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PREPARATION AND CHARACTERIZATION OF TETRASACCHARIDES FROM BEEF-LUNG HEPARIN

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ABSTRACT

Partial *N*-desulfation of beef-lung heparin prior to degradative deamination with butyl nitrite and reduction with sodium borotritide yielded many large fragments. From these, a tetrasaccharide tetra-*O*-sulfate (II-4NH; 8% yield from heparin) and a mixture of tetrasaccharide tri-*O*-sulfates (II-3NHh; 6% yield) were isolated by sequential chromatography on Sephadex G-25 and DEAE-Sephadex. For these and the other tetrasaccharide preparations, the radioactive disaccharides produced by deamination, with and without subsequent relabelling with sodium borotritide, have been quantitatively determined by the methodology described in the preceding paper. In most cases, the results permit a unique reconstruction of the relative proportions of monosaccharide components and of their sequences in the compounds present. Tetrasaccharide II-4NH appeared homogeneous and has the structure (IdoA-SO_4)–(GN-O-SO_4)–(IdoA-SO_4)–(anhMan-SO_4). In tetrasaccharide preparation II-3NHh, the preponderant species (57%) lacks ester sulfate at the terminal L-iduronic residue in the structure just mentioned, and five other species are present. By treatment of the tetra-*O*-sulfate with mild acid, tetrasaccharide preparations with 3, 2, 1, and no ester sulfate were produced and could be isolated. The isomeric tetrasaccharide tri-*O*-sulfate species have been partially resolved. Composition and sequence data are given for all of the preparations. The resolution of numerous small fractions suggests minor irregularities in the fine structure of heparin. Ion-exchange electrophoresis was applied to the acidic oligosaccharides and was found to be a useful technique.

INTRODUCTION

The studies of the preparation, properties, and chemical reactivities of glycosyluronic acid–anhydromannitol disaccharides from heparin that were described in the preceding report[1] are being exploited in a continuing program for isolation and characterization of larger fragments of heparin. Because of close structural similarities between this polymer and heparan sulfate[2–4], such oligosaccharides are considered to be valuable as test substrates for the known hydrolases of heparan sulfate catabolism[5], and for hydrolases yet to be demonstrated. The present report describes the

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preparation, separation, and modification of a family of tetrasaccharides derived from beef-lung heparin, the members of which apparently differ only in degree and location of sulfate ester substitution. The sequence of monosaccharide residues is the same throughout: (α-L-IdoA)→(α-D-GN)→(α-L-IdoA)→(2,5-anhydro-D-Man)*.

As a suitable starting material for preparation of these tetrasaccharides, beef-lung heparin, which is known[6] to have a high L-iduronic acid and ester sulfate, and a small *N*-acetyl content, was selected. The substantial body of literature about the structure of this polymer[7,8] clearly suggested that the most acidic tetrasaccharide species obtainable by partial deaminative degradation would be homogeneous, with no *N*-acetyl, with one *O*-sulfate group on each monosaccharide residue, and with L-iduronic acid as the sole uronic acid. Furthermore, it was anticipated that such a tetrasaccharide might serve as a well-defined starting material for the preparation of additional oligosaccharides of defined structure. In addition to the provision of test substrates for hydrolases, the intention was to furnish reference standards for chromatographic and electrophoretic separations of heparin oligosaccharides. It was expected that these reference standards and the developments in methodology arising from the work undertaken would be valuable in detailed investigations of the ramified structures of heparinoid polysaccharides. Tetrasaccharide preparations derived from heparins have been described before. They include particularly those containing terminal unsaturated uronic acid, resulting from the action of bacterial eliminases[9,10], and those containing internal 2-acetamido-2-deoxy-D-glucose residues[11].

RESULTS AND DISCUSSION

Tetrasaccharides from partial deamination of beef-lung heparin. — To obtain a reproducible, partial-deaminative degradation of heparin, part of the *N*-sulfate groups was first removed by mild acid hydrolysis. Then, use at low temperature and pH of butyl nitrite, a deamination reagent specific for sulfoamino groups[12], avoided deamination of the 2-amino-2-deoxy-D-glucose residues that had been *N*-desulfated. Based on trials with 50mM hydrochloric acid at 80°, an hydrolysis period of 60 min

*The following, simplified abbreviations for monosaccharide residues are used: D-glucuronic acid, GA; L-iduronic acid, IA; L-iduronic acid sulfate, IAS; unspecified uronic acid, UA; 2-amino-2-deoxy-D-glucose, GN; 2-deoxy-2-sulfoamino-D-glucose, GNs; 2-amino-2-deoxy-D-glucose *O*-sulfate, GNS; 2-deoxy-2-sulfoamino-D-glucose *O*-sulfate, GNsS; 2,5-anhydro-D-[1-^{3}H]mannitol, aM; and its *O*-sulfate, aMS. For disaccharides, the abbreviations of the preceding report[1] are used. The tetrasaccharide preparations, which are in most cases complex mixtures of isomeric species, are conveniently classified by specifying the degree of sulfation. Roman II, a class prefix for tetrasaccharides (hexasaccharides are III, octasaccharides, IV, etc.), is followed by an arabic numeral giving the number of sulfate ester groups, then by NH for species with free amino groups, and NS for compounds with *N*-sulfate groups. A lower case letter (and in some cases a prime marking) is used as a suffix to specify an individual preparation, the origin and composition of which are discussed. The suffix "h" indicates preparations isolated directly after deamination of polysaccharides, without subsequent *O*-desulfation ("h" is omitted as unnecessary in II-4NH). For example, a preparation designated II-2NS might contain the tetrasaccharide UA-GNsS-UA-aMS, and any isomeric compound differing only in location of ester sulfate.

