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# THE RELATIONSHIP OF MOLECULAR WEIGHT, AND SULFATE CONTENT AND DISTRIBUTION TO ANTICOAGULANT ACTIVITY OF HEPARIN PREPARATIONS*

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ABSTRACT

Fractions ranging in molecular weight from 4.8 to $12.5 \times 10^3$ daltons and showing wide variations in sulfate and D-xylose proportions were isolated from hog-mucosa and beef-lung heparin by-products. The anticoagulant activity of such fractions was found to be affected in concert by molecular weight, and by sulfate content and distribution. A heparin preparation having nearly half the 2-amino-2-deoxy-D-glucose residues substituted with *N*-acetyl groups retained appreciable biological activity, indicating that proportions of 2-sulfoamino-2-deoxy-D-glucose residues comparable to those present in heparan sulfate are consistent with maintenance of partial anticoagulant activity, provided that the ester sulfate content is similar to that found in native heparin. The presence of multiple repeating units containing 2-acetamido-2-deoxy-D-glucose residues in the interior of the molecule decreases biological activity.

## INTRODUCTION

The correlation of physical and chemical factors to the biological activities shown by heparin is of interest because of the usefulness of this polysaccharide as a therapeutic agent[1–3]. Characteristic parameters under consideration in this relation have included degree of sulfation[3–7], molecular size[8–12] and shape[12–14], dissociation of ionizable groups, and spatial considerations of *N*-sulfate groups[6]. Studies relating the molecular weight of heparin with anticoagulant activity have led to disagreement, and reports indicating a positive relationship[7–9] have been refuted by others[10,12]. Similar uncertainty concerns the relation of *N*-sulfate groups to biological activity. The release of 7–11% of *N*-sulfate groups after acid hydrolysis, under conditions causing little apparent loss of ester sulfate or decrease in molecular weight, has been reported to inactivate heparin[5,6,11]. However, other results[10] suggested that partial

*Dedicated to the memory of Professor W. Z. Hassid.

activity remained after acid hydrolysis under conditions expected to cause increased *N*-desulfation.

In this report, evidence bearing on the specific relation of molecular size and sulfate content to biological activity will be presented. Although relatively large variations in both amino and sulfuric ester groups content occur in heparin, adding to the difficulty in establishing limits of these groups necessary for biological activity, discussions will be provided for defining these limits.

## EXPERIMENTAL

*Materials.* — Beef-lung heparin by-product fractions were obtained from Dr. L. L. Coleman, The Upjohn Laboratories, Kalamazoo, Michigan. Hog-mucosa heparin by-product material was obtained from Dr. H. H. R. Weber, The Wilson Laboratories, Chicago, Illinois.

*Methods.* — Analytical methods for the determination of the content in uronic acid, hexosamine, *N*-sulfate and total sulfate groups, as well as the determination of amino acid and hexosamine content by an amino acid autoanalyzer, and of galactose and xylose by the Technicon carbohydrate autoanalyzer have been described earlier[14]. Anticoagulant activities were performed by Drs. L. W. Van Ness and J. Gallagher of the Wilson Laboratories.

*Fractionation of heparin by-products.* — Methods for preparing fractions from beef-lung and hog-mucosa by-products have been outlined previously[15]. In general, by-products were subjected to column chromatography on Dowex 1 ($Cl^-$) ion-exchange resin and the eluates obtained with 1.5 and 3.0M sodium chloride were used for further fractionation on Sephadex G-75 resin, as described elsewhere[16].

*Estimation of molecular weights.* — This was performed essentially as described by Wasteson[17] by use of a gel-filtration diagram, established from elution values obtained with heparin and heparan sulfate fractions of known molecular weight. The molecular weights of the heparin products used in preparing the elution diagram were determined from viscosity data by Dr. M. B. Mathews.

