduce tissue damage, by improving circulation. Remarkably, despite its widespread use for an impressive list of ailments, heparin structure is still not completely known, and its biological activity is not well understood.

We live in an age in which the introduction of biopolymeric drugs made by recombinant genetics, including interferons, tissue plasminogen activator (tPA) and erythropoietin, has become commonplace. It is useful to remember that heparin, a complex polysaccharide, was the second biopolymeric drug introduced, just after insulin over half a century ago.

Heparin is prepared almost as it was originally, from the extracts of animal tissues. Interest in heparin is growing, despite the absence of new biotechnological methods for its

preparation, and its complex, undefined structure. This interest is fuelled by its diverse biological activities and a consensus that a molecule with such a complex structure must have a very important role within the body.

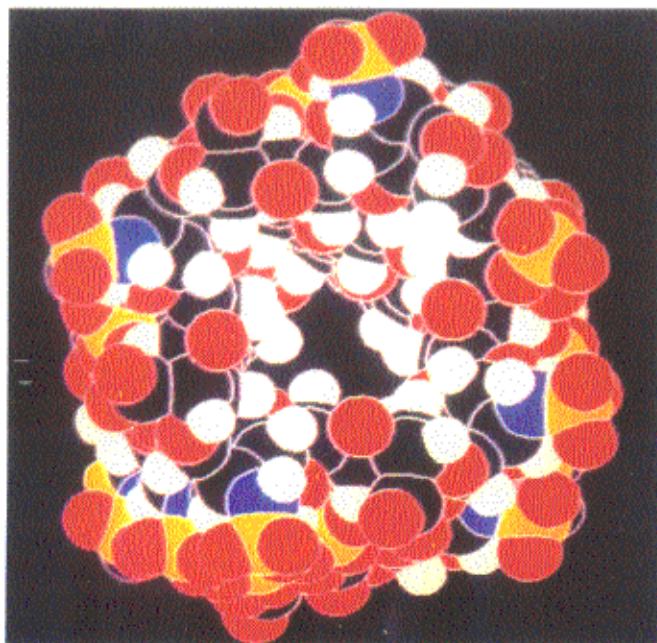
The history of heparin

The heparin story¹⁻⁴ begins during World War I in Baltimore with Jay McLean, a second year medical student at Johns Hopkins University. McLean began his research in 1916, with the physiologist William Howell, on a project directed at discovering substances within the blood which caused it to clot. To his surprise, McLean isolated fractions from mammalian tissues, which, instead of clotting blood, prevented its coagulation.⁵ Howell quickly recognised the importance of his student's discovery, suggesting its therapeutic use to treat coagulation disorders.⁶

This discovery was not without its controversies. First, McLean misnamed the extract 'heparophosphatide'.¹ Heparin, as it was later renamed by Howell, was ultimately established as a sulphated polysaccharide, leaving doubts in the minds of some as to whether McLean had ever prepared heparin.

An understanding of heparin's structure developed slowly from McLean's initial mischaracterisation of it as a phosphate. It did not take Howell long to realise that heparin had no phosphate and was a carbohydrate, identifying one of its sugars as a uronic acid in 1928.⁷ In 1935-1936, Sune Bergstrom (who later won the Nobel Prize for research on prostaglandins) was a student, working with Eric Jorpes in Sweden; he correctly identified glucosamine as the second sugar component in heparin.⁸ Jorpes, and later Arthur Charles, established that heparin contained a high level of covalently linked sulphate, making it one of the strongest acids in nature.⁹ Only in 1950 was Jorpes able to show that the glucosamine in heparin was primarily *N*-sulphated.¹⁰ Heparin's uronic acid was initially identified as *D*-glucuronic acid by Melville Wolfrom in 1946.¹¹ Tony Cinfonelli and Al Dorfman reported in 1962 that they had found *L*-iduronic acid in heparin.¹² Finally, in 1968 Arthur Perlin correctly identified *L*-iduronic acid as the major uronic acid residue in heparin, using a newly developed method called NMR spectroscopy.¹³

