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REVIEW ARTICLE

Chemistry and Pharmacology of Heparin

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Heparin is a mucopolysaccharide composed of partially sulfated units of α-D-glucuronic acid and 2-amino-2-deoxy-α-D-glucose joined by 1 → 4 bonds. It is mainly noted for its anticoagulant activity in the treatment of myocardial infarction, but it is also an antilipemic agent. Heparin is thought to originate in the mast cells of connective tissue and is found in beef, hog, and dog livers; beef muscle, adipose tissue, and spleen; hog mucin and alimentary canal; skin of rats; fish plasma; sea clams; and scales of carp.

Since its discovery in 1916 by McLean, many studies have been made on its chemical structure and pharmacology, but progress has been much slower in the elucidation of the macromolecular properties of the polysaccharide, particularly the relationship of physical parameters to its biological action.

The early history, chemistry, and clinical applications of heparin were described by Jorpes (1), Whistler and Smart (2), Foster and Huggard (3), and Stacey and Barber (4). Some general accounts of the mucopolysaccharides and heparin were later written by Blonde (5), Brimacombe and Webber (6), Whistler and Rowell (7), Engelberg (8), Meyer (9), and Jeanloz (10).

Relative to physicochemical aspects of heparin, it exhibits typical polyelectrolyte behavior in aqueous

solution due to its high negative charge. Accordingly, physicochemical measurements are sensitive to ionic strength and pH. Heparin is polydispersed and molecular weights from 6000 to 20,000 have been reported. Physicochemical properties in relation to blood anticoagulation are discussed later in this review.

The purpose of this review is to furnish an up-to-date, comprehensive coverage of the many aspects of this extremely interesting and important drug. This review, although comprehensive, is not necessarily exhaustive. The literature search was carried up to May 1, 1972, and the main body of the review consists of information from the literature of the last decade.

SOURCES AND ISOLATION OF HEPARIN

Most of the heparin for pharmaceutical use is extracted from bovine lung tissue. However, the literature contains many examples of heparin or heparin-like substances isolated from other sources.

Marine life has yielded heparin-like materials with high blood anticoagulant activity. Thomas (11, 12) obtained such a substance from the common surf clam, *Spisula solidissima*, which might be chemically similar to heparin. Frommhagen *et al.* (13) extracted mactin-A and B from two species of clams, *Mactra spissula* and *Artica islandica*, which had a higher activity than heparin and a lower toxicity. Heparin was also found in the blood (14), liver (15), and muscles (15) of fish.

The skin of many animals has been claimed to be a source of heparin. Bollet *et al.* (16) located it in the human dermis and subcutaneous tissue. Horner (17), in 1971, isolated heparin with a molecular weight of over 1 million from rat skin. Injured animal skin has yielded desulfated heparin (18).

Heparin has been found in normal human serum in small amounts (19). Nilsson (20) described a method of extraction of an anticoagulant, probably heparin, from normal horse blood, which exhibited strong metachromasia. The data of Smith and Von Korff (21) indicate a heparin-precipitable complex of a fibrinogen-like protein closely combined with heparin which was found in human plasma. The origin of heparin in the blood may be the basophil cells, which are almost functionally identical with mast cells and might release small amounts of heparin into the plasma (22). On the other hand, Ringertz (23) found that heparin can be liberated from the mast cells by a number of agents that cause the disruption of these cells, but no heparin was detected in the blood.

The kidney of the rat has yielded a heparin-like substance (24). Allalouf *et al.* (25) isolated this material and discovered that it acted similarly to heparin in anticoagulant activity (8 units/mg.), protamine antagonism, and the recalcification test. The major component of the mucopolysaccharides extracted from this source was heparin (26).

Healthy human liver was shown by Schmitz-Moorman (27) to contain uronic acid mucoids consisting of approximately equal amounts of hyaluronic acid, chondroitin, chondroitin sulfate, and heparin. In cases of amyloidosis, the mucopolysaccharides of the amyloid liver were found to contain small amounts of heparin (28, 29), which was 60 times as much acidic mucopolysaccharide content as in healthy liver (30). The capsule surrounding the ox liver was shown by Serafini-Fracassini *et al.* (31) to contain heparin in single chains or as a protein–polysaccharide complex rather than as a multichain proteoglycan. The ox liver capsule also yielded a heparin–protein complex (32).

Other sources of heparin include the aorta (33–35), the loin and ham muscles of the pig (36, 37), the sweat (38), the thyroid (16), and the heart (39). Heparin-related glycosaminoglycans also were isolated from the acid-soluble fraction of Chinese hamster fibroblasts (40), from HeLa L mouse embryo, and from rat embryo cells grown in culture medium in the presence of radioactive inorganic sulfate (41).

The method of Charles and Scott (42), with modifications, is used for most extractions of heparin. In their technique the animal tissues are treated with alkali to remove proteins. Homan and Lens (43) did this removal by shaking with phenol. Jeanloz (44) reported other methods of heparin extraction involving enzymic degradation. Barlow *et al.* (45) described a technique of isolating heparin from sheep, dog, beef, and hog tissues by selective fractionation with quaternary ammonium compounds. They claimed that the heparins were biologically, chemically, and physically similar.

CHEMICAL STRUCTURE OF HEPARIN

Many reviews have sections on the nomenclature and classification of the mucopolysaccharides (46, 47) and the chemistry of heparin (3, 4, 6, 48–53).

The elucidation of the structure of heparin has been concerned with the type of glycosidic linkage, the nature of the uronic acid component, the sulfur content and sulfamino group, the carboxyl and other groups, and the amount of branching.

Cifonelli and Dorfman (54) showed that the major linkage in heparin is uronosyl 1 → 6 hexosamine, while that in heparitin sulfate is mostly 1 → 4. Durant *et al.* (55) and Danishefsky and Steiner (56), on the other hand, found a 1 → 4 linkage also for heparin. This was confirmed by Wolfrom *et al.* (57), who also found that the configuration of the 2-amino-2-deoxy-D-glucuronic acid linkage is α-D. The possibility of some β-configuration might also exist (51, 58). Lloyd and Evans (59) reported a method of linkage analysis of heparin and related mucopolysaccharides by forming *N*-(2,4-dinitrophenyl) derivatives of de-*N*-acylated mucopolysaccharides, rather than affecting the labile sulfamino groups, by breaking acid-resistant 2-amino-2-deoxyglycosidic bonds to achieve a graded depolymerization.

The sulfur content of heparin has been shown to be associated with its anticoagulant activity (60). A method of determination of sulfur as sulfate is precipitation with barium (60, 61). Another technique, proposed by Braselmann (62), permits the determination of 0–12 mcg. of sulfate, with an error of ±0.2 mcg., by heating the heparin sample with hydriodic acid, hypophosphorous acid, and hydrochloric acid to reduce sulfate to hydrogen sulfide. A rapid and sensitive method using GLC to analyze sulfate in mucopolysaccharides, including heparin, in micrograms was devised by Srini-

vasan *et al.* (63). This method was based on the ability of mineral acids to form stable salts with the mucopolysaccharides, which, after reaction with sodium hydroxide, liberate amines that are detected by GLC. Even more recently, Wessler (64) measured the sulfate content in less than a microgram of glycosaminoglycans with a method based on the fact that the electrophoretic mobility in 0.1 *M* HCl is proportional to the sulfate content. Hiyama *et al.* (65) indicated that the anticoagulant activity of heparin is, in general, dependent on the degree of sulfation and the number and position of the *O*-sulfate groups as well as the size of the skeletal polysaccharide molecule.

Durant *et al.* (55) claimed that approximately one-eighth of the hexuronic acid units have no *O*-sulfate groups. The evidence of the report by Wolfrom and Wang (66) establishes that the C-6 hydroxyl group of the 2-amino-2-deoxy-D-glucose unit of heparin is sulfated. In a later work, Wolfrom *et al.* (67) definitively placed the two sulfate groups (per tetrasaccharide unit) on C-6 of the 2-amino-2-deoxy-D-glucose residues and demonstrated that D-glucuronic acid residues are not sulfated. Danishefsky *et al.* (68) investigated the position of the sulfate groups by methylation procedures, and they concluded that about one-third of the glucuronic acid moieties in heparin are sulfated on C-2 whereas the rest are not sulfated. Additionally, most of the glucosamine is sulfated on C-6, but nonsulfated glucosamine units and 3,6-di-*O*-sulfoglucosamine units are also present in small proportions. Helbert and Marini (69), on the basis of elemental analysis and titration studies, found that not all of the sulfate forms an integral part of the heparin molecule but it may be closely associated with it.

The extent of carboxylation may be important in the anticoagulant activity of heparin, as reported by Stivala and Liberti (70) based on copper binding studies. It is possible to convert the carboxyl groups of heparin and other mucopolysaccharides into substitution acids of amino acids (71). These were obtained with glycine methyl ester, phenylalanine methyl ester, and alanine amide.

Heparin is usually considered to be an extended linear molecule. Some authors, however, such as Freeman (72) and Bettleheim (51), still believe in the possibility of a branched structure.

The question of whether heparin contains acetyl groups is still unsettled. Rhadhakrishnamurthy *et al.* (73) found varying amounts of acetyl groups in heparin samples from various sources. The presence of these groups might be due to the presence of heparitin sulfate, as shown by NMR studies (74).

It was concluded by chemical means that D-glucuronic acid is the chief uronic acid component of heparin (75, 76). Wolfrom *et al.* (77) isolated L-iduronic from heparin by a periodate oxidation study. Perlin *et al.* (78–80) showed with NMR spectra that heparin contains a substantial proportion (perhaps one-third or more) of L-idopyranosyluronic residues, in addition to the D-glucosyluronic acid and 2-amino-2-deoxy-D-glucose units. Lindahl and Axelsson (81), through a nitrous oxide degradation of heparin and separation by gel permeation and paper electrophoresis, found that almost all of the uronic acid present in certain fractions was iduronic acid. Rhadhakrishnamurthy *et al.* (82) investigated the nature of the uronic acid by the use of GLC and found that the ratio of iduronic acid to glucuronic acid was 1:2.6.

Through degradation studies with *Flavobacterium heparinum* (83), it was concluded that the heparin molecule is composed largely of a repeating sequence of (1 → 4)-linked 4-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-2-(deoxy-2-sulfamino-α-D-glucopyranosyl-6-sulfate) biose residues. Another proposed structure is that of the tetrasaccharide unit of Helting and Lindahl (58) with both uronic and iduronic residues (Structure I).

A reaction thought by Sampson and Meyer (84) to be important in structural studies is that of heparin or heparitin sulfate with alkali to form 3,6-anhydroglucosamine groups, in which the 6-position is sulfated and the 3-position is unsubstituted.

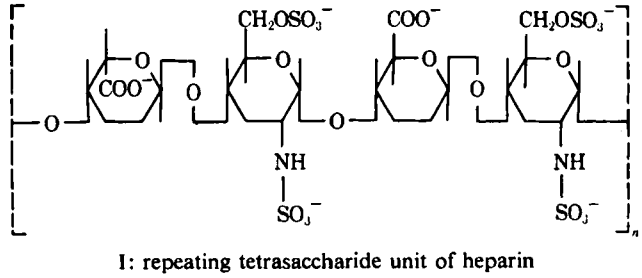
It is evident from the numerous studies conducted on the structure determination of heparin that its true configuration remains to be established unequivocally. The defiance of heparin to be clearly identified may be due to the fact that the heparin derived from different tissues within a given species or from the same tissues from different species may have subtle differences in its structure. Accordingly, it is not surprising that differences in chemical structures have been observed, since heparin is a protein–polysaccharide complex before extraction. The extraction procedure can often leave some undiscernible impurities, which undoubtedly compounds the difficulty in structure determination.

ELECTROPHORESIS AND CHROMATOGRAPHY OF HEPARIN

The techniques of electrophoresis and chromatography of heparin and other acid mucopolysaccharides for separation, identification, fractionation, characterization, and binding studies have been investigated by many workers.

Separation Techniques—One of the oldest separation methods used was electrophoresis. In 1951, Favre-Gilly (85), using the moving boundary method, discovered that the electrophoretic mobility of heparin is 17 × 10^{-5} cm.^{2} v.$^{-1}$ sec.$^{-1}$. Also using the same technique and employing data available on the heparin–streptomycin complex, Barlow and Coen (86) separated heparin free from streptomycin.

Paper and gel electrophoresis have now gained much popularity in separation methods. Bifani and Marcialis (87) discovered that heparin had a higher migration velocity than serum proteins on paper. Conti *et al.* (88) detected heparin in the presence of chondroitin sulfuric



I: repeating tetrasaccharide unit of heparin

acid, hyaluronic acid, or nucleic acid by staining the paper with Auramino O, Malachite green, or Brilliant green. Starch gel electrophoresis has been carried out for the separation of heparitin sulfate from chondroitin sulfate A and B and hyaluronic acid (89). Another sensitive method is zone electrophoresis on cellulose acetate, with alcian blue as a stain (90).

Horner (91) separated heparin and chondroitin sulfate by their differences in charge density on agarose gel. He also noted that heparin had two components on this gel; the slower moving component had the higher anticoagulant activity, whereas the faster moving component had the higher sulfate–carboxyl ratio (92). Jaques *et al.* (93, 94) found that most heparins showed two components by microelectrophoresis on agarose, except for those from the sheep and rat. The use of a microzone cellulose acetate cell[1] enabled Herd (95) to separate heparin; hyaluronic acid; chondroitin sulfates A, B, and C; and keratosulfate. Heparin and heparan sulfate can be separated from other glycosaminoglycans with barium acetate electrophoresis, where the electrophoretic mobility depends primarily on the polysaccharide backbone (96). The relative mobilities of heparin, chondroitin sulfate A (or C), and hyaluronic acid in electrophoresis by polyacrylamide gels were 1.11:1.00:0.67 (97), which are the same as in free solution electrophoresis (98). Another type of electrophoretic separation was performed by Mashburn and Hoffman (99), who used a continuous-flow electrophoretic flow separator for the separation of heparin, chondroitin sulfate, hyaluronic acid, and protein.