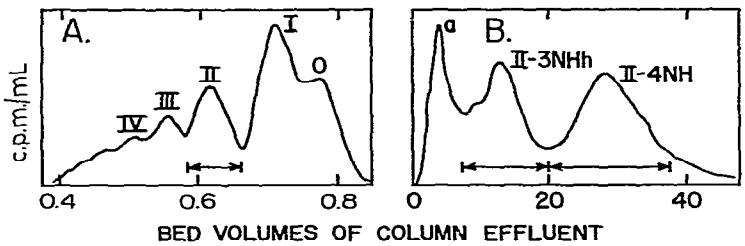

Fig. 1. Column chromatography of oligosaccharides obtained by partial deamination of beef-lung heparin: (A) Sephadex G-25 gel chromatography of the reaction mixture. (B) Chromatography on DEAE-Sephadex at pH 9.4 of the tetrasaccharide fraction II, from A (0.35M total acetate in developer).

was found to remove about half of the *N*-sulfate groups. Heparin so-modified was treated with butyl nitrite. The degradation products were treated with an excess of sodium borotritide of known specific activity, to reduce the newly formed 2,5-anhydro-D-mannose end-groups to radioactive 2,5-anhydro-D-mannitol residues. The mixture of tritium-labelled oligosaccharides was fractionated by gel chromatography, as illustrated in Fig. 1A. Peak 0 was a complex mixture of small fragments[1]. Peak I contained the disaccharides previously described[1]. Peak II, the starting material for the tetrasaccharides of the present report, contained 21% of the total radioactivity and ~20% of the total mass. The remaining, poorly resolved peaks, which obviously contained higher-mol.wt. oligosaccharides, were not examined.

By chromatography on a column of DEAE-cellulose, as shown in Fig. 1B, the peak-II material from the Sephadex column was resolved into four fractions. Peak II-4NH (42% of total radioactivity) was subsequently shown to contain a tetrasaccharide of net charge −6 at pH 9.4 (−5 at pH 6), homogeneous in first approximation. A second peak from the DEAE-cellulose column, peak II-3NHh (30% of total radioactivity), was subsequently shown to contain tetrasaccharides of net charge −5 at pH 9.4 (−4 at pH 6). Peak a (18%) apparently contained less-acidic tetrasaccharides, and any disaccharide included by overlap of peaks in gel chromatography. Larger fragments (10%, not shown) were eluted only with buffer containing M acetate. These two fractions were not studied in detail.

Preparations II-4NH and II-3NH contained (butyl nitrite-resistant) free amino groups, as shown by their mode of preparation. They were re-*N*-sulfated with sulfur trioxide–trimethylamine to give the preparations named II-4NS and II-3NSh, respectively (8% and 6% yield from heparin). In an earlier series, re-*N*-sulfation had preceded chromatography. Except for the expected influence of the *N*-sulfate groups on the chromatographic pattern, the results were quite analogous. The behavior in electrophoresis and column chromatography of the pair of tetrasaccharide preparations obtained through this alternate route (II-4NS′ and II-3NSh′) was indistinguishable from that for the later preparations. Accordingly, no distinctions were made between data obtained with one set of preparations or with the other.

Preparation II-4NS′ contained 5.62 mol of total sulfate and 2.28 mol of uronic acid per mol of 2,5-anhydro-D-mannitol, on the basis of the known specific activity

of its 2,5-anhydro-D-mannitol end-group. For preparation II-3NSh', these analytical ratios were 4.14 mol of total sulfate and 2.39 mol of uronic acid. The ratios for II-4NS' may be compared with those expected (five sulfate groups, and two uronic acid residues) for a tetrasaccharide with four ester sulfate groups and one *N*-sulfate group, and the ratios for II-3NSh' with those (four sulfate groups and two uronic acid residues) for tetrasaccharides with three ester sulfate groups and one *N*-sulfate group. The agreement seems reasonable if the cumulative errors in the specific activity of sodium borotritide, and the radioactivity and colorimetry of the tetrasaccharides are considered.

Direct colorimetric analysis of II-4NH, II-3NHh, or the additional tetrasaccharides to be described was not performed. The composition of these preparations was inferred instead from their direct relationship, by chemical manipulation, to the two preparations described in the preceding paragraph, from the regularity of electrophoretic patterns observed, and from identification of the disaccharide products obtained by degradative deamination. Such radioactivity-based data, particularly for small-scale preparations that had been subjected to repeated chromatography, were considered most reliable because they circumvented any problems arising from progressive contamination of the minute amounts of oligosaccharides with extraneous carbohydrate or other chromogenic impurities from the separation media used.