By the 1920's, several groups were manufacturing heparin. At Hynson, Wescott & Dunning Co. in Baltimore, another of Howell's students, Dr. Dunning, had designed the first commercial process for manufacturing heparin from dog liver, a particularly rich source.² In Toronto, Charles Best, a colleague of McLeod and Banting (who received the Nobel Prize for their discovery of insulin), began a research programme aimed at the commercial production of beef lung heparin.³ Eric Jorpes, then a graduate student, transferred this technology



Space-filling model of heparin: black = carbon, white = hydrogen, red = oxygen, blue = nitrogen, yellow = sulphur

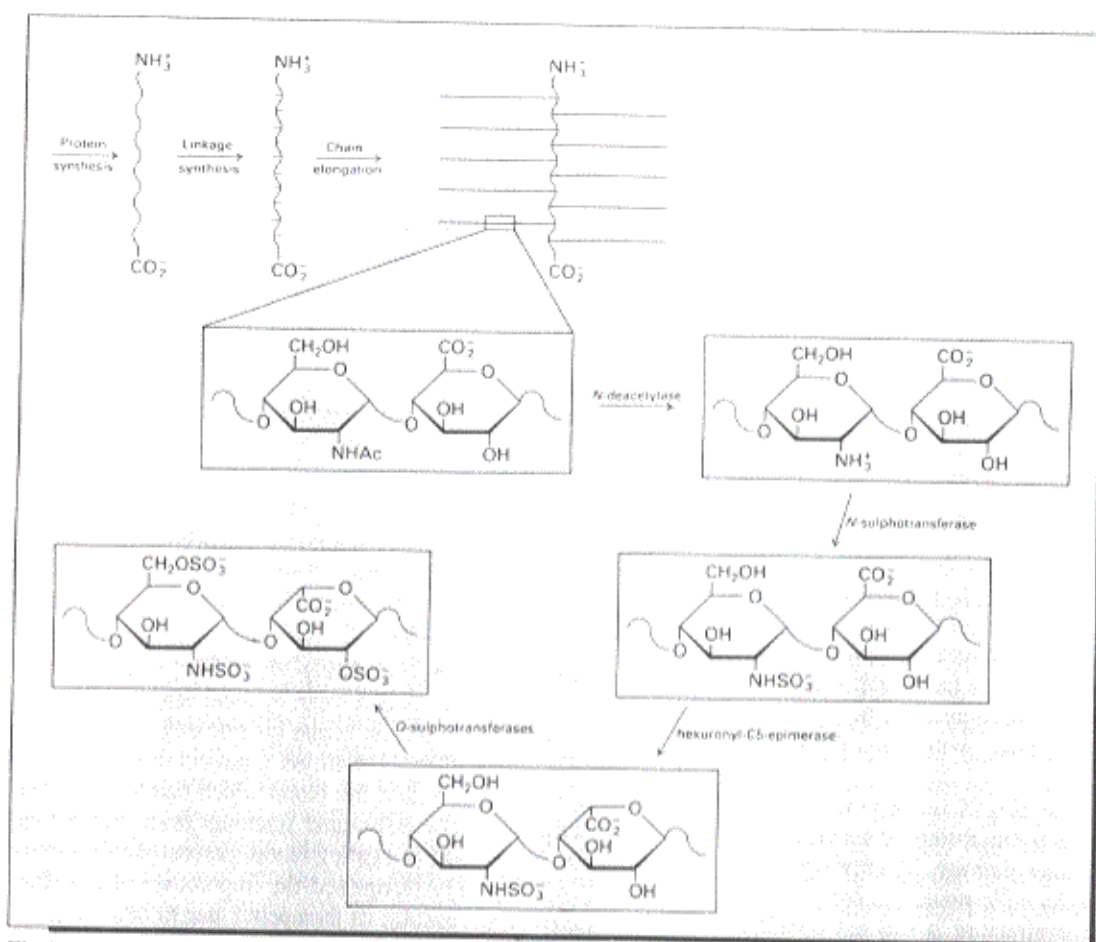


Fig 1 Proposed mechanism of heparin biosynthesis

from Toronto to Stockholm.⁴ By 1935, Jorpes in Stockholm, along with Arthur Charles and David Scott of Connaught Laboratories at the University of Toronto, had prepared sufficient heparin for human trials. In 1935, Clarence Crafoord successfully began using heparin post-operatively in surgery patients, a medical practice that continues to this day.⁴