Liquid and ion-exchange chromatography are rather old techniques but recently gained new impetus for separations. In 1962, Schmidt (100) obtained a clear separation of the acid mucopolysaccharides on a DEAE-Sephadex anion exchanger. Using the same type of column, Braselmann and Ramm (101) did a microseparation of hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate, chondroitin-6-sulfate, and heparin. In a column chromatographic study, Greiling *et al.* (102) and Egardo *et al.* (103) separated heparin from hyaluronic acid and chondroitin sulfate, utilizing ECTEOLA-cellulose with gradient elution. This type of column was also used for the quantitative estimation of acid mucopolysaccharides in vascular tissue (104). A cellulose microcolumn technique to separate mucopolysaccharides was described by Svejcar and Robertson (105). Microcolumns of Sephadex G-25 (fine) were used to carry out the separation of heparin from D-galactose by Stouffer *et al.* (106), with a hydrogen flame-ionization detector. Another interesting application of this method was the differential precipitation of individual polyanions with quaternary ammonium salts in solutions of specific ionic strength and the subsequent formation of red ion association complexes with the bound quaternary moieties; thus the individual mucopolysaccharides could be identified (107).

Two other major techniques are paper chromatography and TLC. Spolter and Marx (108) separated heparin and chondroitin sulfate by paper chromatography with a solvent of buffer and isopropanol. Bayer (109) used a solvent system containing *n*-propanol. An ascending paper chromatography method with acetate buffer allowed Good (110) to separate heparitin sulfate, heparin, keratosulfate, and chondroitin sulfate B. Aliev and Alekperov (111) used ascending paper chromatography for stepwise control of heparin production. They (112) also reported that commercial heparin gave two spots which might be due to chondroitin sulfate as an impurity. Chondroitin sulfate B (dermatan sulfate) has been identified as a contaminant of bovine heparin during fractionation due to solubility differences of the two substances (see *Physicochemical Studies*). Other methods included microseparation on paper based on the formation of insoluble complexes with cetylpyridinium chloride (113) and the use of salt-gradient ion-exchange paper (114).

In the last few years, TLC has gained support in the separation of mucopolysaccharides. Wustemann *et al.* (115) successfully separated heparin from heparitin sulfate, chondroitin-6-sulfate, and chondroitin-4-sulfate on silica gel, with nitrous indole as a spray specific for detection of the *N*-sulfated aminopolysaccharides. Bischel *et al.* (116) used Sephadex and cellulose as layers, with toluidine blue staining. Other investigators (117) obtained two spots for heparin on cellulose, using ninhydrin as a detection agent.

Fractionation—Chromatography and gel permeation are potent methods for fractionating according to molecular weights and for estimating heterogeneity. In 1958, Payza (118) fractionated heparin on Amberlite IR-45 resin by elution with different molarities of sodium chloride. An ECTEOLA-anion exchanger was later used by Whiteley (119). Laurent (120), after precipitating heparin with cetylpyridinium chloride, used the same system to obtain four fractions. Lasker and Stivala (121) likewise obtained the same number of fractions by ECTEOLA elution, but got 12 fractions by differential solubility in alcohol–water mixtures. (Nine fractions have been obtained by fractional precipitation, using ethanol and dioxane as precipitants; see *Physicochemical Studies*.) The efficiency of DEAE-cellulose as an adsorbent in fractionation was proven by Cleland *et al.* (122), and recently DiFerrante and Popenloe (123) fractionated heparin on Dowex-1.

Further work on fractionation was carried out by Hall *et al.* (124), who submitted pork heparin to gel filtration on a column of Sephadex and found that the heparin was polydispersed with respect to molecular size. Constantopoulos *et al.* (125) and Mathews and Decker (97) determined the degree of heterogeneity of a mixture of acid mucopolysaccharides of different molecular sizes by means of gel filtration.

Characterization—Since exclusion by the gel particles depends on molecular shape, the study of the behavior of heparin on a Sephadex column with changes in the ionic strength of the eluant gave indication that the heparin molecules change in shape and size as well as in solvation, since they are retarded on the column with solvents of higher ionic strength (126).

The work of Hall *et al.* (124) suggests that the anticoagulant activity of a sample could be relatively homogeneous even though it is polydisperse in size. Ferrari and Boffi (127), on the other hand, claimed that

[1] Beckman model R-100.

it was impossible to correlate anticoagulant activity with chromatographic behavior since all samples gave lengthened spots, similar in shape and with the same R_f.

Hilborn and Anastassiadis (128) recently demonstrated how acrylamide gel electrophoresis could be used to: (*a*) estimate the molecular weight of many acidic mucopolysaccharides (including heparin) and (*b*) measure the variations that may exist in their molecular weights and/or the degree of sulfation.

Binding Studies—Boundary electrophoretic studies were conducted by Barlow (129) on the binding of heparin with histamine, using paper electrophoresis. The finding of two different electrophoretic patterns as a function of anticoagulant activity indicates that there is a difference among samples as to their ability to bind histamine; this result could be due to a difference in anticoagulant activity.

Another study of binding was conducted by Accary *et al.* (130) on polysaccharide–sulfate–protein complexes, using paper electrophoresis.

PHYSICOCHEMICAL STUDIES

Deactivation of Heparin and Structure Determination—It has been suggested that anticoagulant activity of heparin is related to sulfation (6, 131–134), to carboxylation (6, 131–134), and to various physical parameters such as molecular weight, shape, and size (6, 131–135). These have been studied by mild hydrolysis of heparin, which results in desulfation (without chain cleavage) accompanied by a decrease in biological activity (deactivation).

Wolfrom and McNeely (136), in 1945, discovered that very mild acidity causes the heparin molecule to lose its blood anticoagulant activity. The rate of loss of activity is proportional to the rate of appearance of a free amino group in the molecule, and substantially complete inactivation occurs when somewhat more than one-half of the nitrogen is released as free amino nitrogen. Jensen *et al.* (135) reported that reaction of heparin with warm acetic acid resulted in a strong decrease in anticoagulant activity without any decrease in molecular weight, sulfate ester, or glucosamine content. There was also an increase in the sedimentation constant and the degree of polydispersity but a decrease in the frictional ratio. They suggested that the amino nitrogen that was first freed during recrystallization of heparin after acid treatment played an important role in anticoagulant activity. The findings of Gauthier *et al.* (137) showed that if the pH was adjusted with acid to 1 or 2 at 25°, there was no change in the biological activity of the heparin within the first 24 hr. Substantiating this finding is the evidence presented by Stock and Warner (138), who observed deterioration of heparin only after 60 hr. at pH 4.4 and 23°. Swarte and Huizinga (139) showed that heparin solutions can be sterilized by steam at 100° for 1 hr. at pH 7.0 with no changes in anticoagulant activity. On the other hand, it is possible to degrade heparin in a strong alkali medium with a rapid loss of antilipemic activity (140).

Foster *et al.* (141) maintained that the anticoagulant activity does not necessarily follow the content of sulfamino groups, since they observed that some heparin preparations with high *N*-sulfate content exhibited low anticoagulant power. In most cases, however, the anticoagulant properties of the sulfated polysaccharides containing no sulfamino groups amounted to only a fraction of that of heparin itself.

Ricketts (142, 143) concluded that the molecular weight of the polysaccharide was more important than the sulfate content, since little activity was found in low molecular weight species of polysaccharides (degree of polymerization = 9) even when the sulfate content was high (2–2.8 sulfate groups per glucose residue). Sulfated dextrans showed a higher anticoagulant activity for the polymer possessing the higher molecular weight. Patat and Elias (144) likewise found that there is a definite dependence of the biological activity of heparin on molecular weight. Laurent (120) claimed a correlation between biological activity and molecular weight in heparin fractions from a single source. Barlow *et al.* (145) and Braswell (146) did not find this relationship; however, their studies were based on unfractionated heparin from various sources. Lasker and Stivala (121) reported that the biological activities of low molecular weight fractions (performed by differential solubility) were uniformly low. Liberti and Stivala (147) discovered that the anticoagulant activity of heparin with molecular weights of 4500–15,500 (fractionation done by precipitation with ethanol and dioxane) increased with molecular weight up to 10,000 but then showed no appreciable change in activity on further increases of molecular weight.

Lasker and Stivala (121) demonstrated that the samples obtained by deactivation of unfractionated heparin with dilute hydrochloric acid showed no detectable changes in molecular weight, intrinsic viscosity, IR spectra, or optical activity. They also found that partially inactivated heparin behaves like active heparin in that there is a drop of activity in an environment of 0.5–1.0 *M* NaCl (148).

The treatment of α-heparin (bovine) and ω-heparin with 40% acetic acid at 37° for almost 1 day, by Yosizawa *et al.* (149), almost completely deprived these heparins of anticoagulant properties, with a loss of 7–8% of *N*-sulfate groups. These results indicate that the anticoagulant activity of α-heparin is more labile and its lipolytic activity less labile than those of ω-heparin. Braswell (146) also degraded heparin with acid, but only until one-half of the activity was removed. There was little change in molecular weight, intrinsic viscosity, or elemental analysis. He postulated that these results are due to the formation of internal esters because acidity is destroyed without losing sulfur.

Opposed to these latter results are the findings of Helbert and Marini (150) who used self-hydrolysis and titration techniques. They found that *N*-sulfate groups were removed twice as fast as *O*-sulfate groups, but they did not perform elemental analyses or anticoagulant assays throughout the course of degradation; therefore, it is difficult to compare their work with that of others.

Stivala *et al.* (133), following the method of Helbert and Marini (150), did a graded hydrolysis on unfractionated heparin and measured the sedimentation co-

efficient, intrinsic viscosity, molecular weight, and axial ratio as functions of hydrolysis time. The data suggest that: (*a*) the decrease in molecular weight is due to desulfation rather than depolymerization, (*b*) hydrolysis results in both *N*- and *O*-desulfation, but the former is greater, and (*c*) the degree of sulfation and size and/or shape are responsible for the biological activity of heparin.

Yuan (151), in a study with Ehrlich (152) on the self-hydrolysis of a heparin fraction, found that the decrease in the anticoagulant activity of the partially hydrolyzed heparin corresponds to the amount of sulfate groups removed. There is also a decrease in the intrinsic viscosity in water, molecular weight, and the axial ratio. Yadlowsky (152A) and Stivala *et al.* (152B) studied the kinetics of the acid hydrolysis of heparin from its Cu (II) complex. They showed that hydrolysis during 5 hr. at various temperatures obeys first-order kinetics, and they obtained specific rate constants of 3.3–26.6 $\times$ 10^{-5} sec.$^{-1}$ in the range of 50–80°. Arrhenius plots of the data yielded 14.7 kcal. as the energy of activation. Yuan (151) also obtained similar results on the self-hydrolysis of heparin. These investigators found that the anticoagulant activities of heparin and the Cu (II) complex were not appreciably different and that the deactivation parallels Cu (II) release from the complex, which in turn parallels desulfation.

Optical rotatory dispersion, NMR, and gel permeation also were utilized to follow deactivation. Stone (153) demonstrated that the induced Cotton effects of the dye diminished in a graded hydrolysis. Changes in conformation were also followed through optical rotatory dispersion by Hirano and Onodera (154). They concluded that *N*-desulfation does not change the original conformation, but complete desulfation causes the original helical conformation of the heparin–dye complex to disappear. Findings by Sumyk and Yocum (155) on elution volumes were consistent in the conformation difference between native and desulfated heparin. Using NMR, Jaques *et al.* (156) found that *N*-desulfation causes a downfield displacement of the signal at 4.75 p.p.m.

The degradation of heparin by UV light and fast electrons was studied by Balazs *et al.* (157) and Sundblad and Balazs (158). Irradiation with UV light gave a decrease in anticoagulant activity and cationic dye binding power of the polyanion. Irradiation with electrons causes depolymerization. The use of γ-irradiation on dilute solutions of heparin by Jooyandeh *et al.* (159) produced depolymerization but no desulfation.

Interaction of Heparin with Dyes, and Optical Rotatory Dispersion—When dyes such as azure A, methylene blue, basic fuchsin, Brilliant green, and other metachromatic dyes are reacted with heparin, there is an absorption at a wavelength that is shorter than with the dye itself. Stone and Bradley (160), in 1967, showed that spectrophotometric titration of mucopolysaccharides with dyes was possible and, because of its high charge density, heparin would best be titrated with proflavine. A review was presented by Stone (161) on the use of metachromasy to induce Cotton effects in glycosaminoglycans.

According to Yen *et al.* (162), two dye molecules react with each tetrasaccharide unit, and apparently the reaction occurs preferentially between two carboxylic or sulfamino groups. Young *et al.* (163), in agreement with the latter investigators, believed that there is an electrostatic interaction between the dye and polyanion sites. Thermodynamic studies on heparin–azure A complexes indicated that the complexation produces a loss of energetic symmetry in the chromaphoric system, resulting in a shift to a lower wavelength. Various other methods, such as electron paramagnetic resonance (164) and pulse radiolysis (165–167), were used by Balazs *et al.* to examine the electrostatic interactions between methylene blue and heparin. Through this latter method, Phillips (168) unequivocally showed that cationic dyes are bound at anion sites of polyanions in such a way that an electronic exchange occurs, which is directed to the metachromasy.

