

Heparin Sodium—A Review

(*The Continuing Battle for Standardization*)

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Heparin is the name applied to a heterogeneous group of sulfated mucopolysaccharide polymers associated with anticoagulation and antilipemic activity *in vivo*. It has been under investigation since the period 1881-1910 when several workers studied a substance with anticoagulant activity which was found in the blood and tissues of dogs after injection of antigen or peptone.¹ Heparin research received impetus in 1916 when Jay McLean, a second year medical student studying with Howell at Johns Hopkins, incidentally isolated alcohol insoluble phosphatides with anticoagulant activity from heart and liver tissue in his studies of the coagulation-accelerating properties of cephalin.² In 1918 the name heparin was given this material by Howell and Holt. During the next ten years Howell worked on the purification of heparin and in 1928 was able to report its chemical properties in some detail. Much has been done since that time to elucidate the structure and mechanisms of action of this substance, but it continues to be a chemical, biological and pharmacological enigma.

Although the chemical structure of heparin has not been completely established, most sources agree that it is a polysaccharide composed of alternating units of D-glucosamine and D-glucuronic acid, with seven anions per tetrasaccharide unit (2 COO⁻, 2 N sulfamino, and 3 O-sulfo groups on the hydroxyls of both sugar moieties).^{3, 4, 5} Recently, however, investigators have indicated that L-iduronic acid is also an important component of heparin, and may be its major sulfated uronic acid. This source also points out that most glucosamine groups are of the sulfamino type, and that the rest are N-acetylated. They indicate that the majority of glucosamine substituents are also sulfated at C6 and that approximately one-half of the uronic acid groups are sulfated at C2.⁶ The sulfaminic linkage is a rare naturally occurring linkage and is associated with heparin anticoagulant activity. Clearing factor activity, on the other hand, seems to depend on the O-sulfate groups.^{1, 7} The molecular weight of heparin is given as 6,000-20,000 for various preparations, with anticoagulant activity generally increasing for increasing molecular weight. The structure of heparin is related to beta-amylase: after desulfuration and reduction heparin can be hydrolyzed by beta-amylase.³

Heparin is found in the tissue mast cells, where it accounts for their metachromatic staining. The highest concentrations of heparin are in pulmonary and hepatic tissues. While investigators have been able to demonstrate high blood levels of heparin after experimentally induced peptone or anapylactic shock, it has been very difficult to demonstrate heparin in normal blood. However, specific methods of detection have been developed and have yielded positive results. These methods are complicated by the fact that heparin is distributed among platelets, WBC's, plasma-bound, and plasma-free fractions *in vivo*. Determination of human free heparin by one investigator revealed values of 0.1-0.24 microgm./ml. The smallest quantity of free heparin that can be demonstrated by anticoagulant activity is 1 microgm./ml., while for lipemia clearing it is 0.1 microgm./ml.; it can therefore be seen that the trace amount of heparin present in normal blood is probably not significant in terms of coagulation, but may be of some significance in relation to serum cholesterol levels.³

Heparin is equally distributed among the mentioned blood fractions, is metabolized by liver heparinase to uroheparin and excreted in the urine (by an unknown renal mechanism). No heparin is found in fecal material, nor does it cross the placenta. High doses of heparin lead to the excretion of free unmetabolized heparin in the urine; small molecular weight forms are also quickly excreted. When measuring the *in vitro* effect on clotting or lipolytic activity of a single IV dose of heparin, it is noted that activity generally peaks within a few minutes (10-20), and rapidly disappears, so that those responses are generally gone within two hours.^{1, 3, 9}

Article available at <https://ject.edpsciences.org> or <https://doi.org/10.1051/ject/197464207>

While identical on elementary chemical analysis, heparin from different mammalian species has different anticoagulant activity (e.g. dog $2\frac{1}{2} \times$ beef, the commercial preparation). There is a tissue difference in reactivity also, with gut-derived heparin (mucosal) having more anticoagulant activity/mg. than lung-derived heparin, with this tissue difference generally greater than the species difference (some have speculated that the lower activity of lung heparin might be a function of contamination with a mucopolysaccharide that interferes with the assay). When examining figures reporting differences in anticoagulant activity it is important to consider differences in methods of preparation and purity and their possible effect on heparin activity.^{7,8} Many commercial preparations of heparin are available. Some of these are as follows:¹⁰

