Structural Studies of Heparin. I. Hydrolytic Cleavage of Sulfates*

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Molecular weight, titration, and elemental analytical data led to $(C_{12}H_{16}O_{16}NS_2Na_3)_{20}$ for the molecular formula of sodium heparinate. Six to seven equivalents of sulfate per mole of heparin were found, which were not an integral part of the heparin molecule. After conversion of sodium heparinate to heparinic acid, titration data indicated forty ionizable sulfate groups, twenty ionizable carboxyl groups, and one ionizable amino group per mole. These values indicate that there were no diester sulfate linkages in the heparinic acid. Although the possibility of one $-NH-SO_2-O-$ cross linkage per mole of *sodium heparinate* has not been excluded by the present findings, it seems improbable. Unequivocal stoichiometric evidence for the *N*-sulfate group in heparin has been given, showing a one-to-one correspondence between the appearance of free amino groups and released sulfate (corrected for *O*-desulfation) during self-hydrolysis. From an analysis of the kinetics of self-hydrolysis, it was concluded that the reaction is complex and can be described by either first- or second-order plots. In either case, however, an abrupt change in the rate is indicative of differences in the environment of the *N*-sulfate groups.

Although many hydrolytic studies of heparin have been made (Foster and Huggard, 1955; Gibbons and Wolfrom, 1962) a number of difficulties remain. While titration studies by Wilander (1939) indicated no free amino groups in heparin, later investigations (Meyer and Schwartz, 1950; Foster *et al.*, 1953; Gibbons and Wolfrom, 1962) involving reaction of heparin with nitrous acid and 2,4-dinitrofluorobenzene, respectively, showed 6–10% of the amino groups to be unsubstituted. Much qualitative and semiquantitative evidence exists (Jorpes *et al.*, 1950; Wolfrom *et al.*, 1953; Foster *et al.*, 1954; Foster and Huggard, 1955; Gibbons and Wolfrom, 1962) for the *N*-sulfate group in heparin, but a quantitative demonstration has been wanting.

Improvements in isolation methods (Foster and Huggard, 1955; Scott, 1960) have made available to us heparin of significantly higher purity than was available to Wilander (1939), or to Meyer and Schwartz (1950). The advent of improvements in titrimetric equipment and techniques (Marini and Wunsch, 1963; Levy and Petracek, 1962), and more reliable methods for determining the molecular weight of heparin (Barlow *et al.*, 1961), led us to reexamine the problems cited above.

Moreover, recent investigations of various methods for hydrolyzing heparin (Brown *et al.*, 1961; Helbert *et al.*, 1961; Rosenthal *et al.*, 1962) have indicated that one of the salient difficulties in such studies is the quantitative evaluation of simultaneous reactions. The combination of techniques employed in the present study provides an approach to such problems which should be generally useful.

EXPERIMENTAL

Materials.—The heparin was a highly purified specimen prepared by a commercial firm from porcine intestinal mucosa; it was obtained as a sodium salt with an anticoagulant activity of 160 IU/mg (U. S. Pharmacopeia XVI, p. 317). Although the details of preparation are not known to us, we do know that purification was achieved by fractionating a quarternary salt complex. Moreover, acetic acid was not used in the preparation, thus avoiding the degradative effect of this reagent (Wolfrom and McNeely, 1945). This material was dialyzed at 4° for 48 hours against frequent changes of distilled water and then lyophilized. Visking tubing prepared according to Hughes and Klotz (1956) was used for dialysis.

Approximately 1.0 N solutions of carbonate-free KOH were prepared in freshly boiled distilled water, standardized against potassium acid phthalate, and stored in small, paraffin-lined, rubber-stoppered bottles. HCl of the same concentration was standardized against the KOH.

Dowex-50 × 4 (200–400 mesh) was converted from the sodium to the hydrogen form, boiled in several changes of distilled water until color throw-out was absent, and finally washed on a column with distilled water. IRA-400 was converted repeatedly from the chloride to the hydroxyl form and back again with KOH and KCl. This resin was used in the hydroxyl form, after washing on a column with distilled water until free of excess hydroxyl ion.