Heparin biosynthesis and preparation

The biosynthesis of heparin has been well studied.¹⁴⁻¹⁵ Heparin is biosynthesised as a proteoglycan (PG) consisting of a small central core protein, from which long polysaccharide chains extend (Fig 1).¹⁴

The core protein, rich in serine-glycine residues, is first synthesised in the rough endoplasmic reticulum. In the Golgi apparatus, short linkage regions containing neutral sugars are attached to serine residues in the protein core. On this linkage region a repeating copolymer of 1-4 linked glucuronic acid and *N*-acetylglucosamine is assembled through the stepwise addition of activated-sugars. These linear polysaccharide chains are extended by approximately 300 sugar units before their synthesis terminates. Each chain is partially de-*N*-acetylated and sequentially *N*- and *O*-sulphated (Fig 1). The structural variability in the heparin polymer is primarily the result of the incomplete nature of these post-polymerisation modifications.

Shortly after PG biosynthesis, specific peptidases release the polysaccharide chains. Endoglycosidases present in the tissue act on these freed polysaccharide chains, reducing their average molecular weight to 13,000.¹⁶

These polysaccharide chains are called glycosaminoglycan (GAG) heparin. GAG heparin is prepared commercially from animal tissues that are rich in mast cells, such as porcine intestinal mucosa and bovine lung. Other mammals, birds and

even invertebrates such as lobsters and clams contain heparin.¹⁶

The methods for the commercial processing of heparin vary between manufacturers and are generally trade secrets.^{2,17} The basic approach involves the collection of the appropriate tissue, proteolytic treatment, extraction and complexing with ion pairing reagents, and fractional precipitation. Treatment with base and bleaching with oxidising agents, removes residual core protein and results in the pure white drug form of heparin. The major criterion for purity is a high specific anticoagulant activity, expressed as units per mg.⁴

The structure

GAG heparin has three levels of structural complexity. On the first level, heparin is a polydisperse mixture of chains of molecular weights ranging from 5000 to 40,000.¹⁶ On the second level, each heparin chain has a discrete primary structure represented by its saccharidic sequence. On the third level, heparin's secondary structure, and its solution conformation, is responsible for its biological activities. The presence of these levels of structural complexity explains why a drug that has been used for over 50 years still has an undefined structure.

Methods developed to examine heparin's properties as a mixture include viscometry, laser light scattering, gel exclusion chromatography and polyacrylamide gel electrophoresis.^{18,19} Each technique measures heparin's molecular weight and polydispersity. Computer and mathematical simulation can treat these data to understand their effect on heparin's physical, chemical and biochemical properties.^{20,21} Characterising molecular weight and polydispersity has become increasingly important, since clinical studies show that low molecular weight heparins have improved biological properties.¹⁹

The incomplete nature of post-polymerisation modifications in heparin biosynthesis results in a great deal of sequence diversity in the heparin polymer. The major trisulphated disaccharide repeating unit accounts for 60-90 per cent of heparin's sequence, while a variety of minor disaccharide variants comprise the remaining 10-40 per cent of the polymer.^{16,22}

The sequence of disaccharide building blocks in the heparin polymer represent an alphabet through which information can be stored. This is the case for the antithrombin binding site, within heparin, which is defined by a particularly rare saccharidic sequence (Fig 2). Until recently, there was little information on the sequence of the heparin polymer. It was not known whether heparin even had a sequence. Heparin had been

viewed instead as a random polymer. Biosynthetic studies,^{14,15} and the frequent occurrence of a rare sequence associated with the antithrombin binding site, have changed this perception. Computer and mathematical simulation studies on heparin have contributed to our understanding of heparin's sequence.²⁰

Classical sequencing methods have also been applied to studying heparin's primary structure. GAG heparin can be partially depolymerised (either enzymatically or chemically) to prepare transient oligosaccharides (ie, oligosaccharides not found in the final products because they still contain cleavable sites). These oligosaccharides are then purified to homogeneity and exhaustively depolymerised to smaller final oligosaccharide products of known structure.²¹ Sequence can be determined by introducing a tag, such as a radiolabel, at one end of the transient oligosaccharide.²¹ For more complex transient oligosaccharides, a series of partial depolymerisations with various enzymes can be carried out and analysed by gel electrophoresis. This represents the polysaccharide equivalent of the Maxim-Gilbert approach of sequencing nucleic acids.²⁴