N-*Sulfation and* N-*acetylation of heparin by-product preparations.* — The procedure for *N*-sulfation followed that described by Lloyd *et al.*[18]. No prior hydrolysis was performed before treatment with trimethylamine–sulfur trioxide, except as indicated in the next paragraphs. Completion of reaction was determined by trinitrophenylation[6]. Preparations were isolated, after dialysis, by precipitation with 2 vol. of ethanol.

*N*-Acetylation was accomplished by addition of 5% sodium hydrogencarbonate (10 vol.) and acetic anhydride (3 vol.) in acetone (20 vol.) to a 1% solution of the heparin compound (100 vol.) and by stiring occasionally over a period of 0.5 h at room temperature. After adjusting the pH to 7.0, the above treatment was repeated. The mixture was kept for 2 h at room temperature, then dialyzed, concentrated 3 fold, and ethanol was added (2 vol.). After centrifugation, the precipitate was washed with absolute ethanol and ether, and air dried.

Preparations of partially *N*-acetylated heparin were obtained by hydrolysis of a 1% solution of hog mucosa heparin with 0.05M hydrochloric acid for 4 or 12 min in a boiling water-bath. The solutions were cooled, neutralized with dilute sodium carbonate, and *N*-acetylation performed as just described. Aliquots of the 4-min hydrolyzate were also *N*-sulfated or isolated without further treatment after the neutralization step.

*Treatment of heparin preparations with organic nitrites.* — This procedure follows the method reported previously[19]. After reaction, the products were subjected to gel filtration on a column of Sephadex G-25 resin and eluates were analyzed for uronic acid, hexosamine, and 2,5-anhydro-D-mannose contents.

## RESULTS AND DISCUSSION

In comparing the biological activities of heparin fractions, a prime consideration is the purity of the preparations. Purification of commercial heparin samples is not difficult with established procedures[15]. In this study, however, the wide differences in molecular size and chemical composition of the heparin fractions isolated from by-product material increased the problem of purification by the usual methods. Although dermatan sulfate, a common contaminant of the by-product material, was removed readily as the copper complex[15], fractionation on Dowex 1 ($Cl^-$) ion-exchange resin, followed by gel filtration on Sephadex G-75 resin, was found necessary to give fractions of satisfactory homogeneity for use in this investigation.

*Fractionation of beef-lung heparin by-products.* — Gel filtration on Sephadex G-75 resin[16] of a by-product fraction of beef-lung heparin, obtained by elution from Dowex 1 ($Cl^-$) resin with 3M sodium chloride, gave the pattern illustrated in Fig. 1 (bottom panel). The polydispersity of the fraction is evident. Subjecting the eluates, pooled as indicated in Fig. 1, to a second gel filtration on a calibrated column of Sephadex G-75 resin gave relatively narrow peaks (Fig. 1, top panel), which indicates that fractions with a range of molecular sizes were present in the original by-product material.

Chemical, physical, and biological properties of the beef-lung subfractions are shown in Table I. Only the 3M subfractions obtained from the material eluted from Dowex 1 resin with 1.5M sodium chloride[15] and showing anticoagulant activities are listed. The preparative gel-filtration pattern of the 1.5M fraction was similar to that of the 3 fraction (Fig. 1). All of the subfractions had relatively high proportions of sulfoamino and ester sulfate groups, the ratios of the former to hexosamine varying from 0.71 to 0.99, and of the latter from 0.97 to 1.67.