The effect of temperature on polyanion metachromasy was studied by Kelly *et al.* (169). The metachromatic ratios, A_{575}/A_{628}, were found to be inverse linear effects of temperature. Stained sections gave the same results (170).

Dyes have been employed in the identification of heparin. In tissue, the staining with ruthenium red was found by Pihl (171) to be stoichiometric and the bulk of the stain was taken up by heparin in a molar relationship of one dye cationic complex per sulfate group of heparin. Sasai (172), after staining tissues containing acid mucopolysaccharides with alcian blue, discovered that the binding of the dye to mucopolysaccharide decreased as the salt concentration was raised.

The study of the optical rotatory dispersion of heparin has been possible after binding with dyes. Stone (173), in 1969, presented an excellent review on conformations of hexose polysaccharides in solution, dealing with optical rotatory dispersion. In contrast to the views of the previous investigators mentioned, Stone (153) believed in a theoretical card-stack aggregation of dye molecules, with a helical conformation for the heparin–dye complex, since an induced rotation was observed showing anomalous dispersion in the absorption band of the bound dye. Furthermore, the Cotton effect was dependent on helical conformation, since no induced rotation was observed when the dyes were bound to polyglutamic acid in its random coil conformation. This helical structure agrees with that proposed by Velluz (174) for heparin itself. The phenomenon of induced rotation was investigated by Stone and Moss (175), as a function of pH, in the range of ionization of the carboxyl group of heparin, with the result that metachromasy decreased with a decrease in anionic site density. Further investigations by Stone (176) on dye binding indicated that the binding of methylene blue to the negative sites of heparin gives rise to a negative doublet (circular dichroism) in the metachromatic absorption band, indicating a net left-hand chirality for the neighboring dye molecules.

The effect of urea in the destruction of hydrogen bonds that serve to maintain the conformation of the mucopolysaccharides was found (154, 177) to increase slightly the optical rotation of those mucopolysaccharides possessing helical structure. Also, the induced

Cotton effect was lost in urea and a positive plain curve was obtained. A later study by Hirano (178) showed, by optical rotation measured in urea and guanidine hydrochloride, that the tertiary structure of polysaccharide chains is almost destroyed while the tertiary structure remains in water.

Other optical rotatory measurements by Stone (153, 179) on the interaction of histamine and heparin with dye-stacking techniques indicate that there is a decrease in the metachromasy of dye–heparin complexes on the addition of histamine; also, conformational changes in heparin may accompany the release of histamine, which causes anaphylactic shock concomitant with degranulation of the mast cell *in vivo*.

A method of using the optical dispersion effects of mucopolysaccharides was presented by Stone *et al.* (180) for distinguishing between San Filippo and Hurler syndromes, since the UV optical rotatory dispersion and circular dichroism of dermatan sulfate are greatly different from those of heparin sulfate because dermatan sulfate is present only in Hurler's syndromes.

Binding and Complexing of Heparin—Since heparin is a highly charged polysaccharide, binding and complexing with both large and small ions and molecules become possible. It is thought by many to exist in nature bound to proteins as a protein–polysaccharide complex in the mast cells and released when necessary. Also, its anticoagulant and clearing action may be due to binding or complexation.

The binding of heparin to various proteins has been studied by many investigators. Recent reviews were written by Louisot (181) and Meyer (182). Cifonelli *et al.* (183) discovered that concanavalin-A, a globulin protein of Jack-bean meal, combines with heparin to form a precipitate. More recently, DiFerrante and Hrgovcic (184) claimed no interaction between the two, but thought that the presence of other proteins could be responsible for the interaction. However, Doyle and Kan (185) maintained that there is a precipitin-like complex with heparin, but that the interaction is pH, salt, and sugar sensitive.

The binding of heparin to collagen was found to occur in definite proportions by Delaunay and Bazin (186), and an ionic linkage was suspected because of the pH dependence. They (187) also found that other substances, which were unable to form stable combinations with collagen A, had the power to affect the normal combination of collagen–mucopolysaccharide linkage, depending on their acidity. Curves obtained by Serafini-Fracassini (188) of the amount of polyion fixed *versus* pH of heparin and chondroitin sulfate were similar to titration curves of collagen. Németh-Csóka (189) found that the activation energy of collagen at pH 7.35 was increased by heparin. Mathews and Decker (190) showed that heparin increased the rate of fibril formation from collagen solutions. They believed that this is due to electrostatic binding of heparin to collagen.

Kudryashov *et al.* (191, 192) found that complexes of heparin with the blood proteins such as fibrinogen, thrombin, and plasmin had antithrombotic and fibrinolytic activity. The existence of a complex between fibrinogen and heparin was confirmed by Bernfeld (193) with the aid of moving boundary electrophoresis. The mixing of radiolabeled heparin and normal serum was shown by Becker *et al.* (194) to give complexes of heparin in two major electrophoretic regions in starch zone electrophoresis. Lages (195) and Lages and Stivala (195A) quantitatively studied the binding of heparin to fibrinogen in the presence and absence of Cu ions, using equilibrium dialysis and sedimentation velocity. They found that the sedimentation coefficient of the fibrinogen–heparin complex is affected by the Cu ions.

The binding of heparin to other proteins also includes insulin, vasopressin, oxytocin, and corticotropin (196). A heparin–trypsin complex had fibrinolytic activity and showed prophylactic effects against development of thrombosis (197). Aborg and Uvnäs (198) described a protamine–heparin complex which has the ability to bind histamine and monoamines electrostatically. In addition, whale heparin can bind protamine sulfate (199).

The nature of the linkage of heparin to protein has been the subject of much investigation. The findings of Lindahl and coworkers (200–205) indicate that galactose and xylose are both involved in the linkage of heparin to protein. A glycosidic linkage between xylose and the hydroxyl group of serine appears to constitute the carbohydrate–protein linkage. The proposed sequence, given by Helting and Lindahl (58), is shown in Scheme I. Stern *et al.* (206) discovered that dermatan sulfate is similarly bound to proteins. Opposed to this view is that of Scott (207, 208), André *et al.* (209), and Aborg and Uvnäs (198), who believed in an electrostatic linkage.

The binding of neutral pH sulfated polysaccharide to serum low density lipoproteins was observed by Burstein *et al.* (210). Srinivasan *et al.* (211) showed that serum pre-β- and β-lipoproteins formed insoluble complexes with acid mucopolysaccharides in the presence of Ca^{+2}. A mechanism of formation of a complex through Ca^{+2}, acting as a bridge between *N*-sulfate groups of heparin and phosphate groups of the phospholipids of the lipoproteins, was suggested. Similarly, Olivecrona *et al.* (212) presented evidence for an ionic binding of milk lipoprotein lipase to heparin.

The binding of histamine to heparin will be considered in conjunction with the mast cells because heparin may be the histamine binding agent in these cells. The granules of mast cells are known to contain both heparin and histamine (213), and strong evidence has been presented that mast cells produce the heparin. A complex between heparin and histamine was discovered by Werle and Amann (214), which was suggested to be present in the mast cells. Lysis of granules of the mast cells by Hill (215) caused destruction of the protein component and released heparin. The mechanism of binding suggested by Kobayashi (216) was the same type as an ion exchanger. Lagunoff (217) presented evidence that the mast cell granule is a heparin–protein matrix to which histamine and perhaps other nitrogenous bases are attached electrostatically. The findings of Lloyd *et al.* (218, 219) indicate the natural occurrence of mast granule heparin linked to protein *via* alkali-labile covalent bonds. The pH dependence of the binding mechanism of the granules led Aborg and colleagues (220, 221) to suggest that the

histamine is bound ionically, which is easily broken on exposure of the granules to other cations. A study of the heparin–protein complex from ox liver capsule by Serafini-Fracassini *et al.* (222) led to the observation that the mast granules are composed of a random network of elongated heparin–protein macromolecules surrounded by the other components.

The exact correlation between heparin content and mast cell count is not a simple one, according to Jaques and Debnath (223). Also, Drucker-Colin (224) suggested that large amounts of heparin present in the rat intestine are not associated with mast cells. In a study of mast cells in mastocytosis by Zimmer *et al.* (225), it was discovered that heparin, unlike histamine, is only rarely released into the circulation in clinically significant amounts. Experiments by Slorach (226) revealed that both histamine and heparin are released when rat mast cells are exposed to the histamine releasing agent, Compound 48/80. As in the case of mast cells, histamine was shown by Aborg and Uvnäs (227) to be bound to carboxyl groups in a heparin–protein complex in rat thrombocyte granules. The binding of histamine to heparin was used by Barlow (228) to follow desulfation of heparin.

It is possible for heparin to bind with many other organic compounds. Woodside *et al.* (229) interacted heparin with glycogen to form insoluble complexes. Organic dyes such as azure A, toluidine blue, Bismarck brown, and brilliant cresyl blue combine with heparin (230). Acridine orange forms a reversible complex with heparin (231), and alcian blue is fixed to the available binding sites of heparin (232). Heparin combines with many enzymes such as trypsin, chymotrypsin, pepsin, and lysozyme (230). The reaction with lysozyme, according to Bychkov and Kharlamova (233), involves primarily electrovalent forces. Bergman *et al.* (234) mentioned that enzyme–heparin complexes can cause artificial enzyme polymorphism, which can be used both as a diagnostic tool in clinical medicine and in the study of genetic polymorphism. Heparin combines with histones and displaces deoxyribonucleic acid from a bond in deoxyribonucleoprotein (235). It forms complexes with polymyxin B (236), streptomycin (145, 236, 237), monomycin (237), kanamycin (237), and neomycin (237). Popova (238) demonstrated that the binding of chlorpromazine to heparin replaced the storage receptor in the molecule of the acid mucopolysaccharide and released the biogenic amines. In a study of the binding of histamine, carbaminoylcholine, and 5-hydroxytryptamine to heparin, Mordelet-Dambrine *et al.* (239) observed that only histamine was fixed at pH 4, probably because it is most strongly ionized. Evidence was given by Kudryashov and Lyapina (240, 241) that a heparin–adrenalin complex exists which has a possible role in blood coagulation. The complex dissolved nonstabilized clots of fibrin, exhibited antipolymerase activity, and blocked the fibrin-stabilizing activity of factor XIII.

Heparin was shown by Ascoli and Botré (242) to have a high binding capacity for Na^+ and Ca^{+2} ions. Both Jaques (230) and Salminen and Luomanmaki (243) agreed that potassium ion is bound to heparin in preference to the sodium ion. The order of increasing cation activity for heparin was given by Dunstone (244) as Na^+, K^+, Mg^{+2}, Ca^{+2}, Sr^{+2}, and Ba^{+2}. Ohkuma and Furuhata (245) felt that Na^+ ions, in addition to toluidine and albumin, form a complex with heparin, with the suggestion that heparin possesses a cation-exchange behavior. In an investigation of heparin's polyanion affinity to $^{22}Na^+$ and $^{137}Cs^+$, Dorabialska and Plonka (246) discovered that the affinity was greater to the one with the lower total concentration.

The binding of $Co(NH_3)_6^{+3}$ to connective tissue mucopolysaccharides and heparin was investigated by Mathews (247), who concluded that ion-pair formation with $Co(NH_3)_6^{+3}$ is influenced by local binding factors, electrostatic interaction of neighboring charged groups, and competition with other cations for binding sites. Anticoagulant activity was generally associated with affinity for $Co(NH_3)_6^{+3}$. This was also experienced by Moore *et al.* (248) in the interaction of connective tissue and other polyanions with cationic counterions. The binding of Cu^{+2} to heparin forms a basis for studying the anticoagulant activity of heparin (134). It is pH dependent because of competition for sites and is ionic strength dependent because of a change in conformation. In desulfation, reduction in binding sites results in decreased binding of Cu^{+2}. In the work (134) on the Cu^{+2} binding from polarography, it was also shown that the ability of heparin to bind copper is related to blood anticoagulant activity.

The order of calcium binding to hyaluronic acid, chondroitin sulfate, heparin, and heparitin sulfate was examined by Lászlo (249) with the result that chondroitin sulfate had the highest and hyaluronic acid the lowest binding capacity. It was found that most of the Ca^{+2} was bound to the carboxyl groups. The effect of Ca^{+2} ions on the scattering of light by heparin was investigated by Loucas *et al.* (250) who found that the interaction constant was influenced by the ionic strength of the solution.

A study by Quintarelli *et al.* (251) showed that binding between phosphotungstic acid and desulfated heparin only took place between the metal and the desulfated hexosamine amino groups through electrostatic forces. On acetylation of the *N*-desulfated products, no phosphotungstic binding occurred.

Effect of Ionic Strength and pH—An attempt was made by Barlow *et al.* (145) to study the effect of ionic strength on the sedimentation constant and molecular weight, but no previous fractionation was performed on these samples of heparins of different sources. Their results indicated that there is a probable increase of the sedimentation constant with ionic strength until a maximum is reached and then it remains constant. However, they did not use an extrapolated value for the sedimentation constant to allow for concentration dependence. They also found that the molecular weights obtained from light scattering did not change with ionic strength.

Liberti and Stivala (147) found that there is a definite decrease of intrinsic viscosity with an increase of ionic strength. This same effect was experienced by Braswell (146). Yuan (151) also obtained similar results with unfractionated heparin from one source, but the influence of pH was not studied under controlled conditions.

Ehrlich (152) used controlled conditions of pH and ionic strength on a particular fraction and had results for the sedimentation constant that were similar to Barlow's *et al.* (145). Also the axial ratio decreased, the intrinsic viscosity decreased, the molecular weight did not change, and the partial specific volume increased between the ionic strength of 0.5 and 1.0 *M*. This behavior can be explained in that the addition of by-ions results in a lower field strength, with a resulting change in shape offering less frictional resistance to transport.