Degradative deamination of II-4NS'. — The number and location of sulfate ester groups in the tetrasaccharides, and additional information on the chemical structures, were obtained by cleaving each tetrasaccharide into two readily identifiable, disaccharide fragments. This was performed with nitrous acid, as all the tetrasaccharides considered contained a remaining nitrous acid-susceptible 2-amino- or 2-sulfoamino-deoxy-D-glucose residue. (As in the case of heparin itself, such deamination generally left 15–20% of uncleaved tetrasaccharide, which was of necessity disregarded in evaluating the results).

The sole radioactive disaccharide obtained from the treatment of II-4NS' with butyl nitrite was the disaccharide disulfate[1] SIMS, as shown by electrophoresis at pH 5.3. Furthermore, on reduction of the reaction mixture with sodium borotritide to label the 2,5-anhydro-D-mannose end-groups newly formed by deaminative cleavage, this disulfate was still the only radioactive disaccharide present. Consequently, the structure of II-4NS' is IAS→GNsS→IAS→aMS.

Degradative deamination of II-3NSh'. — As expected from its presumed smaller sulfate content, more complex results were obtained with the tetrasaccharide preparation II-3NSh'. The composition data for disaccharides from the butyl nitrite treatment of this preparation, without and with subsequent sodium borotritide reduction, are summarized in Table I. The analyses on which these data are based involved several stages of separation, but permitted direct calculation of the originally labelled ("old") disaccharides in the deamination reaction mixture. The calculated composition of the pool of newly labelled ("new") disaccharides was much more sensitive to cumulative errors, since it resulted from subtraction of a correction factor for the

TABLE I

DISACCHARIDES FORMED BY TREATMENT OF TETRASACCHARIDE PREPARATION II-3NSh′ WITH BUTYL NITRITE[a]

Disaccharide	*Deamination mixture*		*Newly labelled (calc.)*	*All disacch. (calc.)*[b]	*Newly labelled (adjusted)*[c]
	Original	*Relabelled*			
SIMS	85.3	59.6	28	57	15
IMS	8.9	26.6	48	29	57
SIM	1.7	6.7	13	7	15
GMS	4.1	7.1	11	7	13

[a]Except as noted, numerical values represent percent of total radioactivity in disaccharides. The specific activity of the tetrasaccharide was 13.6 Ci/mol, and that of the newly labelled disaccharides was 10.8 Ci/mol. [b]These values are given in mol/100 mol. [c]These values have been adjusted by equating the newly labelled SIMS to the total radioactive monosulfate in the reaction mixture before relabelling, and by equating total newly labelled monosulfate to radioactive SIMS in the mixture before relabelling.

original radioactivity from the new, net radioactivity of each disaccharide present. Obviously, for best accuracy, the second label should have a far higher specific-activity than the first. Calculations were, however, greatly simplified when the two specific activities were identical, and this was preferred. Table I represents an early experiment meeting neither criterion, and is presented as the least accurate example.

Definite limits to the compositions must be met for tetrasaccharide preparations homogeneous as to charge type. For a tetrasaccharide having three sulfate ester groups, half of the total products is expected to be SIMS, and half disaccharide monosulfates. For II-3NSh′, the SIMS content was 57%, and disaccharide monosulfate content 43% of total disaccharides (see Table I). This agreement was considered satisfactory in view of errors in the sequential analyses and indirect calculation. Also, for tetrasaccharides having three sulfate ester groups, "old" SIMS content was expected to equal "new" disaccharide disulfate content, and "old" monosulfates equal "new" SIMS content. The values of 28 and 72%, calculated by difference for "new" SIMS and monosulfates (Table I), corresponded moderately to the more directly measured values of 15 and 85% for "old" monosulfates and SIMS, respectively. To simplify further calculations, total "new" monosulfates component was adjusted to 85% in the last column of Table I (to correspond with "old" SIMS content), and the values for individual monosulfate species were taken in the same proportion as those calculated before adjustment.

Based on these adjusted values, and the consideration that only a single monosulfated disaccharide unit could be accommodated per molecule, a unique composition was determined for II-3NSh′: IA-GNsS-IAS-aMS, 57%; IAS-GNs-IAS-aMS, 15%; GA-GNsS-IAS-aMS, 13%; IAS-GNsS-IA-aMS, 9%; IAS-GNsS-GA-aMS, 4%; and IAS-GNsS-IA-aMS, 2%.

General remarks on structural determination by deamination. — It has been

assumed here that only one *O*-sulfate group could be substituted on any monosaccharide residue. Any major departure from this assumption would be noticed upon deamination of the tetrasaccharide tetra-*O*-sulfate (or heparin itself), as the disaccharide cleavage-products would include either a tri-*O*-sulfate, or a novel disulfate, such as L-Ido*p*A→(2,5-anhydro-D-Man di-*O*-sulfate). The presence of the first compound would be obvious; that of the second has not so far been detected in this work, or in the published work of others. If monosaccharide disulfate residues are accordingly excluded as a major component, deamination of tetrasaccharide tetra-*O*-sulfates would give only disaccharide disulfates, and tetrasaccharide tri-*O*-sulfates would give disulfate and monosulfates in equal proportion, as previously considered. Disaccharide mono- and di-sulfates, and non-sulfated disaccharides may result from tetrasaccharide di-*O*-sulfates, but compounds of the last two-charge types would be produced in equal proportion. Non- and mono-sulfated disaccharides would be produced in equal proportion from tetrasaccharide mono-*O*-sulfates. Nonsulfated tetrasaccharide can give only nonsulfated disaccharide. In summary, the degree of *O*-sulfation of tetrasaccharides may be uniquely determined by their gross deamination-patterns, independent of sizeable technical errors. As seen, the monosaccharide composition and location of sulfate ester groups can also be determined approximately from such data.