Ultracentrifugation.—Sedimentation velocity measurements were made with the Spinco Model E analytical ultracentrifuge. Runs were made at 59,780 rpm in 1.0 M aqueous NaCl at room temperature (Barlow *et al.*, 1961). Values of the sedimentation coefficient, $s_{20,w}$, were plotted against concentration, a straight line was fitted to the points by the method of least squares, and $s^\circ_{20,w}$ was obtained by extrapolation to infinite dilution. Deviation from linearity was not observed over the concentration range examined, i.e., 2.0–10.0 mg/ml.

Diffusion.—Measurements were made with the Spinco Model H electrophoresis apparatus at 1° in 1.0 M aqueous NaCl (Barlow *et al.*, 1961). Values of the diffusion constant, $D_{20,w}$ were estimated by the area-height method with a zero-time correction of 200 seconds; $D^\circ_{20,w}$ was obtained in a manner analogous to that described above for $s^\circ_{20,w}$.

Elemental Analysis.—Since thoroughly dried heparin is hygroscopic, while heparin with 10–12% moisture is not, elemental analyses were made on a material containing moisture, and subsequently were converted to a dry-solids basis. Elemental analyses were obtained

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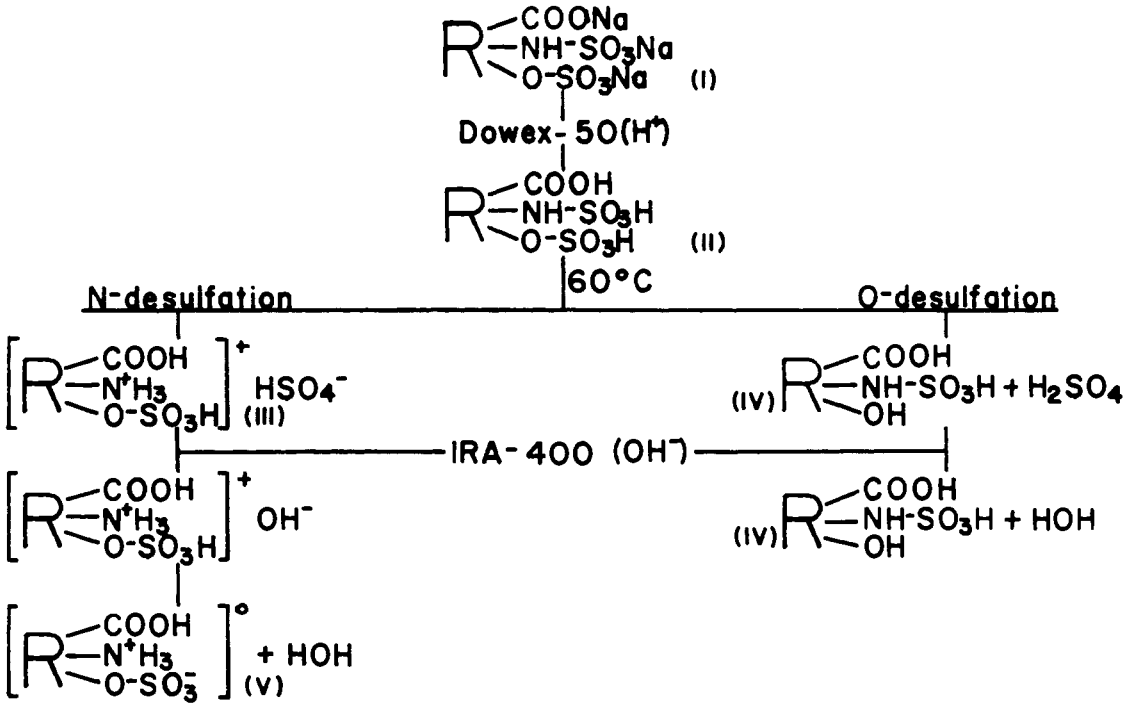

FIG. 1.—Transformations of dimeric repeating unit with treatments used. R represents hexuronic acid glycosidically linked to hexosamine, exclusive of functional groups explicitly shown.

from Micro-Analysis, Inc. (P.O. Box 5088, Wilmington 8, Del). Specimens were transported in gas-tight vials. At the time that samples for elemental analysis were withdrawn, samples were also taken for the determination of dry solids.