Size and Shape of Heparin—Stivala *et al.* (252) studied molecular weights, radii of gyration, and persistence lengths of a fractionated sample of heparin by means of low angle X-ray scattering. The molecular weight of the heparin fraction was found to be 12,900. This value is in excellent agreement with the values of 12,600 and 12,500 obtained for the same fraction from sedimentation equilibrium (121) and intrinsic viscosity (147) measurements, respectively. A persistence length of 21.1 Å was calculated. Based on low angle X-ray scattering, the heparin fraction in water (1%) at room temperature may be described as a Gaussian coil molecule.

Since heparin is a polyelectrolyte, plots of specific viscosity *versus* concentration in water are nonlinear. Linearity may be achieved by use of various empirical relationships. Some researchers (121, 133, 147) measured the intrinsic viscosity of numerous heparin fractions of varying molecular weights in water and as a function of pH and ionic strength. Based on various solution theories of linear polymers, heparin exhibits substantial flexibility, approximating a Gaussian coil. From partial specific volume, sedimentation coefficient, molecular weight, and intrinsic viscosity measurements, where charges were swamped (by means of pH and/or ionic strength), Ehrlich (152) and Ehrlich and Stivala (252A) reported that heparin is best approximated as a random coil (Gaussian).

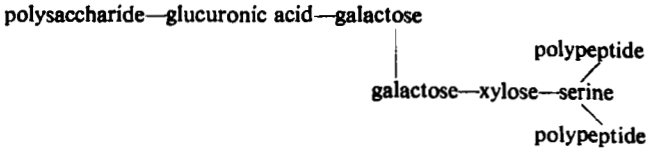
Yuan and Stivala (252B) studied the intrinsic viscosity as a function of dielectric constant and found that the shielded intrinsic viscosity (which is that of a highly shielded polyion) increases linearly with the increasing dielectric constant of the solvent. Furthermore, based on an interaction parameter of a semiempirical relationship containing the shielded intrinsic viscosity, Yuan and Stivala (252B) showed that the interaction parameter is independent of the dielectric constant of the solvent of the various heparin solutions. The significance of this interaction parameter is that it contains a term associated with the equivalent freely jointed segment length when the backbone of a polyion is represented by a Gaussian coil for which the interaction parameter is independent of the dielectric constant.

ASSAY AND ESTIMATION OF HEPARIN

The various methods of heparin determination can be essentially divided into two groups, those involving the reaction of heparin with substances such as protamine or toluidine blue and those measuring directly the specific anticoagulant activity with respect to total blood or plasma.

Metachromatic and Protamine Methods—A recent review of metachromasy of dyes in solution was presented by Padday (253). Awe and Stüdeman (254) found that quantitative measurement of the course of protamine titration and metachromatic dye change from blue to red-violet conformed with USP XV standards, but heparinoids gave erroneous results. They presented two methods for the spectrometric evaluation of the toluidine blue complex of heparin, both involving the metachromatic shift of the complex. The use of azure A and toluidine blue O (tolonium chloride) by Serafin (255), to test activity, gave much larger amounts of heparin in normal blood than previously reported. The ratio between metachromasy and uronic acid content was measured by Bianchini and Osima (256), who found that neither the anticoagulant activity nor the clearing activity is linear with the uronic acid content. According to Jaques *et al.* (257), there is no correlation between activity and chemical analysis except for the finding that increasing metachromatic activity accompanied an increase in sulfur content. A sensitive, accurate, quantitative, colorimetric assay[2] with azure A, with absorption at 505 nm., was presented by Jaques and Wollin (258). A new method for fractionation and determination of heparin by differential colorimetry of metachromatic substances formed by toluidine blue was devised by Satake (259). A method utilizing very small amounts (0.5–5 mcg.) of acidic polysaccharides, employing the shift in the visible absorption spectrum of carbocyanine dye when bound to a polyanion, was given by Edstrom (260). Two colorimetric methods were described by Plekhan *et al.* (261). They involved the: (*a*) reaction of heparin and azure A, and (*b*) reaction with carbazole and concentrated sulfuric acid. A procedure for studying stoichiometry of complexes like heparin and toluidine blue was presented by Singh (262). Qualitative limits of the sensitivity of toluidine blue and azure A as compared with alcian blue and mucicarmine were established by Rahman *et al.* (263). The carbazole method is used widely for determining heparin concentrations. Galambas (264, 265) pointed out that the ratio between maximum absorptivity in sulfuric acid and the carbazole color at 525 nm. is higher for heparin than for the other mucopolysaccharides, probably because of the lack of *N*-acetyl groups in the former. Tsuji *et al.* (266) presented a novel spectrophotometric method for the microdetermination of 2-deoxy-2-sulfamidohexose and 2-acetamido-2-deoxyhexose residues in heparin, using an ion complex which absorbs at 653 nm.

For the determination of tissue heparin, Freeman *et al.* (267) mentioned a method of releasing the heparin from the protein complex that facilitates proteolytic digestion, since proteins interfere with metachromasy of applied dyes (268). The possibility of using acridine



Scheme I—Linkage of heparin to protein

[2] Using a Beckman spectrophotometer.

orange fluorescence for the determination of heparin was explored by Zelenin and Stepanova (269), who found that it had a red fluorescence to pH 7.0 and was resistant to sodium chloride solutions. Zugibe (270) gave many histochemical methods, one of which involves formation of the insoluble polyanion–quaternary salt complex with the mucopolysaccharide, followed by treatment with ferric thiocyanate. Other methods precipitate the acid polysaccharide with dyes such as toluidine blue and alcian blue and then differentially remove the polysaccharides by adding varying amounts of sodium chloride. The critical concentrations of electrolytes for heparin were measured by Sasai (172) by precipitation of alcian blue on cellulose acetate membrane, and he found 0.9 *M* for magnesium chloride and 1.1 *M* for sodium chloride. Scott (271) reviewed the critical electrolyte effects in interactions between acid glycosaminoglycans with organic cations and polycations.

Titration of heparin with protamine sulfate, followed by measurement of the coagulation after a period of time, is another possible method (272). Vincke and Benfey (273) found that 1 mole of protamine sulfate is equivalent to 629 g. of heparin. But Nagasawa and Kimura (274) claimed that there is no relationship between anticoagulant activity and protamine binding capacity. A possible reason for this, given by Lowary *et al.* (275), is that the number of heparin units neutralized by protamine sulfate varies with the source and specific activity of the sodium heparin. Recently, Godal and Gjeugedal (276) found that a transient coagulant effect is observed when heparin is incubated with protamine, possibly due to an excess of protamine. Another method was proposed by Barkagan and Barkagan (277) based on comparison of anticoagulation rates with and without inhibition of heparin by protamine sulfate.

Methods of Determining Heparin Activity in Blood—Reviews on heparin assays were written by Quick (278), Warren and Wysocki (279), Jaques (230, 280), Biggs and MacFarlane (281), and, recently, Jorpes (282). These methods measure directly the specific anticoagulant activity with respect to total blood or plasma.

Blombäck *et al.* (283) determined the heparin level in blood by titrating with thrombin and cofactor. Quick (278) mentioned a thrombin titration method which involves the inactivation of thrombin by heparin and its cofactor albumin X. Another titration method, described by Herbain (284), is a photometric estimation of the specification rate of plasma after the addition of thrombin. Godal (285) argued that the prolongation of the thrombin time is very sensitive to a variation of pH, temperature, and ionic strength; furthermore, calcium ions counteract heparin, especially at alkaline pH. According to Cameron (286), however, the thrombin clotting time is an efficient method of controlling heparin therapy.

The recalcification time was suggested by Belko and Warren (287) to be both useful and timesaving for appraising the heparin effect. The prothrombin time (Quick) method (288) measures the clotting time of recalcified plasma with added thromboplastin. Messerschmidt and Peter (289, 290) used this method with a photometer to measure the beginning of clotting.

Heparin will prolong whole blood clotting time (Howell) to three times the normal level (291). According to Jorpes (282), the main advantage of this technique is that it ensures the presence of the heparin cofactor and all the labile factors entering into the coagulation system without the introduction of additional salts or thromboplastin. Jaques *et al.* (257) found that the results given by whole blood were better than the USP XIV assay which is done in plasma, since the conditions of the former are closer to those used in the clinical control of heparin anticoagulation. The BP assay likewise suffers from the same disadvantages as the USP assay.

Other tests mentioned by Jaques (230) for the anticoagulant activity of heparin include: (*a*) whole blood collected in silicone-clotting tubes, (*b*) plasma time with chicken plasma that does not require recalcification, and (*c*) two-stage tests that are modifications of the previous tests given, with final measurement made on the coagulation time of a separate fibrinogen solution on the addition of an aliquot from the single-stage test.

Other Techniques—Some techniques are not as well known as the ones already discussed. Rosencrantz *et al.* (292) described a method that permits automatic quantitative recording of the formation of clots at 250 nm. An assay of heparin, employing diatomaceous silicate, modified plasma, and a precision coagulation timer[3], was given by Seip *et al.* (293). Attempts to use concanavalin A(I) as a specific reagent for the identification of heparin in biological fluids was found unsuccessful by DiFerrante and Hrgovcic (184). A quantitative method of estimation of acid polysaccharides in the blood of rabbits after the injection of papain, by complexation with cetyltrimethylammonium bromide, was given (294). The determination of heparin activity by an IR spectroscopic method was described by Zhbankov *et al.* (295), where potassium cyanide is introduced as an internal standard and the absorption ratios of 1240–1150 $cm.^{-1}$ and 1240–2065 $cm.^{-1}$ are measured, the latter frequency being due to the CN group. Bohn *et al.* (296) presented an automatic procedure for the determination of mucopolysaccharides (including heparin) based on a turbidity reaction with cetylpyridinium chloride. The thrombo test was described by Owren (297) as differing from earlier methods by measuring the coagulation activity of the absorbable factors in both the intrinsic and extrinsic coagulation systems, thus being insensitive to depression of factor IX during anticoagulant therapy.

For the assay of whale heparin, Matsuno and Hashimoto (298) modified the BP assay by using ox and human sulfated blood and ox thrombokinase. The USP method was more specific than a chemical method based on the shift of the isoelectric point for the estimation of polyanionic compounds like polyestradiol phosphate and polymethylene salicylate (299).

Much controversy exists as to the effectiveness of the various methods. A discrepancy of up to 40% was observed by Walton *et al.* (300) between potencies ex-

[3] Fibrometer.

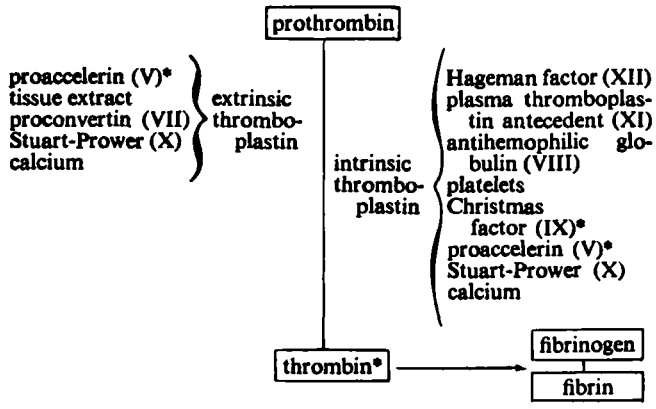
amined by the BP and USP assay procedures. These findings led to a British standard of mucosal heparin of mucosal origin. The results of Bangham and Woodward (301), however, indicate differences between the estimated relative potencies of lung and mucosal heparin, but they were not so consistent as to necessitate a separate international standard for each. The use of animal plasma or human plasma can also alter results (302). The correspondence between the *in vitro* and *in vivo* anticoagulant activities of beef and whale heparin was studied by Hashimoto and Matsuno (303), with the result that the BP unit of whale heparin and its *in vivo* potency in dogs was 1.5-fold more potent than those of beef heparin, whereas USP units of whale and beef heparin were the same. This led to the suggestion that the BP assay method may be better for clinical evaluation than the USP method when the whale lung or intestine is used in the place of the ox lung as the source of heparin.

Andreenko *et al.* (304) compared the method of titration with thrombin in the presence of heparin-cofactor (Blombäck), a colorimetric method based on precipitation of heparin with azure A (Warren–Bozioni method), with the titration with protamine sulfate (Warren–Vysockii). They found that the most suitable for laboratory practice was the first. In a report by Rezansoff and Jaques (305) comparing USP colorimetric, Howell, spectrophotometric, and microelectrophoresis techniques, no assay method consistently described the activity of heparin in another test system, *in vitro* or *in vivo*. This report conforms with that of Magnusson and Nilsson (306) who received different values of anticoagulant activity when using human, bovine, dog plasma, and whole ox blood in one-stage prothrombin, recalcification, and thrombin systems. Kavanagh (307) and Kuo (308) recently discovered no significant correlations between nitrogen, sulfur, acetyl, glucosamine, and other parameters with biological activities determined by Howell assay, USP assay, *in vivo* clotting time, partial thromboplastin time, and *in vivo* lipolytic activity values. Estes (309) claimed that, on comparison of four tests, the whole blood activated partial thromboplastin time provided the greatest precision, information, and economy, followed by (in order) the activated plasma partial thromboplastin time, the whole blood clotting time, and the partial thromboplastin time of plasma. The indication is, nevertheless, that more uniform systems of anticoagulant activity assay must be devised.

PHARMACOLOGY

Pharmacologically, heparin is noted for its anticoagulant action in the treatment of myocardial infarction and in the induction of the clearing reaction as an antilipemic agent in the blood. In addition, many other actions of heparin (antimetastatic, antiviral, and antienzymic) have been reported. Some general surveys of the pharmacology of heparin were written by Jorpes (1), Engelberg (8), Jaques (230, 280), and Gastpar (50).