Ion-exchange paper electrophoresis. — The technique of high-voltage, anion-exchange paper electrophoresis, which was developed for peptides and oligonucleotides[13], has been adapted for high mol.wt. acidic oligosaccharides. The principal innovation was the introduction of the trivalent, citrate buffer anion to compete

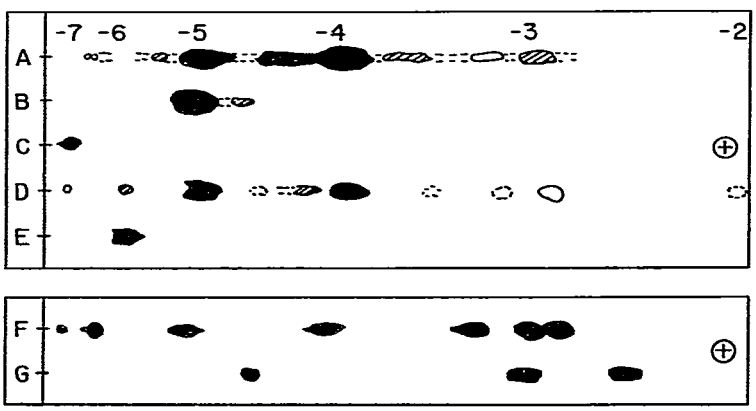

Fig. 2. DEAE-cellulose paper electrophoresis, pH 6.0, 1150 V, 110 min (lanes A–E), and 80 min (lanes F–G). The net ionic-charges at pH 6 of the main compounds in lanes A–E are shown at the top. (A) Crude tetrasaccharide fraction (II, Fig. 1A). (B) II-4NH, purified from tetrasaccharide fraction. (C) II-4NS, obtained by resulfation. (D) Hydrolysis products from action of 50mM hydrochloric acid on II-4NS for 15 min at 100°. (E) Preparation II-3NS. (F) Artificial mixture of purified tetrasaccharides (from cathode end), II-4NS, 3NSh′, 4NH, 3NHc, 2NH, ONH, and 1NH (incompletely resolved). (G) Di- and mono-saccharide markers: (from cathode end) aM, IM and SIMS (not resolved), and aMS and IMS (not resolved).

more effectively with the tight binding of the polyprotic oligosaccharides to the ion-exchange medium. (With monovalent buffers, migration rates of such oligosaccharides are small.) Fig. 2A illustrates the electrophoresis in this medium of II-4NS, II-3NSh, II-4NH, and II-3NHh, oligosaccharides that have, at pH 6, a net charge estimated as —7, —6, —5, and —4, respectively. A regular progression of the tetrasaccharides with decreasing net-charge is the pattern seen, which is extended by the electrophoresis of mixtures produced by mild acid-hydrolysis of II-4NS (Fig. 2D). Since the *N*-sulfate group is the most readily lost, initially the most prominent spots represent II-4NH (charge —5 at pH 6; labile *N*-sulfate group lost) and II-3NH (charge —4; one *O*-sulfate group also lost). Additional spots are visible, however. These were initially presumed to represent compounds having two and one residual sulfate ester group (charge —3, and —2, respectively, at pH 6), a structural assignment that was later confirmed (see below).

Experience with this electrophoretic systems suggests that migration rates depend on a complex balance of several variables. These include, in probable order of importance: ion-exchange equilibria; electrophoresis; electroendosmosis; and, possibly, partition-chromatography effects. Accordingly, although the regularity of the pattern for tetrasaccharides is probably not fortuitous and is, in any case, extremely valuable, it is not surprising to encounter the irregularity seen for a tetrasaccharide having no sulfate ester group and one with a single, negative net-charge (Fig. 2 F), the preparation of which will be described presently. A similar irregularity is seen for disaccharides (Fig. 2G).

Preparation of tetrasaccharides of lesser charge from II-4NS. — As a sequel to hydrolysis studies, some results of which are illustrated for a short reaction-interval in Fig. 2D, a hydrolyzate of II-4NS (50mM hydrochloric acid, 45 min, 100°)