The large variation of D-xylose content of subfractions having similar molecular sizes illustrates the range of linkage region constituents in preparations derived from the same by-product material. As noted in Table I, some of the fractions possessed free amino groups in ratios to hexosamine of as much as 0.05. Since amino acids were generally present in trace amounts, the unsubstituted amino groups appeared to be present in hexosamine residues. However, the relative ease of hydrolysis of

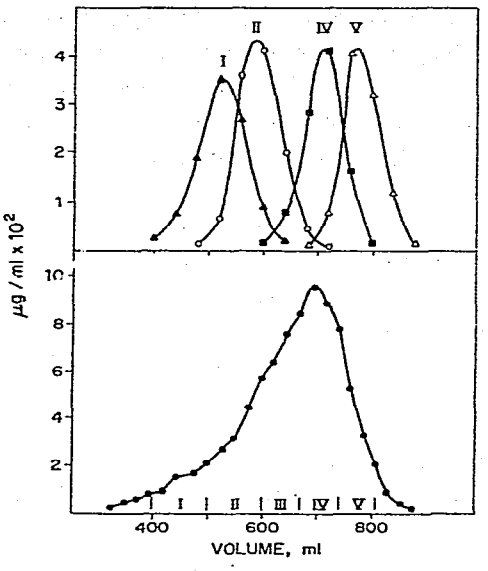


Fig. 1. Preparative gel-filtration pattern, on a column (145 × 33 cm) of Sephadex G-75 resin, of a by-product fraction of beef-lung heparin, which had been previously purified by chromatography on Dowex 1 ($Cl^-$) ion-exchange resin. The fractions were pooled, as indicated in the bottom panel, and were re-subjected to gel filtration on Sephadex G-75 resin. Patterns for four of the pooled fractions are given in the top panel. All values are given as concentration of uronic acid per ml of eluate.

D-xylose and its presence in some of the preparations in approximately theoretical proportion suggests that the free amino groups may not have been produced by chemical degradation during isolation and purification.

Galactosamine was an insignificant contaminant in the subfractions shown, generally representing less than 1% of the total hexosamine content, except for the first subfractions where the concentration was 3 to 5% of the total hexosamine.

*Fractionation of hog-mucosa heparin by-products.* — Chromatography of by-products of hog-mucosa heparin on a column of Dowex 1 resin gave a fraction eluted with 3M sodium chloride that was similar in heterogeneity, when subjected to gel filtration on a Sephadex G-75 column, with the analogous beef-lung material. Preparative gel-filtration of this sample provided three main fractions (Table II) having molecular weights ranging from 6.2 to $11.5 \times 10^3$ daltons. Preparations eluted from a column of Dowex 1 ion-exchange resin with 1.5M sodium chloride were similarly fractionated by gel filtration on a column of Sephadex G-75 resin and Table II also lists several such subfractions. Galactosamine represented less than 1.5% of the total hexosamine in the preparations shown in Table II. The major differences in composition of these preparations from those obtained from beef-lung are in the lower contents of both sulfoamino and ester sulfate groups of the hog-mucosa products, with ratios of these groups as low as 0.63 and 0.93, respectively, in the latter subfractions. Such ratios approach those observed in certain of the heparan sulfates[15].

TABLE I

PROPERTIES OF GEL-FILTRATION FRACTIONS FROM BEEF-LUNG HEPARIN BY-PRODUCTS

| *Fraction* | *Uronic acid* (%) | *Hexosamine* (%) | N-*Sulfate groups*[a] | *Ester sulfate groups*[a,b] | *Amino group*[a] | D-*Xylose*[a] | *Mol. wt.* ($\times 10^{-3}$) | *Anticoagulant activity* (*I.U./mg*) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *Before* N-*sulfation* | *After* N-*sulfation* |
| BL, 3[c] | | | | | | | | | |
| I | 39.6 | 22.1 | 0.85 | 1.46 | 0.015 | 0.035 | 12.5 | 158 | |
| II | 40.1 | 21.7 | 0.81 | 1.58 | | 0.031 | 9.9 | 163 | |
| III | 41.2 | 22.2 | 0.92 | 1.67 | 0.020 | 0.015 | 7.6 | 167 | |
| IV | 39.9 | 22.5 | 0.96 | 1.54 | 0.028 | 0.004 | 6.4 | 114 | 125 |
| V | 38.6 | 21.6 | 0.93 | 1.51 | 0.045 | | 5.2 | 29 | 40 |
| BL, 1.5[c] | | | | | | | | | |
| III | 42.2 | 24.7 | 0.80 | 0.97 | 0.051 | 0.058 | 8.0 | 71 | 86 |
| IV | 43.6 | 25.1 | 0.71 | 1.22 | | 0.071 | 6.9 | 79 | |
| V | 32.7 | 18.0 | 0.99 | 1.56 | 0.043 | 0.002 | 4.8 | 27 | 24 |