Effects on Circulatory System—The most important action of heparin is the inhibition of blood coagulation. Surveys on blood anticoagulation were presented by



*Scheme II—Scheme of blood coagulation (*Here it is thought that heparin affects coagulation)*

Wessler and Gaston (310), Rodman (311), Buri and Schneeburger (312), Divald and Jouillié (313), Yen *et al.* (314), and Haas-Weston (315).

Anticoagulants may be divided into two groups: (*a*) the direct type like heparin and the heparinoids, which act rapidly by their presence in the blood, and (*b*) the indirect type (called antivitamin K), the derivatives of coumarin and indandione, which act after some delay. The first class of compounds interferes with the formation of thrombin and thromboplastin, whereas the second class interferes with the formation of prothrombin. The antagonists of the first class are protamine sulfate types, and the antagonists of the second are vitamin K types.

To date, no one has clearly elucidated either the mechanism of blood coagulation or anticoagulation. A recent review on blood coagulation was written in 1971 by Mammen (316). Many theories for blood coagulation have been proposed. A generally accepted scheme of coagulation is presented in Scheme II, according to Fremont (317).

The system offered by Wessler and Gaston (310) is similar to that of Fremont; however, they carry the clotting mechanism to the gelation stage (Scheme III).

Anticoagulant Effects of Heparin—Antithrombic Action—Prothrombin is unaffected in heparinized blood and, therefore, heparin must act on the first part of clotting (318). It is thought to have "antithrombic" activity in the presence of a certain plasma protein named "heparin complement" by Ziff and Chargaff (319) or cofactor (Howell) (317), which is close or identical to antithrombin III (320, 321). The protein forms a complex with heparin which is capable of binding rapidly with thrombin and thus inactivating it. Serotonin and tryptamine can interfere with this reaction (322). Henstell and Kligerman (323) felt that, more likely, three factors are involved: a plasma antithrombin (antithrombin III), a plasma antithrombin inhibitor, and a platelet factor with heparin acting as a catalyst. Porter *et al.* (324) indicated that the anti-

$$\text{fibrin monomer} \xrightarrow{*} \text{fibrin polymer} \quad (1)$$

$$\text{fibrin polymer} \xrightarrow{\text{calcium}} \text{fibrin gel} \quad (2)$$

Scheme III—(Here it is thought that heparin affects coagulation)*

thrombic action of heparin itself is weak in comparison with that of the heparin–cofactor complex.

Inhibition of Thromboplastin—In addition to its antithrombic action, heparin is capable of inhibiting several blood factors for the formation of thromboplastin, which is necessary for the conversion of prothrombin to thrombin, through depression of factors V, IX, XI, and XII (317). The data of Pitlick *et al.* (325) indicate that heparin and hirudin interfere with factor IX*a*, which is the heparin- and hirudin-sensitive factor in plasma when coagulation is initiated *via* factor XI or XII, and that subsequent clotting factors remain in inactive forms. Heparin also inhibits the activation of factor VIII by factor IX*a* (315). Josso (326) pointed out that an important discovery in recent years is that the enzyme activities that operate to transfer prothrombin into thrombin are attached to particulate lipoprotein complexes with which the inactive plasma profactors are transformed into active factors. The interaction between heparin and thromboplastin, observed by Chargaff *et al.* (327), results in a complex formed by heparin and the protein moiety of the lipoprotein. According to Yen *et al.* (314), the scheme of complex formation may be as shown in Scheme IV, where A is the polyanion and C is the polycation. In reaction (4) there is a two-electron transfer. These reactions could also hold for complexation with dyes and heparin antagonists.

Fibrinolysis—In addition to being an anticoagulant, heparin usually enhances fibrinolysis. It was found by Buluk and Januszko (328) to reduce the inhibition of antifibrinolysin. Lackner and Mersk (329) reported markedly accelerated fibrinolytic activity after intravenous injection of heparin. Increased blood heparin in leukemia was thought by Efendiev *et al.* (330) to be responsible for the greater fibrinolytic activity. Heparin and fibrinogen were shown by Godal (331) to react in stoichiometric proportions. Evidence was presented (332) that heparin in solutions of low ionic strengths breaks the bonds holding the fibrin monomers together in clots. It is also able to inhibit the polymerization of fibrinogen. The concentration, according to Silver and Hall (333), in the blood must be greater than 2 mg./kg. to potentiate fibrinolysis.

Olesen (334) suggested that the fibrinolysis is caused by plasminogen activation. However, Holemans *et al.* (335) found that the presence of a cofactor is necessary. Heparin is known also to accelerate catalytically the neutralization of thrombin by antithrombin III and thus prevent reclotting following fibrinolysis (336). The preparation of heparin must be free of protease inhibitors to enhance fibrinolysis (337). Eleparon, a mucopolysaccharide polysulfuric acid ester like heparin, increases fibrinolytic activity (338). Boros (339) discovered that "heparin rebound," the reappearance of heparin in the circulation in open-heart surgery despite its previous complete neutralization, was caused by enhanced fibrinolysis.

$$A:{}^{-}Na^{+} + C^{+}Cl^{-} \rightarrow A:{}^{-}C^{+} + NaCl \quad (3)$$

$$A:{}^{-}C^{+} + A:{}^{-}Na^{+} \rightarrow CA_{2}{}^{-}\,Na^{+} \quad (4)$$

Scheme IV

Effects on Platelets—Platelets contain a factor with some antiheparin activity (320, 340). This was described by Shanberge and Furuta (341) as platelet factor 4. Heparin was shown by Sawyer *et al.* (342) to prevent conversion of degenerated platelets in solution from forming a gel.

According to Jaques (280), heparin inhibits platelet adhesion to intercellular cement. Aznar (343) and Silver (344) found that small doses of heparin had no effect on platelet adhesiveness, but with larger doses the antiaggregating effects increase in proportion to the dose. Moreover, Gröttum (345) reported that the negative charge of the platelets was reduced by the positive charge of heparin and lowered the electrophoretic mobility, but increasing the concentration of heparin increased their electrophoretic mobility but did not aggregate them. A method was developed by Hohage and Hiemeyer (346, 347) for quantification of the aggregation curves of platelet-rich citrate–heparin plasma.

The adhesiveness of platelets to tannic acid-treated red cells was shown by Zbinden and Tomlin (348) to increase when heparin was added. If adenosine diphosphate is added to unstirred, plasma-rich platelets, no aggregation occurs and platelet-bound heparin-neutralizing activity develops immediately (349). The addition of adenosine diphosphate to platelets leads to shape and volume changes, to the development of stickiness, and to heparin-neutralizing activity (platelet factor IV) (350). O'Brien (351) reported that alcian blue also binds heparin and may influence heparin-neutralizing sites and probably the charge on the platelet; thus it can influence platelet aggregation. Eika (352, 353) found that washed human platelets were aggregated by heparin in the presence of divalent cations, but heparin inhibited the thrombin-induced platelet aggregation and release of adenine nucleotides. In the presence of calcium chloride and also in barium sulfate-adsorbed serum, heparin was shown by Murase *et al.* (354) to produce platelet clumping activity, but this was not the case in citrated plasma, edetate plasma, or serum.

Another action of heparin is to prevent platelet disintegration (355, 356) and release of phospholipids (355). Platelet instability, however, is not affected by heparin (357).

The ability of a heparin-like sulfonic polysaccharide to counteract platelet adhesiveness in rabbits and intravascular red cell aggregation in cats was discovered recently by Bicher (358).

Other Actions of Heparin—According to Jaques (280), heparin decreases blood sugar, the fall in lymphocytes and eosinophils, and hemorrhage from stress and hypertension. Hougie (359) reported that heparin gives prompt relief from acute myocardial infarction. Heparin facilitates the flow of blood by vasodilation (280, 360) and so could be important in Buerger's disease (361). The viscosity of blood was reported by Alberto *et al.* (362) and Meda *et al.* (363) to be lowered by heparin because of its anionic character. According to Hoak *et al.* (364), heparin prolongs the time to produce thrombi. *In vitro*, heparin was found to cause hemolysis (365), possibly because there is a decrease in the mechanical resistance of the red blood cells due to nucleoprotein mobilized from leucocytes under the influence of hepa-

rin, but *in vivo* it inhibits hemolysis and alters the erythrocyte sedimentation rate (315). Heparin prevents decrease of lymphocytes and eosinophils caused by ACTH or cortisone (366, 367) and activates alkaline phosphatase and esterase in the blood (367). Another biological effect is its enhancement of vascular permeability (368).

Pathological conditions exist, termed acquired and hereditary hyperheparinemia, where unusually high concentrations of heparin in the blood produce prolonged clotting or thrombin times. These can be corrected by treatment with protamine sulfate, toluidine blue, or polybrene (288).

Clearing Action of Heparin on Blood Lipids—In addition to the anticoagulant action of heparin, another major effect is the clearing of lipemic plasma. Early reviews were written by Levy (369) and Engelberg (370). Heparin stimulates the release of lipoprotein lipase (the clearing factor), an enzyme that catalyzes the hydrolysis of triglycerides associated with chylomicrons (lipoprotein molecules rich in neutral fat), and thus promotes the clearing of lipemic plasma (361, 367). The possible connection between the pathogenesis of atherosclerosis and heart disease and the clearing action of heparin is of great interest.

Antonni *et al.* (371) found that the clarifying action is more evident in atherosclerotic subjects than in normal ones. Also, myocardial infarction is usually accompanied by an increase in plasma lipoproteins and free fatty acids, which are damaging to the myocardial cells, in addition to an accumulation of tissue triglycerides and increased synthesis of fibrinogen (372).

Some properties of the clearing factor were examined by Korn (373), with the conclusion that it is primarily a tissue enzyme, a heparin-activated lipoprotein lipase that catalyzes the hydrolysis of chylomicrons but not simple triglycerides. The possibility of a separate monoglyceridase activity was also suggested by Vogel *et al.* (374). According to Engelberg (375), heparin is a component of the enzyme and its dissociation from the apoenzyme can be readily brought about. The clearing factor showed activation by low concentrations of heparin and inhibition by higher concentrations and protamine. This, with density centrifugation evidence, led Payza *et al.* (376) to suggest that heparin is not a necessary requirement or a cofactor for the action of the clearing factor.

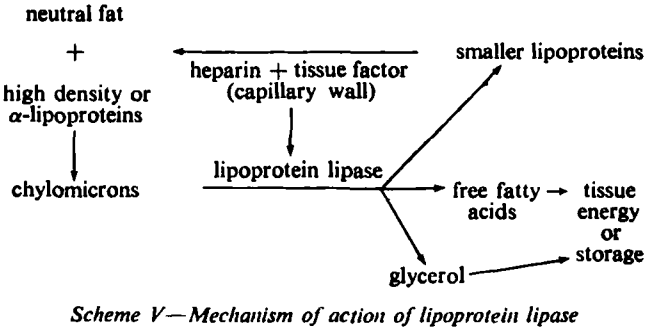
In addition to being found in the plasma, lipoprotein lipase can also be located in the thoracic duct lymph of dogs (377) and near the vascular wall after heparin injection (378). Heparin released lipase from rat epididymal fat pads (379), but Ho *et al.* (380) claimed that heparin only increases the lipolytic activity of the stroma cells of the pads. Ballard *et al.* (381) claimed that heparin increases the release of venous plasma lipolytic activity from perfused canine subcutaneous adipose tissue. From their findings, Gero *et al.* (382) made the observation that heparinoids may act on the tissue level by interfering with the lipid deposition and by locally increasing the lipolytic activity of the vessel wall. Payza *et al.* (383) found that injection of heparin into rats induced an increase in lipase activity of the plasma and a decrease in lipase activity of the tissue. Davis and Heimberg (384) thought that the uptake of chylomicrons from the blood probably is accelerated by a direct action of heparin on the liver.

A mechanism for the action of the postheparin lipidemia clearing factor was given by Engelberg (370) (Scheme V). He believed that the lipoprotein lipase is the major way for the removal of triglycerides from the blood. Rutstein *et al.* (385) argued that heparin leads to increased lipid deposition and that this can be correlated with increased serum levels of nonesterified fatty acids but not with the decreased concentration of triglycerides, cholesterol, and phospholipids. The high nonesterified fatty acid levels are associated with arrhythmias and death in patients with myocardial infarction. Davis and Davis (386) disagreed, their reason being that even though emboli contain lipids, fibrinogen is more important in their formation than lipids. The interpretation of lipase stimulation given by Patten and Hollenberg (387) was that exogenous heparin increases the binding of the enzyme to chylomicrons and then has no effect on either the stability of the extracted enzyme or enzyme activity after the formation of enzyme–chylomicron complexes has occurred. The clearing activity was found by Bianchini and Osima (388) to be independent of the number of sulfate radicals and the presence of uronic acid groups. Also, it does not parallel the anticoagulant activity (389).

Evidence was given by Pav and Wenkeva (390) of the appearance in the blood of another serum esterase in addition to the clearing factor after the injection of heparin. The quantitative inhibition of triton lipidemia by heparin was determined by Haikonen *et al.* (391). Finkelstein *et al.* (392) isolated an extracellular lipase from the culture medium of *Pseudomonas aeruginosa*.

A naturally occurring acid mucopolysaccharide was isolated by Grossman *et al.* (393); it had good lipoprotein lipase-activating effects and cholesterol-reducing ability, with little anticoagulant activity. Sulfovernan, a new heparinoid from a lichen moss, was shown by Stefanovich *et al.* (394) to have anticoagulant activity, but it increased the triglyceride level of the lipids of aortic tissue. The latter effect is not thought to be associated with heparin.