SOME COMMERCIALY AVAILABLE PREPARATIONS OF HEPARIN

<u>Generic Name</u>	<u>Proprietary Name</u>	<u>Mfg.</u>	<u>Animal Source</u>	<u>Organ</u>	<u>Preservative</u>
Heparin Na ⁺	Panheprin	Abbott	Pork	Mucosa	Benzyl Alcohol
Heparin Na ⁺	Heparin Sodium	Lederle	Pork	Mucosa	
Heparin Na ⁺	Liquaemin Sodium	Organon	Beef		
Heparin Na ⁺	Bio-Heprin	Riess	Pork	Mucosa	Benzyl Alcohol
Heparin Na ⁺	Lipo-Heprin	Riker	Pork	Intestine Mucosa	Benzyl Alcohol & Na Bisulfite
Heparin Na ⁺	Hepathrom	Testagar	Pork	Mucosa Lung	Benzyl Alcohol
Heparin Na ⁺	Dopo-Heparin Na ⁺	Upjohn	Beef	Lung	Thimersol
Heparin Na ⁺	Heparin Sodium	Upjohn	Beef	Lung	Benzyl Alcohol
Heparin Na ⁺	Heparin (Tubex)	Wyeth	Beef & Swine	Liver Lung	Benzyl Alcohol

When considering the biologic functions of heparin it must be remembered that many of the actions ascribed to it may possibly be related to the particular preservative used or to peptide and polysaccharide contaminants of the preparation. In this regard it is interesting to note that polysaccharide fractions from a gram-negative proteus have antilipemic activity around 1,000 times that of heparin. Thus it is possible that a similar polysaccharide contaminant might be responsible for this action of heparin.¹

With this consideration in mind, the many actions ascribed to heparin can be examined. While the intention here is to concentrate on its anticoagulant activity, many more functions have been described, as follows:⁸

ACTIONS OF HEPARIN IN VITRO

Enzymes inhibited	Enzyme Activity Increased
Fumarase	Alkaline Phosphatase
Hyaluronidase	Blood Esterase
Lysozyme	Fibrinolysis (? plasma)
RNAase	Ag/ Ab Inhibition
Serum Protease	Bronchospasm test
Trypsin	Hemolytic complement
Acid Phosphatase	Sensitized Uterine Strip
Stabilizes	Inhibition of Coagulation
Leukocyte Peroxidase	Platelet contact
Pyruvic Acid	Platelet agglutination
RNA	Action of thrombin on platelets
Inhibits	Action of thrombin on fibrinogen
Hemolysis by Cobra venom	Formation of thromboplastin (intrinsic)
Histamine on g. pig ileum	Formation of thrombin
Serotonin on rat colon or uterus	

ACTION *IN VIVO*

Decreases

coagulation
 blood sugar
 excretion of K⁺
 fall in lymphocytes and eosinophils
 by ACTH or cortisone
 hemorrhage from stress
 hypertension
 lipemia
 platelet adhesion to intercellular cement
 Serotonin effect of CVS and in
 pulmonary embolism
 thrombus on endothelium
 (gross) thrombus formation

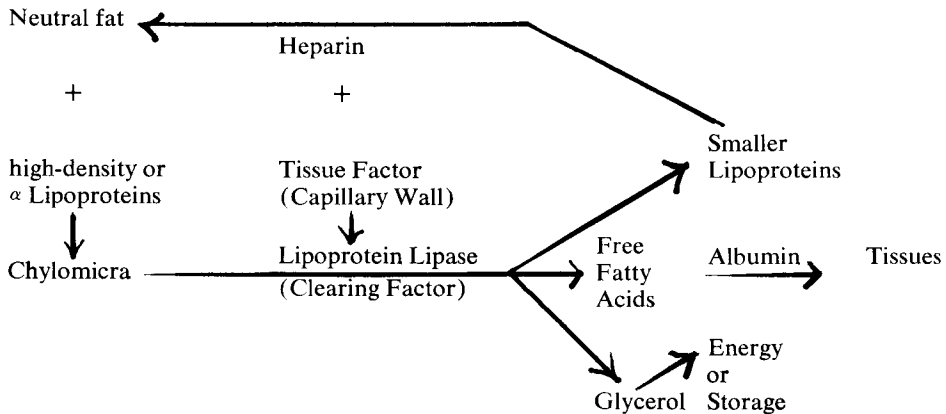
Increases

blood flow
 connective tissue formation
 eosinophilia
 excretion of Na⁺
 fibrinolysis
 vasodilation