[a]Expressed as molar ratio to hexosamine. [b]Estimated from total sulfate less *N*-sulfate content. [c]The starting material used for gel filtration was obtained by elution from a column of Dowex-1 ion-exchange resin with 3.0M (BL, 3) and 1.5M (BL, 1.5) sodium chloride, respectively[15].

TABLE II

PROPERTIES OF GEL-FILTRATION FRACTIONS FROM HOG-MUCOSAL HEPARIN BY-PRODUCTS

| *Fraction* | *Uronic acid* (%) | *Hexosamine* (%) | N-*Sulfate groups*[a] | *Ester sulfate groups*[a,b] | D-*Xylose*[a] | *Mol. wt.* ($\times 10^{-3}$) | *Anti-coagulant activity* (*I.U./mg*) |
|---|---|---|---|---|---|---|---|
| HM, 3[c] | | | | | | | |
| II | 43.1 | 24.1 | 0.77 | 1.41 | 0.020 | 11.5 | 147 |
| III | 39.9 | 22.7 | 0.72 | 1.32 | 0.019 | 9.1 | 148 |
| IV | 38.0 | 22.7 | 0.80 | 1.22 | 0.023 | 6.2 | 62 |
| HM, 1.5–3[c] | | | | | | | |
| II | 36.7 | 22.5 | 0.73 | 1.33 | | 12.5 | 128 |
| III | 40.7 | 22.6 | 0.68 | 1.64 | 0.058 | 8.6 | 45 |
| IV | 37.6 | 23.4 | 0.68 | 1.29 | 0.070 | 7.0 | 53 |
| HM, 1.5–2[c] | | | | | | | |
| II | 42.0 | 22.8 | 0.63 | 1.09 | 0.082 | 8.1 | 59 |
| III | 38.7 | 22.8 | 0.63 | 0.93 | 0.095 | 6.7 | 20 |
| IV | 42.1 | 23.2 | 0.68 | 1.21 | 0.026 | 4.8 | 8 |

[a]Expressed as molar ratio to hexosamine. [b]Estimated from total sulfate less *N*-sulfate content [c]Preparations eluted from a column of Dowex 1 ion-exchange resin with 3.0 (HM, 3), 1.5–3 (HM 1.5–3), and 1.5–2M (HM, 1.5–2) sodium chloride fractions, respectively[15].

The large variability of D-xylose concentration, in hog-mucosa subfractions having similar molecular weights, parallels the results for the corresponding beef-lung subfractions and suggests the possibility that endoenzyme activity may be responsible for the results rather than degradative effects during isolation. No evidence, however, is presently available on this point. In contrast to the beef-lung preparations, none of the hog-mucosa products possessed an appreciable proportion of free amino groups.

*The effect of* N-*desulfation on anticoagulant activity.* — Previous reports[5–7] have stated that *N*-desulfation of heparin to the extent of as little as 10 or 12% of the 2-amino-2-deoxy-D-glucose residues is sufficient to abolish anticoagulant activity. However, the isolation of heparin preparations from whale and other mammalian tissues[16] with one-fourth of the 2-amino-2-deoxy-D-glucose residues substituted with *N*-acetyl groups suggested that simple *N*-desulfation to the degree reported could not alone account for the decreased bioactivity. Fractions of heparin by-products, in which 3–5% of the hexosamine residues contained free amino groups, showed little change in anticoagulant activity after *N*-sulfation (Table I).