Effects of Heparin on Tumor Growth and Metastasis—Much experimental investigation has been attempted to solve the question of whether heparin actually alters tumor growth and metastasis, but to date this problem remains unsolved. Reviews were written by Balazs and Jacobsen (395), Shats (396), and Regelson (397). The results of Lippman (398) on the Ehrlich ascites tumor in mice show that heparin is a mitotic inhibitor in this



Scheme V—Mechanism of action of lipoprotein lipase

tumor. No effect on the growth of a transplantable lymphosarcoma in mice, however, was found by Kreisler (399). Csaba *et al.* (400) agreed with these results and even thought that some tumors require heparinoid substances for their growth. The findings of Montes de Oca and Dietrich (401) in the incorporation and fate of heparin in L, Le, HeLa, and mouse embryo tissue culture indicate that there is no inhibition by heparin of the four cell lines.

Fisher and Fisher (402) believed that their observed decrease of tumor metastases in rats cannot be explained by an inhibition of tumor cell lodgment and microclot formation but by rheological alterations in the blood as a result of the administration of heparin and previous tumor plasmin. The treatment of tumor-bearing mice by Kudryashov *et al.* (403) did not produce a consistent reduction in tumor growth but actually promoted growth in several animals. If, however, the vegetative nervous system was blocked with chlorpromazine during anticoagulant and fibrinolytic treatment, there was a significant regression of tumor growth. Németh (404), using a combination of heparin and degranol[4], found a reduction in the size of tumors rich in connective tissue.

The effects of heparin and fibrinolysins on pulmonary metastasis were studied by Cliffton and Agostino (405, 406), with the finding that the incidence of metastasis was decreased. Suemasu and Ishikawa (407) discovered a similar result on pulmonary metastasis which they attributed to indicate that tumor cells were flushed away by the anticoagulant through the lung by blocking the formation of fibrin clots around the cells in capillaries that enmesh them. Likewise, Rutstein *et al.* (385) found that heparin has a disseminating influence on pulmonary metastases of experimental tumors. On the other hand, Hagmar and Norrby (408) reported that the average volume of pulmonary metastases became less but their number rose. This was also experienced by Boeryd and Rudenstam (409).

Miscellaneous Effects—The antiviral action of heparin on *Herpes simplex* was mentioned by Jaques (230). The observation of Vaheri (410) was that heparin has a potent reversible inhibitory action on the early interaction of *H. simplex* virus and cells but that it does not have an irreversible effect on the virus. Horváth and Hadházy (411) found that heparin inhibits only the absorption of free virus and does not influence the penetration and cell-to-cell transfer of viruses carried by infected cells. Treatment of chick embryo cells by Mentkevich and Zhdanova (412) inhibited H and B_1 strains of Newcastle disease virus from producing interferon. The stimulation of cell locomotion and pseudopodium formation of the amoeba by heparin was seen by Bell and Jeon (413). Bell (414) suggested that heparin has a depolarizing effect on the cell surface. The antibacterial action of heparin in the presence of blood from regions of injury was mentioned by Regelson (397).

Heparin inhibits the action of many enzymes such as β-glucuronidase (415, 416), renin (417), hyaluronidase (418), alkaline phosphatase (418), chondroitin sulfate glycanhydrolase (418), elastase (418), and collagenase (419). The inhibition of hyaluronidase in the gingiva by heparin was cited by Nord (420) as an important defense mechanism in periodontal disease. Heparin and rifampicin were acclaimed by Neuhoff *et al.* (421) as being the most effective known inhibitors of DNA-dependent RNA polymerase from *Escherichia coli*. It also reduces the action of ribonuclease (367, 421), fumerase (367), serum protease (367), and lecithinase (422). Heparin was found by Wu (423) to inhibit polynucleotide kinase, which catalyzes the transfer of orthophosphate from ATP to the 5^1-hydroxyl terminus of polynucleotides. Freeman (424) suggested that the ability of heparin to inhibit a rather large number of enzyme systems may be due to its acidic character which allows it to combine rather tightly with almost any basic protein. There are some enzymes, such as staphylococcal coagulase, upon which heparin has no action, at least under experimental conditions (425). On other enzymes such as pepsinogen (426) and diamine oxidase (427), it has a stimulating action.

Heparin has some activity in secretion and excretion. Gastric secretion was inhibited by heparin and heparinoids in some dogs (428). It has a diuretic effect and this lowers edema (8, 230). It also inhibits the antidiuretic effect of pitressin (230). The excretion of potassium is decreased and that of sodium is increased (230, 367) after heparin administration.

Its interaction with calcium has been of interest. According to the results of Tidball (429, 430), physiologic histamine release is partially inhibited by heparin, the assumption being that the histamine release may result from the binding of calcium by heparin. Cheymol and Gay (431) found that heparin causes an elevation of alkaline reserve in rabbits. According to Reber and Studer (432), the binding of calcium ions by edetate, sodium citrate, ion exchangers, *etc.*, seems to enhance the intestinal absorption of heparin in rats, as measured by clearing factor activation. However, the work of Koh (433) with dimethyl sulfoxide, diethylsulfone, *etc.*, showed that these also enhance heparin absorption but here they act by a process other than calcium sequestration since they are not chelating agents. Additionally, through chelate-like compounds with calcium ions, heparin may deplete capillary walls and decrease wound strength (281). Heparin inhibits calcification *in vitro* and increases calcification *in vivo*. The explanation for this phenomenon given by Harris *et al.* (434) was that there is binding of calcium by heparin *in vitro* and that heparin acts as a template for calcium deposition *in vivo*. Leonard (435) found that it can cause sequestration of calcium. In addition, it induces a stimulatory effect on bone resorption (367).

Heparin caused developmental anomalies in amphibian and sea urchin eggs. This was explained by DeCarli and Brachet (436) as being due to damage to the transcription of DNA. Skalka *et al.* (437) reported the incorporation of labeled thymidine into the DNA of rat thymocyte and spleen cell nuclei. In the presence of large amounts of heparin, this leads to the disintegration of the nuclei.

The action on various tissues and organs has been given much consideration. Garin and Bassa (438) found that heparin eliminates quickly the action of total spleen

[4] Zitofen.

extract which blocks up medullary inhibition. Sasaki (439) showed that there is a marked decrease of lymphocytes in lymph nodes and the white pulp of the spleen after heparin is administered. The decrease in respiration of animal tissue caused by heparin was described by Schuster (440). Clinical investigation by Fegen *et al.* (441) indicated that heparin aids in prolonged ischemia time in an occluded renal artery after kidney injury. Heparin formed a gel when added to homogenates and nuclear fractions of rat liver, thymus, spleen, and kidney (442). Lazzarini-Robertson (443) found that heparin has a strong inhibitory effect on the uptake of labeled serum-bound lipids by human and animal endothelial-type cells. This is probably due to the very strong negative charge, acting directly at the cell membrane level. Xanthomatosis and burns of the skin are improved by heparin (8), and it stimulates growth of connective tissue (367). The cytotoxic effect of a rabbit antiserum on guinea pig myocardium was eliminated by heparin (444).

Heparin has an inhibitory effect on hormones such as aldosterone (445) and testosterone (446). In euthyroid and hypothyroid patients receiving heparin alone, there were no changes in thyroxine-binding globulin and thyroxine-binding prealbumin; according to Schatz *et al.* (447), it is possible that the heparin competes with thyroxine for binding sites.

In various types of shock, there is an outpouring of heparin from the mast cells (367). However, in a study of anaphylactic manifestations in the rat, Salmon and Lecompte (448) found no inhibition to ovalbumin sensitivity. It was found by Vannas (449) to have a beneficial effect on iritis in the rabbit. In addition to bone loss, another deleterious effect of heparin is increased mortality after its administration in the case of fat embolism (450). Also, the telogen phase in the hair may be precipitated prematurely, producing telogen effluvium (451).

HEPARIN THERAPY AND PHARMACODYNAMICS

Heparin has been reported (452) to be an "ideal" anticoagulant in ease of administration. It is especially desirable because of its safety, rapid onset of activity, and reversibility.

Reviews have been presented on anticoagulant and heparin therapy (8, 311–314, 453–455), discussing indications, dosage, adverse effects, and contraindications.

Indications and Clinical Application—Heparin has its main use in acute thrombotic disease, vascular and cardiac surgery, and acute myocardial infarction (92). Its efficiency in reducing mortality in thromboembolism was questioned by Enger *et al.* (456), but it may decrease chest pain and thromboembolic complications (457).

Its use in the treatment of pulmonary embolism was reported by Zilliacus (458), who claimed that it has certain advantages as compared to vein ligation in legs since it acts on undiagnosed thrombi. Moser (459) found heparin to be the drug of choice in this complication because of its (*a*) immediate onset of action, (*b*) potent inhibition of the coagulation system, (*c*) enhancement of fibrinolytic dissociation of fresh thrombi, (*d*) inhibition of platelet breakdown (and, therefore, serotonin release), and (*e*) prompt reversibility of anticoagulant action.

In the treatment of cerebral thrombosis (460), anticoagulant therapy postpones the arrival of an impending stroke, prevents transient ischemic attacks, and sometimes stops the advance of a progressive thrombotic stroke.

Other uses for heparin were mentioned by Engelberg (8), such as lowering of edema by its diuretic effect, neutralization of snake venom, lowering of hypertension, aid in burns and allergies, and prevention of atherosclerotic disease. Some other possible uses of heparin were said by Jorpes (454) to be in Dupuytren's contracture and fat embolism. It is the anticoagulant of choice for patients under general anesthesia (452).

Method of Administration and Dosage—Heparin is ineffective when given orally. According to Estes (309), the minimum dose required to anticoagulate blood to a clinically desirable level is 35 units/kg. The various routes of administration for acute myocardial infarction and sustaining therapy described by Lomax (461) are: (*a*) continuous intravenous infusion—an initial dose of 5000 I.U. and then 20,000–30,000 I.U. in 1 l. of 5% glucose or 0.9% sodium chloride solution given slowly over the next 24 hr., (*b*) intermittent intravenous infusion—5000 I.U. every 3 hr., (*c*) intramuscular—an initial intravenous dose of 5000–10,000 I.U. and then 20,000–40,000 I.U. injected every 8–10 hr., and (*d*) deep subcutaneous injection—20,000–40,000 I.U. injected subcutaneously, which can prolong coagulation up to 16 hr.

For pulmonary embolism, Moser (459) suggested that 7500 units of heparin be given subcutaneously every 6 hr. or 10,000 units every 8 hr. This dosage is maintained until the patient's cardiopulmonary status has stabilized and all traces of venous thrombosis have departed. Miller *et al.* (462) claimed, however, that streptokinase is more effective after 72 hr. than heparin.

The dose in cerebral thrombosis (460) is 50 mg. every 4 hr. in progressive stroke or with transient ischemic attacks. The therapy is maintained for 1–3 weeks, and then oral coumarin therapy is continued for 1 year or more.

Pharmacodynamics of Heparin—Since heparin is used in emergency prophylactic treatment, its pharmacodynamic properties must be understood. It was found that intravenous injection of heparin follows a logarithmic linear regression curve (463). Studies on the relative volume of distribution and half-life (464) revealed that all log plasma heparin concentration–time curves fitted an equation for first-order kinetics, even at the highest doses. The relative volume of distribution varied with the dose but not the half-life. These data agree with the hypothesis that heparin may leave the plasma by uptake into the reticuloendothelial system and could indicate the reason for the problems found in devising practical and reliable dose schedules. In later investigations, Estes (309, 465) found that the half-life of heparin's anticoagulant activity is approximately 1–1.5 hr., and the equation expressing the relationship between dose ratio and dose interval is:

$$R^* = \frac{D^*}{D} = \frac{1}{1 - e^{-k_{elr}}} \qquad \text{(Eq. 1)}$$

where R^* is the dose ratio, D^* is the initial dose, D is the maintenance dose, k_{el} is the rate constant for elimination, and τ is the dose interval. It is found then that maintenance of anticoagulation to a degree meeting common acceptance is met by administering heparin at intervals approximating the half-life (465).

Contraindications—Heparin should not be given to patients with a history of bleeding diathesis, bleeding ulcer, severe hypertension or cerebral hemorrhage (466), jaundice, purpura, hemophilia, acute bacterial endocarditis, and accessible ulcerated lesions (especially of the GI tract) (378).

Adverse Effects—Rodman (311) stated that prolonged use of large doses of heparin (over 200 mg. daily for 6 months or longer) may produce osteoporosis. He also mentioned as side reactions: (*a*) dysesthesis pedia, which is an intense itching of the soles of the feet, and (*b*) the possibility of inhibition of the adrenal cortical synthesis of aldosterone.

Various other adverse effects, described by Lomax (461), include transient alopecia, spontaneous hemorrhage, hypersensitivity, anaphylactoid reactions, thrombocytopenia, fever, and acute adrenal hemorrhage.

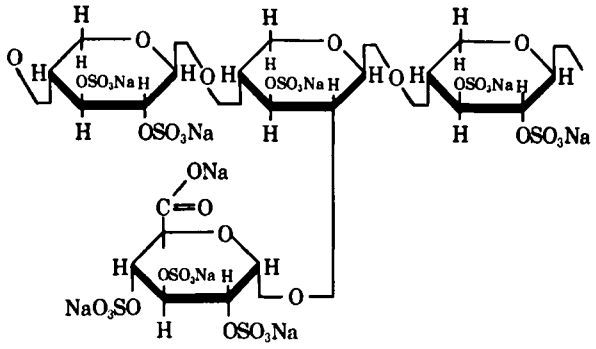
ANTAGONISTS OF HEPARIN

The substances that antagonize the anticoagulant action of heparin are important in the control of heparin therapy and in assay techniques. A review by Agolini (467) discussed the inactivating power of various chemicals on heparin and dextran sulfate. A list of antiheparin agents, given by Stamm (468), includes protamine, clupein, polylysine, lysozyme, polybrene, toluidine blue, fuchsin, and tryptophan.