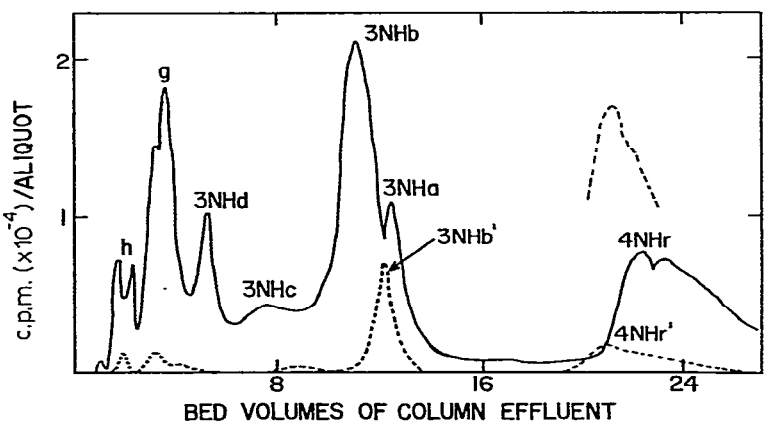

Fig. 3. Separation on a DEAE-Sephadex column (0.35M total acetate in eluent) of the tetrasaccharides obtained by mild acid-hydrolysis of II-4NS. Also shown (lower curve, dashed line, c.p.m. on 1/10 scale) is the chromatography of an hydrolyzate of material recovered from peak 4NHr. A segment of peak 4NHr' is displayed at full-scale. No inferences may be drawn from noncorrespondence of the two curves, which represent columns widely different in size.

TABLE II

TETRASACCHARIDE COMPOUNDS OBTAINED BY MILD HYDROLYSIS OF II-4NS[a]

Monosaccharide sequence	*Proportion (%) in preparation*				
	3NHa	*3NHb*	*3NHc*	*3NHd*	*3NHb'*
IA-GNS-IAS-aMS	88	71	11	16	79
IAS-GN-IAS-aMS	7	23	14	16	13
IAS-GNS-IAS-aM	2	1	11	54	1
IAS-GNS-IA-aMS	3	5	64	14	7

[a]The sequences were determined by deamination, with and without subsequent reduction, giving data corresponding to those of Table I, which are not shown.

was subjected to preparative chromatography, as shown in Fig. 3. Only 4% of the starting material was recovered (eluted with M buffer, not shown). The compounds resulting from *N*-desulfation only (peak II-4NHr) represented 25% of the radioactivity applied to the column. (The pooled material from this peak was subjected to a second hydrolysis, under the conditions used before. Chromatography of the products gave a pattern resembling that of the first hydrolyzate, as shown.)

In the DEAE-cellulose electrophoresis, the earlier peaks II-3NHd through II-3NHa showed migrations similar to, but not identical with, those for II-3NHh, a characterized tetrasaccharide preparation containing only compounds having a charge −4 at pH 6 (three sulfate ester groups). The material in each peak was examined by deaminative degradation into disaccharides, as already described. The composition data are shown in Table II. It is noteworthy that the two clearly distinct peaks, II-3NHa (8% of total radioactivity) and II-3NHb (25%), apparently contained analogous mixtures of compounds, in which IA-GNS-IAS-aMS was the major, and IAS-GN-IAS-aMS the minor component. The small difference between the migration of II-3NHa and that of II-3NHb in anion-exchange electrophoresis corresponds to the difference in chromatographic behavior.

Peak II-3NHc (9%) contained a mixture of compounds in which IAS-GNS-IA-aMS was preponderant and, in peak II-3NHd (6%), IAS-GNS-IAS-aM was preponderant. Thus, although it was not possible to separate the two main compounds in peak II-3NHb, all four sequences expected for the loss of one sulfate ester (and one *N*-sulfate) group from II-4NS were accounted for.

The twin peak g contained a gross mixture of compounds, as judged by ion-exchange electrophoresis. They were resolved partially by rechromatography with an eluent of lower ionic-strength, (see Fig. 4A), giving some 60% of the radioactivity in the earlier, broad peak, still a gross mixture, and some 40% in the later, sharper peak (II-2NH). Material in this peak behaved almost homogeneously in electrophoresis (charge −3, at pH 6). The results of nitrous acid degradation indicated the following composition: IA-GNS-IA-aMS, 72%; IA-GNS-IAS-aM, 12%; IAS-GNS-

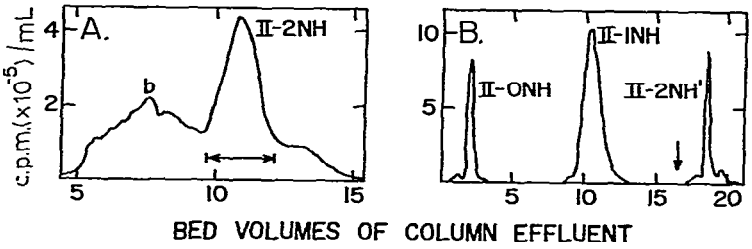

Fig. 4. DEAE-Sephadex column separation of the less-acidic tetrasaccharides. (A) Rechromatography of peak g, Fig. 3 (0.24M total acetate in eluent). (B) Chromatography of the products of action of methanolic hydrogen chloride on II-4NS (0.10M total acetate in eluent, increased to 0.35M at point indicated by the arrow).

IA-aM, 5%; IA-GN-IAS-aMS, 4%; IAS-GN-IA-aMS, 3%; and IAS-GN-IAS-aM, 0%. Reconstruction of the sequence of a tetrasaccharide having two sulfate ester groups was not possible from knowledge of the disaccharides produced on deamination; and it was necessary to assume a random combination of two disaccharide monosulfate units. In the present instance, because of the preponderance of IA-aMS at both ends of the molecules, the calculation of the composition was not greatly influenced by the validity of the assumption.