In contrast, the release of free amino groups by acid hydrolysis appears to have a notable effect on anticoagulant activity. As reported in Table III, a commercial hog-mucosal heparin sample, having 9% of hexosamine units *N*-desulfated (H4-$NH_2$) as determined by the indole reaction[20], showed a loss of over 30% of the activity of the parent compound. *N*-Acetylation of H4-$NH_2$ gave a fraction (H4-NAc) having essentially unaltered activity, although *N*-resulfation of H4-$NH_2$ yielded a product

TABLE III

PROPERTIES OF MODIFIED HEPARIN PREPARATIONS

| *Preparation* | *Hexosamine* (%) | N-*Sulfate groups*[a] | *Total sulfate groups*[a] | *Amino groups* | *Mol. wt.* ($\times 10^{-3}$) | *Anticoagulant activity* (*I.U./mg*) |
|---|---|---|---|---|---|---|
| Original heparin[b] | 22.8 | 0.90 | 2.21 | 0.002 | 11.0 | 145 |
| H12-NAc | 23.7 | 0.55 | 1.85 | 0.006 | 10.5 | 58 |
| H4-$NH_2$ | 21.4 | 0.84 | | 0.090 | | 96 |
| H4-NAc | 21.8 | 0.82 | 1.98 | 0.012 | 11.0 | 83 |
| H4-NS | 21.5 | 0.92 | 1.93 | 0.004 | | 138 |

[a]Given as molar ratio to hexosamine. [b]Commercial hog-mucosal heparin was obtained from Wilson Laboratories.

with activity restored to its original level. These results cannot be correlated with the high anticoagulant activities shown by whale and other mammalian heparins, which have higher proportions of 2-acetamido-2-deoxy-D-glucose residues and will be discussed later. Compound H12-NAc, which contains 45% of the 2-amino-2-deoxy-D-glucose units with *N*-acetyl groups, retained 40% of its original activity, suggesting that *N*-desulfation by chemical means, whether or not this is followed by *N*-acetylation, may influence the biological activity of heparin, though not to the extent previously considered.

The influence of ester sulfate content on the biological activity of heparin is more difficult to assess than that of the *N*-sulfate content because the former cannot be removed under suitably mild conditions that affect only these groups. However, variations in ester sulfate ratios to hexosamine from approximately 0.9 to 1.6 have been found in heparins having reasonably high anticoagulant activities[24]. In contrast, preparations having comparable ester sulfate contents but low molecular weights may show lower activities, as noted in Tables I and II.

*Relationship of molecular weight to anticoagulant activity.* — The molecular weights of the heparin by-products ranged from approximately 4.8 to $12.5 \times 10^3$ daltons for both the beef-lung and hog-mucosal fractions. Beef-lung subfractions from BL, 3 (Table I) having molecular weights down to approximately $7.6 \times 10^3$ showed high anticoagulant activities and progressively lower activities for lower molecular weights. Preparations from BL, 1.5, however, showed activities less than half of those of BL, 3 fractions having similar molecular weights. Differences among the subfractions from the two groups were found primarily in the content of free amino and sulfamido groups, although these differences were within the ranges found commensurate with high biological activity in other heparin preparations.

In contrast to the results obtained for beef-lung fractions, those from hog mucosa (Table II) showed high bioactivities only for the subfractions having the largest molecular size, and fractions having molecular weights below approximately $9 \times 10^3$ daltons exhibited considerably less activity. The relatively high sulfate

contents found for these preparations suggests that other reasons may be responsible for the generally low bioactivities observed. That this was not due to *N*-desulfation is apparent from the lack of significant amounts of free amino groups.