Also—has cation exchange capacity
 antiinflammatory activity—binds histamine

—binds with some drugs (polymyxin, neomycin, viomycin, etc.) to
 their toxicity thus interfering with antibiotic activity

As lipolysis was mentioned at the beginning of this paper as a major function of heparin, its proposed mechanism will be briefly considered. Heparin interacts with a tissue component (apoenzyme) to release an enzyme, lipoprotein lipase, that causes the true lipolysis of neutral fat (triglycerides in lipoproteins), in a system that requires albumin as a receptor molecule. Schematically, this may be represented as follows:¹¹

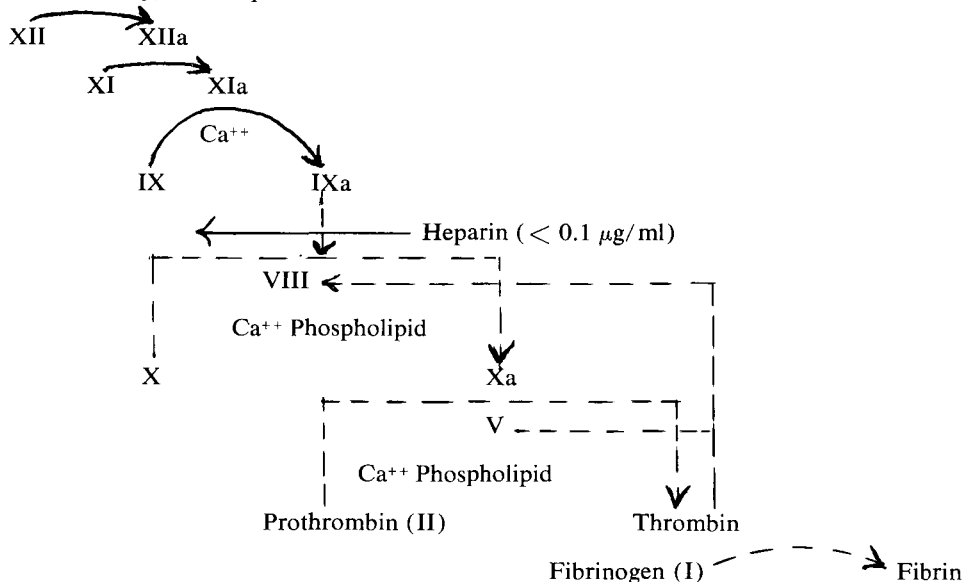


Heparin = a prosthetic group on the enzyme

It has been proposed that this is a major pathway for the removal of alimentary lipemia from the bloodstream.

Focusing on the anticoagulant properties of this drug, it is known that heparin inhibits, in order of increasing effectiveness: thrombin; thromboplastin; Factor V, thromboplastin generation; Factor IX; and the contact effect (Factor XI), all functions which are extremely pH-dependent (activity decreases with decreasing pH). Heparin also has a complicated interaction with platelets: it inhibits their aggregation by thrombin, but not by ADP.^{1,3} There is a suggestion by one investigator¹² that the major anticoagulant action is due to anti-thrombin action, i.e. heparin and its cofactor inhibit the complexing of thrombin with its substrates by competitively complexing with thrombin (without degrading thrombin), a function of the strong negative charge of heparin. He ascribes the anticoagulation action of the strongly positive heparin inhibitors polybrene and protamine to complexing with thrombin substrates. On the other hand, it is known that only 0.003-0.01 mg/ml heparin is required to inhibit thrombin formation, while 0.1-0.4 mg/ml is required to inhibit the action of formed thrombin (plasma levels).¹ This would suggest that the quantitatively most important anticoagulation effect is due to the inhibition of thrombin formation. Other investigators agree with this hypothesis and present data suggesting that activated factor IX is the heparin-sensitive factor in plasma when coagu-

lation is initiated via XII or XI (the intrinsic system) and that subsequent factors remain in inactive forms.⁵ They also go on to cite the action of heparin against factor XI (activated) activation of factor IX and its interference with the action of thrombin. Schematically, this is represented as follows:



It is important to re-emphasize here that the antithrombin action of heparin depends upon a plasma cofactor. The anticoagulation mechanism becomes confused at this point, as it has long been known that plasma has a specific protein associated with the alpha₂ globulins that is able to bring about the irreversible destruction of thrombin in a progressive manner suggestive of enzymatic degradation. This occurs without heparin, but added heparin accelerates the disappearance of thrombin. The exact relationship between plasma antithrombin and heparin cofactor has not been worked out. Some workers^{13, 14} conclude that heparin cofactor activity and plasma antithrombin activity are functions of the same protein, e.g. that anti-thrombin exists in plasma complexed with an inhibitor of its action and that heparin acts to displace the inhibitor and expose antithrombin to its substrate, thrombin. In this model heparin cofactor activity would be ascribed to the antithrombin-inhibitor complex as it reacts with heparin. On the other hand there are those who have claimed to separate these two activities by various electrophoretic methods.¹³ No firm conclusions can be arrived at on the basis of the data presented, and this remains a very important unsolved pharmacologic question, as will be discussed below.