New methods for sequencing heparin that use intact PG are now under study. These sequence-determining methods depend on the presence of a reading frame, such as the GAG's linkage region where it attaches to the core protein. Although these methods could lead to the total sequence of heparin, a potential pitfall is that the sequence could be interrupted by random sequences resulting in a loss of the reading frame.

The heparin chain exists in an extended helical conformation, primarily owing to its high charge density. Heparin is tightly coiled, but is not rigid because of an equilibrium between different conformers (of comparable energy) of the α -L-iduronate residue.¹⁸ Heparin exploits this conformational flexibility in its interaction with proteins responsible for heparin's biological activities.

Heparin's biological activities

Heparin anticoagulates blood, preventing haemostasis, which is defined as 'the spontaneous arrest of bleeding from ruptured blood vessels',²⁵ and is a physiological process, of which the blood coagulation system is just one part. Blood coagulation consists of a cascade of reactions in which inactive protease-precursors are transformed into active proteases. In the cascade's final stages, thrombin (a protease) converts fibrinogen

into fibrin. Spontaneous polymerisation of fibrin monomers produces gelatinous fibres – a clot. The entire process is under the control of a group of protease inhibitors, the most important of which is antithrombin. Heparin binds to thrombin and antithrombin, accelerating the rate of thrombin inactivation.

Heparin has exciting potential as a drug for the treatment of atherosclerosis. When heparin is administered intravenously, it causes the release of lipoprotein lipase from the endothelium lining the vessel wall.²⁶ This results in increased triglyceride lipolysis, lowering the concentration of cholesterol-rich remnant particles in contact with the arterial wall.

The movement of smooth muscle cells into a damaged blood vessel is also an important part of atherogenesis.²⁷ Endogenous heparin-like GAGs, lining the vessel wall, play a physiological role in the regulation of the movement of smooth muscle cells into the vascular spaces.²⁶

Two major problems stand in the way of using heparin as an anti-atherosclerotic agent. These are its potency as an anticoagulant²⁸ and heparin's low bioavailability when administered orally. Low molecular weight heparins are being studied to circumvent these problems.²⁹

Heparin may play a variety of roles in regulating angiogenesis, which is the formation of new blood vessels. This process, originally identified with vascularisation in the placenta, has been extended to other capillary growth including tumour angiogenesis. For instance, after a tumour takes hold it grows slowly, remaining quite small until new capillaries appear. Immediately before capillary ingrowth, heparin-containing mast cells congregate. Their heparin may stimulate endothelial cell migration towards the slow growing tumour.

Heparin also localises various growth factors and enhances their activity and stability. One of these, endothelial cell growth factor (ECGF), stimulates angiogenesis.³⁰ In the presence of certain steroids, heparin can inhibit angiogenesis.³¹ Judah Folkman and Mike Klagsburn proposed that in the presence of such steroids, the heparin-like GAGs lining vessel walls restrain capillary growth.³² This quiescent microvasculature can respond rapidly to heparin-mediated growth factors produced during ovulation, by wounds or by inflammation in immune reactions and tumours.³¹

Specific sequences within the heparin polymer may interact with small highly conserved, consensus peptides found in

many heparin-binding proteins.³⁴ Through these consensus sequences heparin binds to, activates or inhibits many enzymes and proteins.^{16,26}

Particularly noteworthy is heparin's antiviral activity. Heparin binds to a consensus peptide in human immunodeficiency virus (HIV), blocking its replication *in vitro*.³³ Clinical studies are still needed to demonstrate heparin's efficacy in the treatment of AIDS.