*Relationship of* N-*sulfate group distribution to anticoagulant activity.* — In order to explain the unexpected decrease in anticoagulant activity shown by some of the heparin fractions that have high molecular-weights and sulfate contents (Tables I and II), the distribution of *N*-sulfate groups within the molecule was studied as follows. Treatment with butyl nitrite of representative subfractions having various *N*-sulfate contents and anticoagulant activities, gave products that were fractionated by gel filtration on a column of Sephadex G-25 resin (see Fig. 2). The elution pattern of beef-lung subfraction BL, 3IV (Fig. 2A) is similar to that of a highly purified,

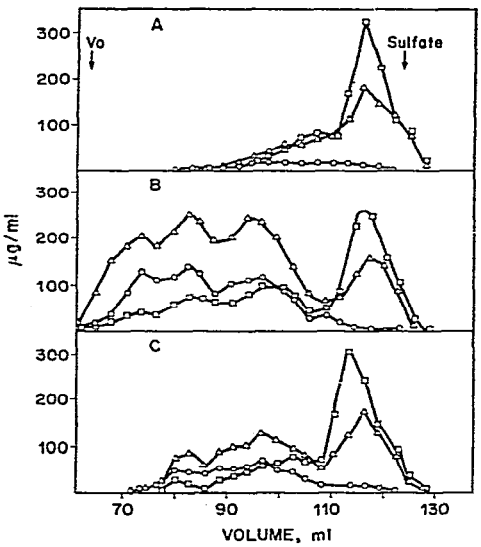


Fig. 2. Gel-filtration patterns on a column of Sephadex G-25 of the cleavage products formed by treatment of the by-product fractions with butyl nitrite: A, fraction BL, 3IV; B, fraction H12-NAc; and C, fraction HM, 1.5–3IV; —△—△—△—, uronic acid; —□—□—□—, 2,5-anhydro-D-mannose; and —O—O—O—, hexosamine.

commercial beef-lung heparin[21], which indicates that the distributions of sulfate groups are similar in both preparations and that the only discernible difference between them is the lower molecular size of the first-named substance. However, treatment of HM, 1.5-3IV (Fig. 2C) with butyl nitrite gave fragments having increased proportions of 2-acetamido-2-deoxy-D-glucose residues in multiple sequence as compared with such residues in commercial hog-mucosal heparin having high biological activity[19]. Furthermore, even larger proportions of these fragments were obtained from H12-NAc (Fig. 2B). The gel-elution volumes of the cleavage products containing 2-acetamido-2-deoxy-D-glucose residues suggest that *N*-desulfation, under the conditions used to produce H12-NAc, yields a compound that possess nearly equivalent

proportions of fragments containing 2-amino-2-deoxy-D-glucose residues in single, double, or triple contiguous repeating units. In contrast to this, commercial heparin preparations have 2-acetamido-2-deoxy-D-glucose residues mainly adjacent to the protein–carbohydrate linkage end[19], which supports the hypothesis that *N*-sulfate groups distribution is a factor in the bioactivity of heparin. Thus, it appears that whereas sequences of single repeating 2-acetamido-2-deoxy-D-glucose residues, separated by adjacent units of 2-sulfamido-2-deoxy-D-glucose residues, do not affect the anticoagulant activity of heparin, as noted by the occurrence of such sections in whale heparin[19], the presence of multiple 2-acetamido-2-deoxy-D-glucose residues in sequence decreases the anticoagulant activity. In agreement with this suggestion, heparin by-product fractions that contained increased proportions of 2-acetamido-2-deoxy-D-glucose residues in sequential repeating-unit arrangement were found to give decreased anticoagulant activities.

*Relationship of* L-*iduronic acid content to anticoagulant activity.* — A previous study[24] of the L-iduronic acid content of heparin preparations obtained from different sources indicated that this content varied from 50 to 90% of the total uronic acid content. Since the anticoagulant activities of preparations having an L-iduronic acid content at the lower end of the range were similar to those having a high content, it is apparent that the effect of variations of the L-iduronic acid content may not be pronounced. However, this assessment does not take into account the possible role, on the biological activity, of sulfated L-iduronic acid residues, or of the distribution along the chain of such residues.

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