Garagnini and Fuccini (469) suggested that the inhibiting action of lysozyme on heparin is due to bond formation between the two substances. Protamine sulfate, a low molecular weight protein with a strong basic charge, is antagonistic to the clearing action of heparin (470). The early stages of blood coagulation *in vitro* are inhibited by protamine and polybrene (471) by interfering with the activation of factor IX (plasma thromboplastin component, Christmas factor). Factor IX is activated in the presence of calcium by activated factor XI, a reaction accelerated by thrombin. Protamine prevents this activation by inhibiting the activated XI. Heparin does this by being antithrombic, whereas polybrene acts directly against the inactive IX. This inhibition is probably due to a competitive complex formation since the inhibitory action of each can be overcome by increasing the concentration of its antagonist.

The ratio of protamine sulfate antagonistic to heparin is given as 1:1 by weight (461). According to Lowary *et al.* (472), this is not always true but is also dependent on the tissue source of the heparin in both *in vitro* and *in vivo* studies. Yen *et al.* (473) gave the activity of the antiheparins as β-lipoprotein < thrombin clotting system < Christmas factor < platelet protein < protamine sulfate.

In comparison to toluidine blue, which is very toxic, polybrene administered intravenously promptly and completely neutralizes the anticoagulant action of heparin, with minimal hypotension and no disturbance in pulse rate (474).



II: chemical structure of a sulfonic polysaccharide

Several other less known inorganic and organic substances also have antagonistic properties to heparin. Hexavalent complexes of cobalt and chromium were quite active and complexes of acriflavine, stilbamidine, and viomycin were slightly active (475). Visconti *et al.* (476) discovered that carbon disulfide showed significant antiheparin activity in blood. Substances such as phosphate and sulfate ions, adenosine triphosphate, adenosine diphosphate, and myosin were able to remove the antihemostatic effect of heparin (477).

HEPARINOIDS AND HEPARIN-LIKE SUBSTANCES

This section discusses those substances that have a similar biological action as heparin. The quest for a substance with a high anticoagulant activity and low toxicity still continues.

Sulfur esters of chitin, starch, and cellulose are effective as anticoagulants *in vitro* but are toxic on injection into animals. Dextran sulfate is harmless, but the maximum activity is 15 units/mg. (478). Wood and Mora (479) found that glucose sulfates (degree of substitution 2.6–3.0) had approximately the same activity as clinical preparations of heparin. Amylose, amylopectin, and guaran, after being sulfated, gave low *in vitro* anticoagulant activity, suggesting the importance of *N*-sulfation (480). A sulfated alginic acid[6] has been used as a heparin substitute in therapy (361).

Other heparin-like substances include a sulfonic polysaccharide (Structure II) made by Aznar *et al.* (481). It has marked fibrinolytic activity and is able to inhibit platelet aggregation but has little anticoagulant effect. Wolfrom and Wang (482) prepared an *N*-sulfated heparinoid from amylose which has negligible activity. Chitosan was modified by sulfation and oxidation by Whistler and Kosik (483), converting some of the sugar units to uronic acid. The highest anticoagulant activity attained (60 I.U./mg.) was after all amino groups were sulfated, *O*-sulfate content was increased, and uronic acid groups were introduced. Still another semisynthetic anticoagulant, a Pentosan-polysulfo-ester (SP 54), was introduced by Walb *et al.* (484). In contrast to heparin, *in vitro* this compound has less influence on coagulation and 10 times as great anticomplementary action. Re-

[6] Paritol.

cently, a synthetic sulfated polygalacturonic acid methyl ester[6] was introduced by Jarrett and Jaques (485) which, when administered in conjunction with edetate, has potential as an oral anticoagulant based on the assumption that the chelating agent would form chelate bonds with ions which would otherwise obstruct the absorption of the heparinoid.

Bhattacharya and Singh (299) conducted a comparative study of polyanionic compounds, such as polyestradiol phosphate and polymethylene salicylate, but discovered that they possess only a fraction of the activity of heparin.

Unmodified heparin-like substances from natural sources have shown some promise as anticoagulants. Heparin-like anticoagulants were extracted from clams (11–13, 486). These have acetyl residues but have a high activity (130 I.U./mg. from mantle tissue), low toxicity, and a higher molecular weight than heparin. The polysaccharide, carrageenin, is not an antithrombin and does not inhibit the viscous metamorphosis of thrombocytes as does heparin, but it does decrease fibrin polymerization (487). Matsuoka *et al.* (488) found that charonin sulfuric acid, a polysaccharide from the mucus of *Charonia lampis*, remarkably prolonged whole blood clotting time after intravenous injection in the rabbit but had a toxicity 3.6–7.2 times as strong as heparin. A list of other natural and synthetic anticoagulants was given by Bernfeld (356).

Whale heparin (ω-heparin) was first isolated by Yosizawa (489) from the lungs and intestines of the finback whale (*Balaenoptora physalus*). It contains *N*-acetyl glucosamine, to which the other groups of heparin are attached (490), and has approximately the same molecular weight as heparin (491). The structure was confirmed with NMR by Inoue and Inoue (492). Masao (493) reported that ω-heparin from lung tissue exhibited higher activity than heparin, and it was also found to be longer lasting (494). The heparin isolated from the small intestine of whales showed a much higher anticoagulant activity than bovine heparin (495). Whale intestine heparin was used therapeutically by Abe *et al.* (496) during and after an operation for occlusion of the bilateral iliac arteries. There was prolongation of blood clotting and recalcification time, but the amount of bleeding during and after the operation became insignificant. Nagasawa (497) found, in addition to whale heparin from the small intestines and lungs, that the whale (mucous) heparin also had high activity, 580 units/mg. (BP).

METABOLISM AND ENZYMATIC DEGRADATION

Reviews on the metabolism of heparin were presented in 1966 by Whistler and Rowell (7), in 1968 by Dodgson and Lloyd (498), and in 1969 by Silbert (499).

Studies with Labeled Heparin—With the preparation of a radioactive heparin, the possibility of quantitative metabolic studies could be attempted. A method was described by Lalich (500) for the synthesis of radioactive heparin using a process of *N*-desulfation and *N*-resulfation. The results of Eiber *et al.* (501) indicate that heparin-^{35}S or ordinary heparin given orally would pass into the blood only to a very small extent and would have no effect on blood coagulation. However, when the labeled heparin was injected intravenously, the radioactivity in the blood increased in proportion to increases in clotting time and clearing activity.

Labeling of heparin with tritium and ^{35}S gives a stable product under a variety of conditions which retains the original physicochemical and biological properties (502). Heparin labeled with ^{3}H was found by Barlow and Cardinal (503) to have an activity of 124 USP units.

Dietrich and Montes de Oca (504) isolated radioactive mucopolysaccharides from HeLa, K., mouse, and rat embryo cells grown in the presence of a culture medium with radioactive inorganic sulfate. A ^{51}Cr-labeled heparin was made by refluxing heparin with $^{51}CrCl_3$ by Varga *et al.* (505). This could be important in investigations of the function of heparin *in vivo* and *in vitro* as well as its metabolic fate.

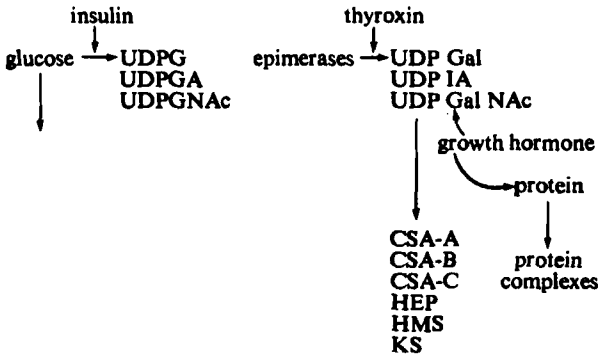
Biosynthesis—Studies by Silbert (506–509) indicate that an *N*-acetylated glycosaminoglycan is transformed into heparin at the polymer stage. Also, the polymerization appears to take place in close proximation to the sulfation in the mast cell. The resulting sulfated polysaccharide remains bound to the same particulate preparation that catalyzes the heparin formation.

In contrast, the results of Rice *et al.* (510) on the mastocytoma showed that there is transfer of sulfate to small molecular weight precursors. Also, the observations of Marx and Eisenman (511) establish that the major portion of the "microsomal" fraction from the homogenates of Furth mast cell tumor was capable of controlling the transfer of radioactive sulfate to *N*-desulfated heparin. Balasubramanian *et al.* (512) concluded that apparently free amino groups are required for the enzymatic *N*-sulfation of glycosaminoglycans.

Most of heparin exists in nature in firm covalent combination with protein and does not appear in appreciable amounts as an extracellular component of connective tissue (498). Helting (513), in 1971, investigated the natural trisaccharide, 3-*O*-β-D-galactosyl-4-*O*-β-D-galactosyl-D-xylose, of the heparin–protein linkage region and found that partial acid hydrolysis of ^{14}C-galactose-labeled endogenous acceptor yielded several fragments with the characteristics of neutral oligosaccharides previously isolated from the heparin–protein linkage regions and, in addition, a compound with the chromatographic qualities of *N*-acetyl lactosamine. This confirmed the biosynthesis scheme of acid mucopolysaccharide production offered by Dorfman (514). Scheme VI (514) shows the biosynthetic pathways of the acid mucopolysaccharides of connective tissue. A more recent scheme (Scheme VII) was presented by Silbert (499).

Studies on Catabolism—Danishefsky and Eiber (515), in 1959, concluded from excretion studies with heparin-^{35}S that the sulfate linkages of heparin were cleaved as a result of metabolic degradation; with small doses, all of the $^{35}SO_4$ in the urine was in the form of inorganic sulfate while larger doses of heparin-$^{35}SO_4$ led to the output of some urinary heparin-$^{35}SO_4$. The findings of Lloyd *et al.* (516) complemented those of Danishefsky and Eiber (515) and also showed the presence in the

[6] G31150.



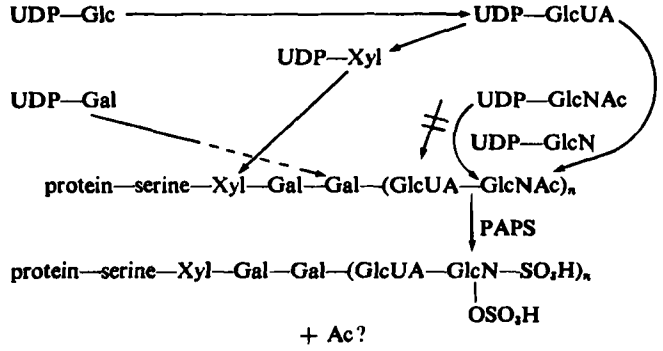
UDPG	=	uridine diphosphoglucose
UDPGA	=	uridine diphosphoglucuronic acid
UDP Gal NAc	=	uridine diphospho-*N*-acetylglucosamine
UDP Gal	=	uridine diphosphogalactose
UDP IA	=	uridine diphosphoiduronic acid
CAS(A,B,C)	=	chondroitin sulfuric acid (A, B, and C)
HEP	=	heparin
HA	=	hyaluronic acid
HMS	=	heparitin sulfate
KS	=	keratosulfate

Scheme VI—Biosynthetic pathways of acid mucopolysaccharides of connective tissue (514)

urine of a material with an electrophoretic mobility similar to heparin but a reduced molecular weight. Thus, it appears that greater catabolism is reserved for higher molecular weight materials.

Lemaire *et al.* (517) found the formation of partially desulfated catabolite in the urine and strongly labeled sulfonucleotides in the liver. The heparin excreted in the urine was termed uroheparin by McAllister and Dennis (518) and was shown to have about half of the anticoagulant activity of ingested heparin; the number of moles of sulfate per repeating unit was 3.94 for uroheparin in contrast to 5.6 for heparin.

Degradation by Enzymes—The change in heparin after absorption in the body is thought to be due mainly to bacterial enzymes. Heparinase, isolated by Walker (519) from *Flavobacterium heparinum*, a Gram-negative soil bacterium, reduces heparin and heparitin sulfate to oligosaccharides, with glucosamine or *N*-acetylglucosamine as the reducing end-group. Linker and Hovingh (520) fractionated a crude heparinase on a Sephadex column and obtained two active fractions. The first had the properties of an eliminase, which degraded heparin and heparitin sulfate nonhydrolytically to di- and oligosaccharides containing α,β-unsaturated glucuronic acid. The second had the properties of a glucuronidase, which hydrolyzed the unsaturated uronides to monosaccharides. Dietrich (521) identified the products of such a bacterial degradation as glucosamine 2,6-disulfate, saturated disaccharides made up of uronic acid and glucosamine and containing two and three sulfate residues, and tetra- and hexasaccharides with the same disaccharide units. From this, he concluded that positions 2 and 6 are the positions for the *N*-sulfate and *O*-sulfate, respectively, in the disulfated glucosamine molecule. He (522, 523) also showed that five enzymes from *F. heparinum*, acting in concert, were able to degrade heparin to its monosaccharides. Karapally and Dietrich (524) isolated a uronic acid isomerase from *F. heparinum* which converts glucuronic acid and galacturonic acid to fructuronic



Scheme VII—Biosynthetic pathway of mucopolysaccharides (499)

acid and tagaturonic acid. Hovingh and Linker (525) found that a heparinase and a heparitinase could be obtained from *F. heparinum*, and the specificity appears to be related to the presence or absence of *N*-acetyl groups. Whale heparin was degraded by Yosizawa (490) by crude heparinase to three major monosaccharides: α-keto acid, *N*-sulfated glucosamine, and *N,O*-desulfated glucosamine. There were also minor products: *N*-acetylglucosamine containing unsaturated oligosaccharides.