Dry solids were determined in an Abderhalden apparatus, using distilled water in the boiling flask, concentrated sulfuric acid in the desiccant flask, and a vacuum of 0.10–0.15 mm Hg. Samples were dried for 24 hours.

Titration.—Radiometer *p*H meter, type PHM 4C, was used to make *p*H measurements using type G-202-B glass electrode (low sodium error) with type K-100 calomel reference electrode.

Misco type 1130 micrometer burets with tips bent 90° were used to deliver titrant below the surface. These burets had glass plungers and a total capacity of about 1 ml, with the smallest division on the micrometer equal to 0.346 μl.

A water-jacketed titration cup was designed to accommodate volumes of 5–10 ml. The contents were stirred with a motor-driven glass agitator, and provision was made to continuously flood the surface of the liquid with nitrogen saturated with water vapor. Water at 20° ± 0.1° was circulated rapidly through the jacket of the titration cup.

Specimens were thermostated at 20° ± 0.1° prior to titration. After 4.00 ml of a specimen was transferred to the clean, dry titration cup, 4 to 5 minutes with rapid stirring were allowed for final temperature equilibration. Titrations were performed by adding successive increments of 1.000 N KOH to effect *p*H changes of 0.2 unit up to *p*H 4.0 and changes of 0.5 unit from *p*H 4.0 to 11.5 or 12.0.

The *p*H meter was standardized at *p*H 2.00 against a calculated amount of 1.000 N HCl in 4.00 ml of 0.15 N KCl at 20.0°. Calculations were based on published values for the activity coefficient of the hydrogen ion under the conditions specified (Harned and Owen, 1958), with correction for liquid junction potential (Bates, 1948). This calibration and other operating conditions were checked by comparing the calculated and empirical amounts of 1.000 N KOH to titrate from *p*H 2.00 to 7.0 to 12.50. Further verification was obtained by reading one or more standard buffers between *p*H 2.00 and 12.50. Readings within the stated tolerance of a standard buffer (usually ± 0.02 *p*H unit) were considered satisfactory. This calibration procedure was carried out before and after each run. If checks were unsatisfactory, the data were rejected. A more detailed account of the standardization and titrimetric procedures has been reported (Marini and Wunsch, 1963).

Hydrolysis of Heparin.—An accurately weighed quantity of the dialyzed and lyophilized sodium heparinate was dissolved in a few ml of distilled water. This solution was slowly percolated through a column of Dowex-50 in the hydrogen form; the quantity of resin used was about five times that estimated to be necessary on the basis of the exchange capacity of the resin and the quantity of exchangeable sodium. The effluent from the column was collected in a volumetric flask of suitable size, so that the concentration of heparinic acid (Foster and Huggard, 1955) was equivalent to about one μmole/ml.

Graded hydrolysis was effected by heating aliquots of the heparinic acid solution at 60° in a constant temperature water bath for various periods of time (Levy and Petracek, 1962). The reaction was stopped by quickly cooling each aliquot in ice-water as it was removed from the 60° bath. When samples were not titrated at once, they were transferred from the ice-water bath to a cold box at 5°–10°.

Some samples were titrated at this point; other samples were passed through a column of IRA-400 in the hydroxyl form (Burson *et al.*, 1956; Levy and Petracek, 1962) to remove hydrolyzed sulfate radicals prior to titration (cf. Figs. 1 and 2).