Having considered heparin from the standpoint of structure, origin, preparations and function, it is now appropriate to consider the assay procedures that have been developed to assess heparin activity. This area perhaps presents more problems than all the previously considered ones, as there exists no general agreement as to which of many assays used most closely reflects the true biological potency of heparin.⁹ The establishment of true chemical assays that might give accurate dose-response data is limited by several factors. It has been mentioned that heparin from different species and even from different tissues of homologous species poses different anticoagulant activity/mg. in spite of giving identical chemical analysis. It also has been stated that heparin represents a family of polymers. This has a direct effect on assays in that *in vitro* assays will be influenced by heparin molecules of all sizes, while *in vivo* assays reflect the activity of only those molecules large enough not to be rapidly excreted by the kidney.⁹ A further complicating factor to such analysis is the contaminating substances accompanying heparin in its preparations. The classic fractionation of Jorpes, et. al. (alcohol, benzidine, brucine, barium, and long chain amines) doesn't separate all contaminants. Paper chromatography does, but is difficult to perform on undergraded polysaccharides. Electro dialysis leads to loss of activity, and ion exchange resins are hydrolytic for polysaccharides.⁸ Recently electrophoresis and microelectrophoresis assays have been developed which may prove to be very useful chemical tools in heparin analysis.¹⁵

Some investigators have exploited the metachromatic activity of heparin in assays. This has met with problems. Different sources of heparin, as indicated, give different

units/mg. However, one mg. of any preparation has the same metachromatic activity (which is associated with polymer size and degree of sulfation). Physiologic inactivation of heparin proceeds in terms of anticoagulant activity and not its metachromatic activity. Thus this assay method is of little significance in the prediction of biologic activity.⁸

With these limitations in mind, investigators have turned to biological assays, feeling that the only acceptable definition of heparin is its anticoagulant activity. Several methods of assaying anticoagulation activity exist, among these the whole blood clotting time (WBCT), the partial thromboplastin time (PTT), the thrombin time, and plasma heparin concentration (antithrombin assay)—all limited by the fact that there is no sure species-dose-response relationship, as plasma cofactor and general host-response remain uncontrolled variables. The WBCT and PTT are further limited by their sensitivity to small amounts of heparin.¹⁶ Nevertheless, several types of assays using these techniques and variants have been developed.^{8, 16-19} Pitney and coworkers in England recently did a study of the clinical effects of heparin therapy compared with anticoagulation as indicated by the WBCT, PTT, and plasma heparin concentration assays. While there was internal consistency within each assay, there was very poor correlation among the assays and the clinical response, a correlation that worsened as the anticoagulation effect became more pronounced.²⁰ This revealing study indicates the unreliability of these tests as clinical standards.

Protamine assays for heparin are likewise somewhat unreliable. These assays involve titration of a quantity of heparin with aliquots of protamine using a suitable clotting system as the indicator; since protamine also inhibits coagulation, the end point is recognized as the clotting time which most closely corresponds to the test system without heparin or protamine (protamine has a much higher affinity for heparin than for blood-coagulation proteins).⁷ Although protamine generally reacts with heparin on a mg for mg basis, there is evidence that more than one mg protamine is required to neutralize one mg of heparin preparations of high specific activity (150 IU and above). This, and the fact that host variability is uncontrolled makes such an assay at best empirically useful, at worst a false indicator.