A group of enzymes from *F. heparinum*, the sulfamidases capable of liberating inorganic $^{35}SO_4^{-2}$ ions, was discovered by Lloyd *et al.* (526). The sulfamidases extracted from bacterial and mammalian sources differed in that the mammalian enzyme liberated inorganic $^{35}SO_4^{-2}$ ions only from ^{35}S-heparin and the bacterial enzyme preferentially degraded authentic 2-deoxy-2[^{35}S]-sulfamino-D-glucose but to a lesser degree [^{35}S]-labeled polymer (527). The actions of sulfamidase and sulfoesterase in the desulfation of the catabolites of heparin were studied by Dietrich (528, 529). Recent work on heparin degradation (530, 531) established the presence in rat tissues of an entirely normal mammalian system which has the ability to degrade the sulfamate groupings of exogenous [^{35}S]-sulfoamino-heparin and thus exhibits the characteristics of a "heparin sulfamidase."

Testicular hyaluronidase was shown by Ogren and Lindahl (532) to digest and degrade heparin from mouse mastocytoma. The results of this investigation suggest that the molecular weight of the newly synthesized heparin is much higher than that of the polysaccharide stored in the mast cell granules; the heparin released from the mast cell *in vivo* is believed to originate from these granules, thus having partially degraded rather than intact polysaccharide chains.

HEPARITIN SULFATE

Heparitin sulfate, also referred to as heparan sulfate or heparin monosulfate, is closely related to heparin. A general review was written by Spiro (533) in 1965. Heparitin sulfate is obtained as a by-product in the preparation of heparin from lung and liver tissue (534). It has a lower sulfate content than heparin (535) and, unlike heparin, the glucosamine residues are partly *N*-acetylated and partly *N*-sulfated. The most important linkage in this mucopolysaccharide is 1 → 4 and there may be branching (536). The major uronic acid of hepa-

ritin sulfate seems to be glucuronic acid, but the presence of L-iduronic acid was also demonstrated as in the case of heparin (537). In contrast to heparin, heparitin isolated from the human aorta has a higher molecular weight (24,000–29,000) and negligible antithrombin activity (534), and it is bound to protein by way of serine (538). On treatment of heparan sulfate with alkali or proteolytic enzymes, it behaves as a multichain proteoglycan such as chondroitin sulfate or keratan sulfate (539).

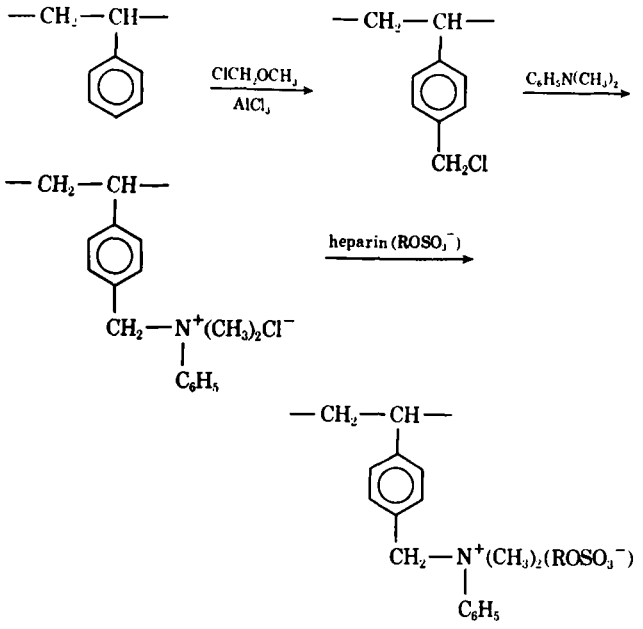
Heparitin sulfate has been isolated in small amounts from human brains (540) and other mammalian brains (541–543). Some evidence (544) indicates that mucopolysaccharides also may be present in the extracellular space in the brain. This could be important in understanding the blood–brain phenomenon, since the distribution of various ions and other high molecular weight substances in the CNS could be influenced by the localization of charged mucopolysaccharides.

Studies report the isolation of small amounts of heparitin sulfate in the dermis of mammalian skin (545) and chicken skin (546). The skin of hogs (547) and that of neonatal rats (548) yielded heparitin sulfate constituents. It was discovered that, in contrast to heparin, the heparitin sulfate content in rat skin increased with age rather than decreased (549).

Other sources of heparitin sulfate include the pig aorta (550), bovine periodontal membrane (551), human umbilical cord (552), and beef lung tissue (553, 554). Normal urine was found to contain heparan sulfate with a molecular weight of 8000–30,000 and calculated Stokes radius of about 60 Å (555). It was separated from dermatan sulfate and chondroitin sulfate in the urine by a cetylpyridinium method (566), electrophoresis (557), and column chromatography (558, 559). Heparitin sulfate was recently isolated from cotton wick granulation tissue of rats (560) and mouse fibroblast line 3T6 (561). It was found also in young mast cells in addition to heparin (562).

Abnormal excretion in the urine of mucopolysaccharides can be related either to disorders of a hereditary character or to a series of conditions where there is a metabolic defect involving the connective tissue. These pathological conditions have been termed the mucopolysaccharidoses, and several reviews have been written concerning them (33, 563–570). Heparitin sulfate was detected in abnormal amounts in the urine of patients with San Filippo's (566), Morquio's (571), Hunter's (565, 567, 571, 572), Hurler's (563, 567, 568, 573–577), and Ulrich-Scheie's (567) syndromes. Some methods of detection of the mucopolysaccharides in Hunter's syndrome were paper chromatography (578) and ion-exchange chromatography (579). Glycosaminoglycans from the liver and spleen of patients with Hurler's syndrome had much lower molecular weights than normal, consisting of single chains of a molecular weight of about 5000 together with multiples of up to four such chains attached to peptide moieties (571).

The mechanism involved in the pathogenesis of mucopolysaccharidosis was indicated by Hers and Van Hoof (580) as: (*a*) the mucopolysaccharides are synthesized in excess by the fibroblasts, released in the circulating fluids, and then excreted in the urine or taken up by the tissues through endocytosis; or (*b*) the mucopolysaccharides are typical inborn lysomal diseases, similar to type II glycogenosis.



Scheme VIII—Reaction for heparinization of quaternary groups on a polymer

BONDING OF HEPARIN TO SURFACES

With the advent of prosthetic circulatory devices, there has been a search for materials that: (*a*) cause no change in the blood, (*b*) stand up for long periods of time, (*c*) are compatible with tissue, (*d*) can be sterilized, and (*e*) are available both as rigid and flexible materials. Of these prerequisites, the first is probably the most urgent, so that clotting is not initiated at the surface and hemolysis of the red cells does not occur.

Heparin appears to be satisfactory for the blood compatibility requirement, but it must be bonded in some way to other materials. In 1963, studies were conducted with colloidal graphite surfaces which had been rinsed with a surfactant capable of bonding heparin (581). The resistance of this new surface was far greater to clot formation and was much better than plastic or silicone, but small amounts of heparin left the surface during a 10-hr. period. Some of the significant features of these heparinized surfaces are that there are no *in vivo* thrombi and the ζ-potential is less than in a benzalkonium surface without heparin (582). Recent reviews were presented by Gott and Furuse (583), Salzman (584), and Yen *et al.* (585).

Polystyrene, polyethylene, butadiene–vinylpyridine, polyvinyl chloride, silicone rubber, and many other materials can be rendered nonthrombogenic by a physical surface bonding of heparin on quaternary groups attached to the polymer (581–591). The procedure involves chloromethylation, quaternization, and heparinization (592) and the typical reaction is shown in Scheme VIII (593). Tests conducted by Fourt *et al.* (594) of some of these heparin-bearing surfaces on nylon and on heparin base with collodion in dilute films present evidence that heparin on the walls may be more effective than heparin in solution.

Milligan *et al.* (595) believed that the antithrombogenic property is not due to heparin, since most of it is eluted and a colloidal graphite coating alone has similar properties to animal tissues. On the other hand, Usdin and Fourt (596) found only partial dilution of heparin from surfaces exposed to whole blood, plasma, or serum. This seems to be dependent on the particular protein fraction used and the nature of the cationic surface (597).

Heparin, chemically bonded to rigid and flexible materials including regenerated cellulose and other hydroxyl-bearing substrates by amination through graft polymerization with ethylene imine, yielded permanently coated surfaces even under high fluid shear (598). Another aminated surface on glass, made from a polymer of 2-hydroxy-3-methacryloyloxypropyltrimethylammonium chloride, showed platelet adhesion but exhibited no attraction of proteins and platelets to the film when exposed to heparin (599). Britton *et al.* (600) demonstrated that heparinized aminoethylated cellulose tubing at low levels of aminoethylation had excellent mechanical strength as well as antithrombogenic properties.

Rubber surfaces that had good blood compatibility were made by chemically binding heparin onto the surface of a diamine-cured polyurethan rubber, but there was a significant thrombosis rate (601). A nonthrombogenic surface on silicone rubber was made by combining graphite, silicone rubber, benzalkonium chloride, and heparin (602). In addition, Hepacone (a combination of heparin and silicone rubber) and Hepacone-6 (a combination of colloidal graphite, heparin, and silicone) were described by Hufnagel *et al.* (603) for intravascular applications.

Other elastomeric complexes were synthesized by Yen and Rembaum (604) from polyether diisocyanates, which had desirable mechanical properties; it was shown that an ionic bond had been formed between the heparin and the quaternized polyether polyurethan. In addition, a new family of linear elastomeric ionenes or cross-linked homo or block polymers containing nitrogen were synthesized, which were capable of bonding with heparin (605, 606).

The use of heparinized polyester fibers in vascular prostheses is finding increased interest. Najjar and Gott (607) suggested that a heparinized, small diameter, Dacron graft would find excellent applications in lower extremity vascular surgery and possibly in coronary artery bypass. Two methods were developed by Hersh *et al.* (608) for heparinizing polyester fiber. One method involves the fixation by heat of a water-insoluble complex of heparin onto the polyester; the second uses an aminosilane coupling agent to bond the heparin ionically to the protonated amine. Conflicting reports as to which is most effective have been issued. According to Hersh and Weetall (609), the thermal method is better since ionically bound heparin eluted from the Dacron quicker. However, in a later report, Hersh *et al.* (610) inferred that the Dacron grafts that had ionically bound heparin yielded the best results on both tissue and blood compatibility.

Membranes for hemodialysis and encapsulation have been constructed from heparin combined with nylon and cellulose. Luttinger and Cooper (611) devised two methods for binding heparin to nylon membranes. In the first, free amino groups are readily quaternized with methyl iodide and the surface is placed in contact with a dilute aqueous heparin solution; in the second, there is surface attachment of quaternary ammonium salt to the membrane surface, followed by heparinization. A heparin-complexed membrane for coating on microcapsules, which might be useful for treating certain enzyme-deficient diseases, was made by combining a benzalkonium–heparin complex with an ether–colloidin solution (612). Promising nonthrombogenic properties were demonstrated by heparinized DEAE CA-cellulose acetate membranes with an indefinite clotting time (613). Measurement of heparin permeability through cellulose membranes gave variable results, possibly because of the polydispersity of commercial heparin (614).

Heparin also may be easily embedded on the surface of biologically derived collagen membranes, which may permit kidney dialysis (615). The effects of heparin on this membrane showed little influence on the recalcified clotting time until levels of 1–10 mg. of heparin/g. of collagen were reached and then there was an increase (616). For extracorporeal connections and membrane assemblies, as well as for potential intravascular implantation, Merrill *et al.* (617) made a hydrogel by heating a homogeneous water solution of polyvinyl alcohol, heparin, glyceraldehyde, and an acid catalyst.

Some other interesting examples of heparin adsorbed physically and chemically on surfaces may find use in cardiovascular surgery. Flexible and rigid epoxy and urethan polymers containing heparin, chemically and uniformly incorporated throughout and/or ionically complexed to quaternized amine groups, were implanted into the vena cava of dogs with no adverse effects on the blood (618). The innominate artery was reconstructed by Murray *et al.* (619) with the use of a temporary heparin-coated shunt bypass. Merenstein (620) flushed umbilical artery catheters with heparinized saline to maintain potency. The absorption of fibrinogen on mica surfaces was inhibited by heparin (621).

An effect that occurs in polymer films after heparin uptake is that only small amounts of protein or oxide and no detectable amounts of fibrinogen or platelets are absorbed at the polymer solid interfaces (622). Another interesting effect is that materials treated chemically so as to introduce negatively charged groups, such as sulfonate, carboxylate, or heparinate, tend to be antithrombogenic, but the surfaces must have a uniform negative charge (623).

The limitation of heparinized surfaces given by Salzman *et al.* (624) is that deposition of platelets occurs on these surfaces which antagonize the action of heparin. Thrombocytopenia, platelet thrombosis, and embolism can complicate heparinized surfaces or extracorporeal surfaces with large surface areas unless methods are developed to reduce heparin platelet interaction. It is also possible for hemolysis to be induced by heparinized polymers (625). According to Friedman and Leonard (626), platelet adhesion on heparinized and nonheparinized surfaces is similar.

According to Salzman (584), some of the problems that remain to be solved are:

1. What is the nature of events that occur at a blood-solid interface?

2. What conditions must be satisfied for a material to be nonthrombogenic?

Since thrombosis is probably most important among the complications with the development of artificial organs, these problems remain foremost in the minds of workers in this field.

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