RESULTS

Molecular Weight.—The value $s^\circ_{20,w}$ was found to be 1.94 S (S = 10^{-13} sec), and $D^\circ_{20,w}$ was found to be 7.90×10^{-7} cm²/sec. The molecular weight of heparin

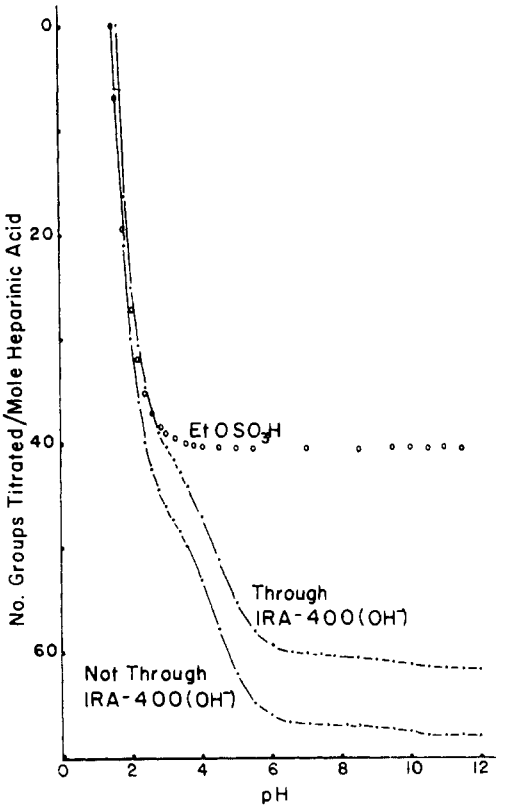

FIG. 2.—Titration curves of heparinic acid prior to hydrolysis, before and after passage through IRA-400 in the hydroxyl form. Titration curve for amount of ethyl sulfuric acid equivalent to sulfate groups estimated to be present in one mole of heparinic acid.

was calculated by the following equation (Ogston, 1956):

$$M = \frac{RT}{(1 - \bar{v}\rho)} \times \frac{s^\circ_{20,w}}{D^\circ_{20,w}}$$

where R is the gas constant (8.314×10^7 erg mole^{-1} degree^{-1}), T is the absolute temperature (293.2°), $\bar{v}$ is the partial specific volume of the heparin (0.47), and ρ is the density of the solvent (1.0403). The molecular weight computed from these data was 11,900 ± 200 (cf. values obtained by Barlow *et al.*, 1961).

Elemental Analysis.—The results of analysis are given in Table I. Although the values in this table have been corrected to a dry-solids basis, the high affinity of dry heparin for moisture makes it reasonable to assume that not all moisture was removed in the determination of dry solids, in spite of the relatively rigorous conditions used. In such case the gram formula weight computed in Table I for a twelve-carbon repeating unit would be high. This consideration and the fact that the molecular weight given above is for the unhydrated molecule (Ogston, 1956) lead to an average degree of polymerization of 20 dimers per molecule.

Although the heparin used in these experiments had been dialyzed against distilled water, titration data (Fig. 2 and Table II, discussed below) indicated the presence of sulfate which was not an integral part of the heparin molecule. Consequently, if the fractional moles of sulfur and sodium (cf. Table I) are considered to arise from Na_2SO_4, the gram formula weight for a twelve-carbon repeating unit of heparin becomes 583. This value differs by one mole of water from the molecular formula $C_{12}H_{16}O_{16}NS_2Na_3$ (fw = 563), derivable from a dimeric repeating unit of hexuronic acid and hexosamine with two sulfate substituents. On this basis, barring disulfate linkages, etc., one would expect heparinic acid to have sixty groups (consisting of twenty carboxyl and forty sulfate groups) titratable on the acid side of *p*H 7.0.

TABLE I
ELEMENTAL ANALYSIS

		Dimeric Repeating Unit	
Carbon	23.35%[a]	12.0 moles	144 g
Hydrogen	3.28	20.0	20
Nitrogen[b]	2.23	1.0	14
Sulfur[c]	11.55	2.23	71.4
Sulfated ash	38.30	—	—
Sodium[d]	12.41	3.33	76.6
Oxygen[e]	47.18	18.19	291
			617 g

[a] All values are on a dry-solids basis (dry-solids = 89.88%). [b] Micro-Kjeldahl method. [c] Schöniger oxygen flask combustion method. [d] Estimated from sulfated ash as Na_2SO_4. [e] Estimated by difference.