The twin peak h showed a small amount of radioactivity and its composition was heterogeneous. To obtain non- and mono-sulfated tetrasaccharides, a separate desulfation of II-4NS was performed (0.06M hydrogen chloride in methanol, 28 h, 25°). The resulting mixture was chromatographed on DEAE-Sephadex (see Fig. 4B). The most acidic product separated (II-2NH') had a behavior identical with that of II-2NH, on paper electrophoresis at pH 1.7 and DEAE-paper electrophoresis at pH 6. The composition indicated by the results of the deamination experiments was grossly different, however. Some 60% of IA-GNS-IAS-aM and 35% of IAS-GN-IAS-aM made up most of preparation II-2NH'. IA-GNS-IA-aMS, the major constituent of II-2NH (prepared by mild acid hydrolysis), was a trace component of the methanolysis product II-2NH'. The major product with methanolic hydrogen chloride (Fig. 4B) was II-1NH containing a single sulfate ester group. Deamination showed II-1NH to contain some 83% of IA-GN-IAS-aM and 12% of IAS-GN-IA-aM. The remaining product isolated was II-ONH, a tetrasaccharide containing no ester sulfate. It presumably had the composition IA-GN-IA-aM, as at least 95% of the deamination product with or without relabelling by sodium borotritide was IM.

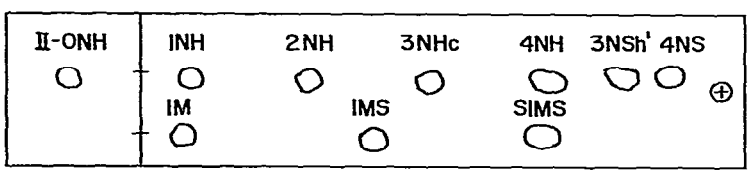

Fig. 5. Paper electrophoresis at pH 1.7 (1100 V, 40 min) of an artificial mixture of purified tetrasaccharide fractions (upper lane). Ionic charges for the tetrasaccharides ranged between +1 and −5 at this pH value. Disaccharide markers have been included for comparison (lower lane).

TABLE III

CALCULATIONS FOR PARTIAL DEAMINATIVE DEGRADATION OF PARTIALLY *N*-DESULFATED HEPARIN

Fraction No.	*Molecular species*	*D.p.m.* ($\times 10^{-9}$)	*End groups*[a] (*μmol*)	*Disaccharide residues*		
				μmol	*Obs. (%)*	*Calc. (%)*
0		8.4	127			
I	Disacch.	15.7	237	237	17	21
II	Tetrasacch.	9.1	139	278	20	28
III	Hexasacch.	5.4	81	243	18	23
IV	Octasacch.	2.7	41	164	12	15
V	Larger	3.3	50			
Total		44.6	675	1370[b]		

[a]Values are based on the specific radioactivity of the sodium borotritide used (119.2 Ci/mol.). [b]Total number of disaccharide residues in the sodium heparinate used (1.01 g), are calculated from a mol. wt. of 614 g per mol of disaccharide residue, and a measured moisture-content of 14.9%.

The correctness of the assignment of charge type to tetrasaccharide preparations was verified, and the utility of these preparations as markers was demonstrated by paper electrophoresis at pH 1.7 (see Fig. 5), which showed regular progression of mobility with number of charges.

Implications for the structure of beef-lung heparin. — Table III shows some estimations from preparative, gel-filtration data (Fig. 1A) for a typical, deaminative degradation of partially *N*-desulfated heparin. It is possible to calculate that some 49% of the 2-amino-2-deoxy-D-glucosyl linkages were cleaved in the deamination, based on the estimated molecular weight of disaccharide residues, weight of polymer treated, total radioactivity of the products, and their known specific radioactivity. From the nominal degree of polymerization of the species in each peak and the radioactivity data, the amount and proportion of each size-class can be estimated. The observed composition and the composition data calculated (by a simple computer model, last column in table) for random cleavage of 49% of the 2-amino-2-deoxy-D-glucosyl linkages in the polymer were compared (Table III). The model takes no account of the presence of some 10% of nonreacting 2-acetamido-2-deoxy-D-glucosyl linkages, of deamination unaccompanied by cleavage, or of secondary reactions leading to formation of the uncharacterized compounds in peak 0. When these limitations are considered, the correspondence between the finding and the random-cleavage model seems satisfactory. This agreement is important because it implies that the fragments are essentially representative of the polymer.

That II-4NS was the most abundant tetrasaccharide isolated (8% yield from the polymer) had been expected from preponderance[1] of the disaccharide di-*O*-sulfate repeating-unit in the beef-lung heparin used. In first approximation, there is no reason to suspect gross heterogeneity of II-4NS preparations, based on the findings already considered. However, the observations made in the experiments on partial

desulfation with acid strongly suggest the presence, in II-4NS preparations, of minor components the fine structure of which may differ from that of the major compounds. For example, peak II-4NHr (Fig. 3) was chromatographically diphasic, a reproducible finding difficult to explain merely on the basis of *N*-desulfation of an homogeneous II-4NS. The chromatographic resolution of two preparations, II-3NHa and II-3NHb (Fig. 3), was previously mentioned. In both of these, IA-GnS-IAS-aMS was the major component, as deduced from deamination experiments, which gave disaccharides indistinguishable by the technique used. A different type of abnormality was noted for II-3NHb′ (Fig. 3) resulting from the treatment of II-4NHr with mineral acid. The disaccharide monosulfates obtained by deamination of this preparation behaved, in ECTEOLA-cellulose chromatography, essentially like SIM and IMS, but the distance between peaks was roughly half of that in the standard pattern[1], so that "SIM", the minor component, appeared as a shoulder on the IMS peak in repeated trials. However, attempts to demonstrate other differences between these compounds and the corresponding standard disaccharide preparations were unsuccessful.