A more profound instance of unreliability is seen when one examines the methods for arriving at unit values for heparin preparations. The USP unit is that quantity of heparin that will prevent 1.0 ml. of recalcified sheep plasma from coagulating for one hour. Sodium heparin, USP, must contain at least 120 USP units/mg. The sample is compared with the USP reference, of mucosal origin, containing 2.8 USP units/mg. The BP has a similar assay and standard with different unit values.⁹ The international standard, established in 1958, is made from ox lung by a process including precipitation as a crystalline barium salt. An extensive study comparing the USP and BP assays of different heparins and standards revealed that USP assays gave values approximately 70% of the BP assay when comparing lung and mucosal heparin. This difference did not exist if different mucosal preparations were compared. When the international standard was compared with mucosal heparin, the investigators often got an invalid assay.⁷ Although each assay system was internally consistent, the fact that they diverged significantly in certain areas is unsettling, particularly when one considers the fact that one is comparing biological variables with a contrived standard that itself must be considered a biological variable, and that therefore no truly objective foundation for establishing heparin unit activity exists. It is also worth emphasizing that this study reveals that lung heparin is an unsuitable standard for heparin of gut mucosal origin.

One further assay system that may prove more useful employs radiolabelled heparin. Radiolabelled heparin S³⁵ can be detected easily after intravenous injection and provides useful information on blood disappearance time and tissue distribution of heparin.¹⁶ However, this method is too hazardous for clinical use. Recently Varga and his colleagues have described a technique for the *in vitro* labelling of heparin with Cr⁵¹.²¹ This tracer is widely used in clinical studies, and this technique should provide a safe and useful tool for elucidating the physiological aspects, if not the chemical determinants, of heparin activity.

Evaluating the above material, it is easily perceived that there is a distressing lack of basic knowledge about this very useful but potentially lethal drug (there are many side effects²⁻¹⁰ but none comparable in incidence or importance to its hemorrhagic potential), without which a precise assay is impossible. It would seem that there are at least three major areas in which clarification is of immediate necessity. These are as follows:

1) What is the precise chemical structure of heparin? Though much is known of its primary structure and its relationship to function, very little is known of its possible secondary and tertiary structures. The extent of intermolecular cross-linking (mainly SO_4^- mediated) and how it relates to function remains to be elucidated. Some investigators suggest a helical structure for heparin, but the sedimentation-centrifugation methods used in studying such structures are interfered with by the high negative charge of heparin that has a profound effect on the arrangement of solvent molecules in its environment. Perhaps when such factors are known it may be possible to synthesize a heparin molecule with factors that affect activity, such as molecular weight and sulfation, well controlled (established synthetic heparinoids can't approach heparin when all parameters are considered). By synthetically controlling the heparin product other variables can be eliminated, e.g. contamination with nonheparin material. An efficient heparin can be established only when all the factors that govern its reactivity are revealed. Getting a handle on the chemical nature of heparin is only half of the dose-response problem, though—the second half being problem #2.

2) What is heparin cofactor? This seems to be, at least at the present time, the prime uncontrollable variable in considering the effect of heparin on the coagulation process. Biochemists and hematologists must continue their efforts to separate if possible the antithrombin-heparin cofactor activities of plasma (there remains the real possibility that these are dual effects of a large polypeptide molecule). Perhaps heparin-labelling experiments may prove to be useful adjuncts to the promising electrophoretic studies in progress. Recovery of labelled heparin cofactor or heparin antithrombin complexes from plasma would do much toward clarifying the conflicting theories presented above. Other important questions in this regard are: where does cofactor come from? what stimuli elicit its synthesis—is it a heparin-specific factor? Only when the nature of cofactor is elucidated can true controlled studies on the heparin anticoagulation system be done. It is also evident that the development of a meaningful assay awaits the control of this as yet unpredictable variable.

3) The third major problem is the determination of just which step is quantitatively most important in the anticoagulant action of heparin. Data have been mentioned that suggest the most important reaction to be at activated factor IX. However, it remains to be determined whether this is the most significant effect at the blood levels attained clinically, which are much higher than those necessary to inhibit this step. Isolated, and hotly debated, reports of rebound phenomena after discontinuing heparin therapy exist, and clarification is needed as to whether or not there is an accumulation of activated cofactors during heparin therapy, which might lead to rebound coagulation on ending therapy. The fact that this is not a widespread phenomena mitigates against this possibility, but intelligent therapy will be possible only if the issue can be completely resolved.

These problems can be consolidated in the question of how this drug can be reliably and consistently controlled in clinical practice. While the microelectrophoretic assay mentioned above offers hope as a consistent quantitative tool (although one taking a relatively long time to perform), the data it, or any other useful assay, may generate will not be completely predictive or reliable until the above questions are answered. Until the basic nature of the drug and the system in which it operates are elucidated, our therapy will be empirical, at best—a shaky empiricism, with too many uncontrolled variables at work.

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