Titration.—The titration data of heparinic acid hydrolysates before and after passage through an anion exchange resin are given in Table II. These results are interpretable in terms of Figure 1, which gives a sequence of transformations of the dimeric repeating unit attending the treatments used. According to this scheme, sodium heparinate (I) is converted to heparinic acid (II), which has three groups per dimer titratable below *p*H 7.0 and none titratable above *p*H 7.0. Hydrolysis of (II), catalyzed by its own acidity, results in both *O*-desulfation and *N*-desulfation. *N*-desulfation leads to (III); this form of the dimer involves no net change in the number of groups titratable below *p*H 7.0, but an increase of one group titratable above *p*H 7.0. On the other hand, *O*-desulfation leads to (IV) plus a mole of sulfuric acid, which involves a net increase of one group titratable below *p*H 7.0, but no change in the number of groups titratable above *p*H 7.0. Therefore, a reaction mixture containing (III) and (IV) will exhibit a net increase in groups titratable below *p*H 7.0 due only to *O*-desulfation, and a net increase in groups titratable above *p*H 7.0 due only to *N*-desulfation.

Passage of (III) through IRA-400 in the hydroxyl form yields the zwitter ion (V), which has one group titratable below *p*H 7.0 and one group titratable above *p*H 7.0. While (IV) is unaffected by IRA-400, the sulfuric acid produced during *O*-desulfation is removed. Therefore, a reaction mixture, after treatment with IRA-400 in the hydroxyl form, will contain (IV) and

TABLE II
TITRATION DATA OF HEPARINIC ACID HYDROLYSATES

Time (hr) at 60°	Titratable Groups per Mole			
	Not through IRA-400		Through IRA-400	
	Below *p*H 7.0	Above *p*H 7.0	Below *p*H 7.0	Above *p*H 7.0
0	66.8[a]	1.0	60.2	1.2
1.5	68.6		51.6	5.1
3.0	68.5		46.1	6.9
4.5	69.6		43.6	8.5
6.0	70.5		40.5	9.6
24	71.1		30.8	12.9
48	72.4		28.5	14.0
72	72.5		27.6	14.8

[a] Includes 6.6 titratable groups, initially present in this preparation and readily removed with anion exchange resin (cf. column 4 of this table).

TABLE III
COMPARISON OF OBSERVED RELEASE OF AMINO GROUPS WITH CALCULATED RELEASE OF *N*-SULFATE GROUPS

Time (hr) at 60°	Sulfate Groups Released: *O*-Desulfation (A)	Sulfate Groups Released: Apparent Total Desulfation (B)	Sulfate Groups Released: *N*-Desulfation[a]	Free Amino Groups
0	0	0	0	0
1.5	1.8	8.6	3.4	3.9
3.0	1.7	14.1	6.2	5.7
4.5	2.8	16.6	6.9	7.3
6.0	3.7	19.7	8.0	8.4
24	4.3	29.4	12.5	11.7
48	5.6	31.7	13.0	12.8
72	5.7	32.6	13.5	13.6

[a] Calculated as (B − A)/2. See text.

(V), and will exhibit a net decrement relative to (II) of three groups titratable below *p*H 7.0—one for each *O*-sulfate and two for each *N*-sulfate released. Treatment with IRA-400 will not change the number of groups titratable above *p*H 7.0. These phenomena provide a basis for separately assessing the extent of *N*- and *O*-desulfation, though the two reactions occur simultaneously.

In the second column of Table II, the titration at zero time represents the total amount of heparinic acid (II) (Fig. 1) plus sulfuric acid from sodium sulfate associated with sodium heparinate (I) (Fig. 1). Titrations at subsequent times represent the respective sums of (III), (IV), and sulfuric acid (Fig. 1). In accordance with the discussion above, the slow increment in titratable groups (second column in Table II) is due to *O*-desulfation. These values are shown as cumulative increments in column (A) of Table III. The decrement with time of titratable groups seen in the fourth column of Table II represents (IV) and (V) of Figure 1, and is due to *O*-desulfation, *N*-desulfation, and neutralization during passage through the IRA-400 column. These values are shown as cumulative decrements in column (B) of Table III. The increment in titratable groups, shown in the last column of Table II, is due to *N*-desulfation as measured by the appearance of free amino groups, and is shown in the last column of Table III corrected for the free amino group initially present.