The sequences demonstrated in a preceding paragraph to be present in II-3NSh′ (the tetrasaccharide tri-*O*-sulfate obtained by action of butyl nitrite on heparin) are of some interest since they must have been present in the polymeric heparin. Some 6% of the polymer sequence was isolated in this preparation. Caution, however, is necessary in extrapolating the sequences as representative, since bias in cleavage of sulfate-deficient regions of the polymer is already evident from the composition data. Thus, IA-GNsS-IAS-aMS makes up some 57%, but IAS-GNsS-IA-aMS only 9% of II-3NSh′. Obviously, if cleavage were completely random, the amounts of these components should be equal (as should those of the two glucuronic-containing compounds). Apparently, a 2-amino-2-deoxy-D-glucosyl residue that is linked to a uronic acid sulfate residue in the polymer tends to resist deamination, presumably because it is preferentially *N*-desulfated in the preliminary hydrolytic step. (As already noted, the free amino group is resistant to butyl nitrite.) The basis for this bias in *N*-desulfation, probably an electrostatic effect, is unclear.

EXPERIMENTAL

Materials and methods. — Most of the materials used have been described in the preceding communication[1], including the specimen of beef-lung heparin used throughout. DEAE-Sephadex A25 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ 08854), sulfur trioxide–trimethylamine complex from Aldrich Chemical Co. Inc. (Milwaukee, WI 53233), [^{35}S]sulfur trioxide–trimethylamine complex and [^{35}S]sulfuric acid from Amersham Corp. (Arlington Heights, IL 68005), DEAE-cellulose paper, DE-81, and "spin thimbles", used for extraction of paper spots by centrifugation, from Whatman Inc. (Clifton, NJ 07014). Most of the methods have been described previously[1]. Additional methods are given in the following sections.

Partial N-*desulfation, partial deamination, and* N-*sulfation.* — A small portion

of the heparin preparation was *N*-desulfated and resulfated with [^{35}S]sulfur trioxide–trimethylamine[14]. Portions of the resulting *N*-[$^{35}SO_4$]heparin, as a solution in 50mM hydrochloric acid (10 mg/mL), were heated for various time-intervals at 80°, neutralized with sodium acetate, and examined by paper electrophoresis (50mM sodium acetate buffer, pH 4.7, 150 V, 50 min) and autoradiography. The radioactivities of excised spots for inorganic sulfate and polymer were counted, and the liberation of [^{35}S]sulfate was calculated:

Time (min)	20	40	60	90	120
Sulfate groups liberated (%)	24.8	39.3	47.1	58.3	63.1

On a preparative scale, a solution of nonisotopic heparin (250 mg) in 50mM hydrochloric acid (25 mL) was heated for 60 min at 80°, and then cooled. The material was treated with butyl nitrite and processed as described[12]. The resulting product was at once reduced with an excess of sodium borotritide at pH 8.6, de-ionized, applied to a Sephadex G-25 column (231-mL bed volume), and eluted with sodium acetate buffer[1]. The tetrasaccharide fractions (II, Fig. 1A) were pooled and de-ionized.

In some experiments, the tetrasaccharide fraction was *N*-sulfated before further separations were undertaken. In other experiments, outlined in the Results section, *N*-sulfation was applied to fractions obtained from additional chromatographic procedures. In a typical *N*-sulfation, a solution of a tetrasaccharide fraction (10 μmol) in 83mM aqueous trimethylamine solution (3 mL) was heated for 18 h at 55° in a tightly closed vial with sulfur trioxide–trimethylamine complex (17.4 mg, 125 μmol)[15]. (Model experiments with phenyl 2-amino-2-deoxy-β-D-glucopyranoside have shown that *N*-sulfation was essentially complete under these conditions.) The reaction mixture, after dilution with water, was applied to a DEAE-Sephadex column for fractionation of the products.

DEAE-Sephadex columns. — DEAE-Sephadex A25 was freed of finest particles by settling in water and converted to the acetate form. Columns of this material were prewashed with a buffer of pH 9.2, containing 32mM 2-amino-2-methyl-1-propanol in 20mM acetic acid. After the columns had been loaded, they were eluted with this buffer to which sodium acetate was added at various concentrations, as required. At the pH value specified, the amount of material tolerated for good performance was limited. For example, the resolution of tetrasaccharides illustrated in Fig. 3 was achieved on a column of bed-volume of 113 mL, which was loaded with 0.03 mmol of tetrasaccharides and developed with a buffer containing 0.35M total acetate. (Chromatography of the corresponding re-*N*-sulfated compounds was performed with an elution solvent containing 0.45M total acetate in buffer; other concentrations of eluent are given in context in the Results section.) Fractions collected from DEAE-Sephadex columns were de-ionized as already described.