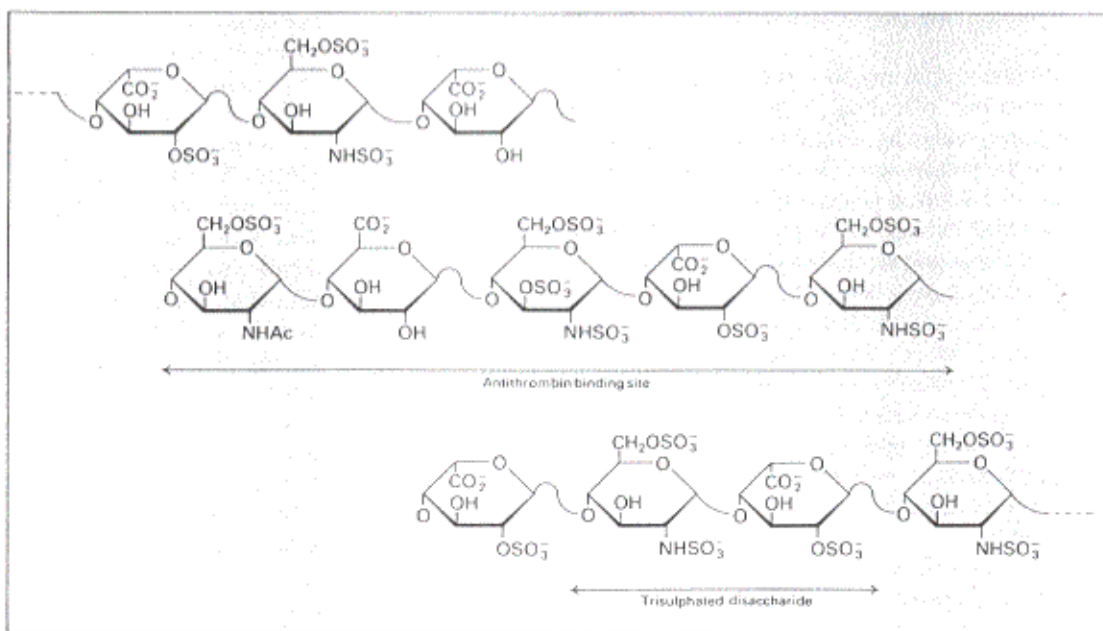


Fig 2 Major disaccharide component and sequence variability within the heparin polymer

Future directions

Low molecular weight (LMW) heparins are prepared by controlled, partial, chemical or enzymatic depolymerisation of commercial GAG heparin. Although each one is different (ie, each has artifacts generated in its preparation), they all have common features. These LMW heparins are polydisperse, with average molecular weights of 5000 and molecular weight ranges of 2000-10,000.¹⁹ Their activity is modified, so they inhibit coagulation early in the cascade and have reduced effect on blood platelets, presumably reducing the bleeding side-effects observed with heparin.¹⁹ LMW heparins are more bioavailable and show an increased half-life. The improved properties of LMW heparins have paved the way for clinical trials of these new anticoagulant/antithrombotic drugs.

A synthetic pentasaccharide, corresponding to heparin's anti-thrombin binding site (Fig 2), was first prepared by Jean Choay and coworkers in 1984 by a low yield, multi-step synthesis.³⁶ This agent has high *in vitro* activity but its *in vivo* potency is lower than heparin's. The cost of its synthesis may preclude its use as a therapeutic agent.

The ideal blood compatible polymer should mimic blood's natural container. This is a vessel lined with heparan sulphate, a heparin-like GAG.^{16,37} The surface should be stable and survive enzymatic and chemical attack from the components present in the blood. Unfortunately, the production of stable heparinised surfaces is very difficult. Simple adsorption of heparin onto a polymer produces a blood compatible surface that only lasts a short period of time.³⁸ Covalent immobilisation offers an alternative, with a chemically stable linkage. These surfaces, however, could still have a short lifetime because of the enzymatic stripping of heparin from the surface.³⁹ The orientation of heparin and linkage chemistry may control enzyme access that would enhance the stability of heparinised surfaces.³⁷ It may even be possible to incorporate heparin directly into the backbone of synthetic polymers.^{37,40}

The use and applications of heparin continue to increase despite its structural complexity, multiple activities and many side-effects. New heparins having high therapeutic indices and increased specificities are currently being developed. The 21st century assures a rising demand for heparinised biomaterials with the introduction of new extracorporeal devices and implanted artificial organs.

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