Diagnostic deamination. — Tetrasaccharides, in the presence or absence of *N*-sulfate substitution, were cleaved with butyl nitrite[12] or buffered nitrous acid[15], respectively. The first procedure is illustrated for II-3NSh′ (specific activity of 13.6

Ci/mol). To a solution of tetrasaccharide (0.5 μmol) in water (250 μL) was added 1,2-dimethoxyethane (200 μL), 1.5M hydrochloric acid (25 μL), and butyl nitrite (25 μL). The mixture was stored for 18 h at −20°, then treated at 0° with water (250 μL) and 0.75M sodium carbonate (25 μL), and evaporated. The residue was treated by several additions and evaporations, *in vacuo*, of methanol, and the aqueous solution de-ionized. Ion-exchange paper electrophoresis showed that some 20% of the radioactivity was present as (uncleaved) tetrasaccharide. The remainder of the reaction mixture was applied to an ECTEOLA-cellulose column of 2-mL bed-volume (elution with 30 mL of 0.02–1.0M ammonium hydrogen carbonate in a linear gradient). The radioactivity of the SIMS and monosulfated disaccharide fractions was counted (nonsulfated disaccharide absent); ~20% of the radioactivity remained on the column.

The disaccharide monosulfate fraction so obtained was de-ionized, lyophilized, dissolved in water (10 μL), applied to a paper chromatogram, and developed with solvent *A*. The GMS spot, and the IMS plus SIM spot revealed by radioautography were excised and counted. The spot for mixed monosulfates was washed with toluene to remove the scintillator agent, dried, and extracted with water. Chromatography on a small ECTEOLA-cellulose column with a shallow elution-gradient resolved IMS from SIM. From the radioactivity data collected, the disaccharide composition of the original reaction mixture was determined.

A second sample of II-3NSh′ was deaminated as just described, de-ionized, and lyophilized. The residue was dissolved in 0.1M potassium borate buffer (pH 8.6), and treated with sodium borotritide (2 μmol). After 1 h at 0°, another portion of sodium borotritide was added. After a further 1 h, the test for excess borohydride was still positive. Excess acetic acid was added. The solution was de-ionized and analyzed as described.

The procedure differed somewhat for the tetrasaccharides derived by hydrolysis from II-4NS. These products lacked an *N*-sulfate group and contained no D-glucuronic acid. Also, because of the minute amounts available, these preparations were particularly prone to contamination by reducing substances contributed by the separation media used. As an example of the modifications used here, a solution of II-3NHa (55 nmol, 1.6 μC) in 35mM potassium borate buffer (150 μL, pH 8.6) was treated for 2 h with nonisotopic sodium borohydride (10 μmol). The mixture was acidified with acetic acid, deionized (final volume 100 μL), and treated[15] with 0.7M sodium nitrite (100 μL) and 5.7M acetic acid (100 μL). After 80 min, nitrous acid was removed by repeated additions and evaporations with methanol and water *in vacuo*, and the volume was adjusted to 225 μL. An aliquot of this solution (100 μL) was chromatographed on a small ECTEOLA-cellulose column, with ammonium hydrogencarbonate used as eluent in a shallow gradient[1]; this chromatography provided a direct measurement of the SIMS, IMS, SIM, and IM content of the reaction mixture. A second aliquot of the deamination-reaction mixture was reduced with two portions (2 μmol, total) of sodium borotritide (positive test for sodium borohydride at end). This reduced product was subjected to ECTEOLA-cellulose chromatography, as described. In later work, the analytical separation of IM, IMS, SIM, and SIMS

was accomplished by t.l.c. electrophoresis[1] at pH 5.3 and 2.9 (two runs were required to account for all species).

Ion-exchange paper electrophoresis. — This was performed on DEAE-cellulose paper in a 18mM sodium citrate buffer (pH 6.0), at an applied potential of ~1100 V, in the horizontal-bed apparatus of Yaron and Sober[13]. The paper rested directly on a separable bed of polypropylene or polyacrylate, which was lowered into position under the layer of solvent naphtha on a plastic rack. The bed width was 20 cm, and the length, 30 cm. DEAE-cellulose paper, precut to the size required, was flooded with buffer in a shallow tray, transferred to a dry, filter-paper surface and blotted, and then transferred to the removable bed. The DEAE-cellulose paper was spotted (5-μL spots) and lowered into the tank on the rack. At conclusion of the electrophoresis, after the wick area had been cut away, the paper and plastic bed were dried in an oven as a unit. Impregnation of the dry paper and autoradiography afforded no difficulty. Use of freshly prepared citrate buffer was found to be essential. Results were poor with old buffer, presumably because of bacterial decomposition.

NOTE ADDED IN PROOF

Studies on the antithrombin-binding region of heparin have recently revealed the presence of a 2-sulfoamino-2-deoxy-D-glucose di-*O*-sulfate residue, apparently separated from a neighboring 2-acetamido-2-deoxy-D-glucose[16] by one uronic acid residue. Such structures would, of necessity, escape observation in our analytical schemes, since they could be present in de-amination mixtures only as *N*-acetylated tetrasaccharides or larger fragments, which would not be cleaved by nitrous acid.

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