In terms of titration data, *O*-desulfation results in the loss of one sulfate group for each —O—SO_3H cleaved, cf. (IV) in Figure 1; but *N*-desulfation results in the apparent loss of two sulfate groups for each —N—SO_3H cleaved. Consequently, if the values in column (A), Table III, are subtracted from the corresponding values in column (B) and the differences are divided by two, the results will be an estimate of *N*-desulfation based entirely on sulfate measurements. If heparin in fact does contain the *N*-sulfate group, and if this group undergoes hydrolysis, then the above estimate of *N*-desulfation should be in good agreement with that based on the appearance of free amino groups. The last two columns of Table III indicate this to be the case. The results thus provide a quantitative demonstration that the free amino groups appearing during acid hydrolysis of heparin are accompanied by an equivalent loss of sulfate from *N*-sulfate groups. It is noteworthy that this argument depends on stoichiometry alone, and does not depend on rate data as such. As shown below, the rate data for this reaction are complex and difficult to interpret.

The changes in the titration curves with time of hydrolysis are shown in Figure 3. These solutions contain no salt other than the potassium heparinate generated during the titrations.

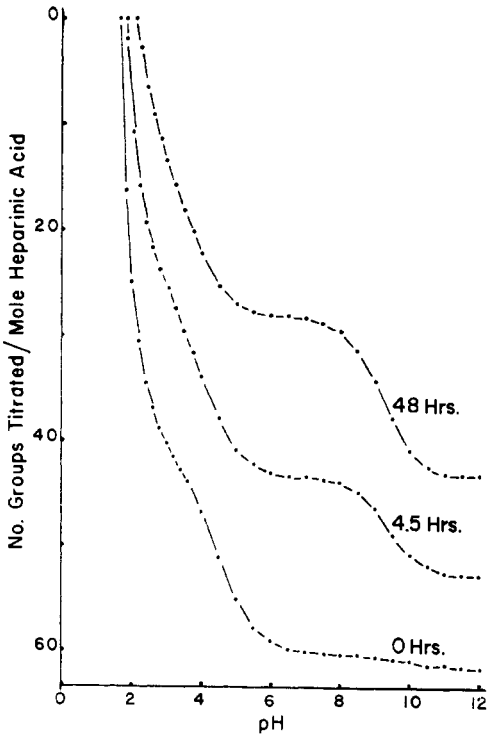

FIG. 3.—Titration curves show the decrease in titratable sulfate groups and the increase in free amino groups as a result of heating heparinic acid at 60° for the indicated periods of time.

DISCUSSION

It is noteworthy that there was no loss of heparin on the anion or cation exchange resins. The fact that sixty groups per mole are titratable in native heparinic acid below *p*H 7.0 is in excellent agreement with the data obtained from elemental analysis and molecular weight determinations. Also, the number of amino groups titratable before hydrolysis is the same before and after passage through IRA-400 in the hydroxyl form (cf. zero time, columns 3 and 5, Table II).

Prior to hydrolysis each mole of heparinic acid contained 6.6 equivalents of anion which were removed by the anion exchange resin (Fig. 2; zero time, columns 2 and 4, Table II). Furthermore, when an aqueous solution of sodium heparinate was percolated through IRA-400 (hydroxyl form), the effluent had an alkaline reaction (F. J. Petracek, personal communication) as a consequence of the sodium hydroxide formed. A quantitative determination, after correction for titration blank and free amino groups, indicated 6.1 exchangeable equivalents of anion per mole of heparin. These findings, together with the evidence that heparin is not retained on the ion exchange resins, corrobrate the view (already advanced on the basis of the elemental analytical data) that these exchangeable anions come from sodium sulfate strongly associated with, but not an integral part of, the heparin molecule. Thus the molecular formula, $C_{12}H_{16}O_{16}NS_2Na_3$, for the dimeric repeating unit, is in good agreement with all the experimental evidence available on this preparation.

The finding of 5% unsubstituted amino groups prior to hydrolysis is at variance with the earlier titration data of Wilander (1939), but agrees with the findings of Meyer and Schwartz (1950) and Gibbons and

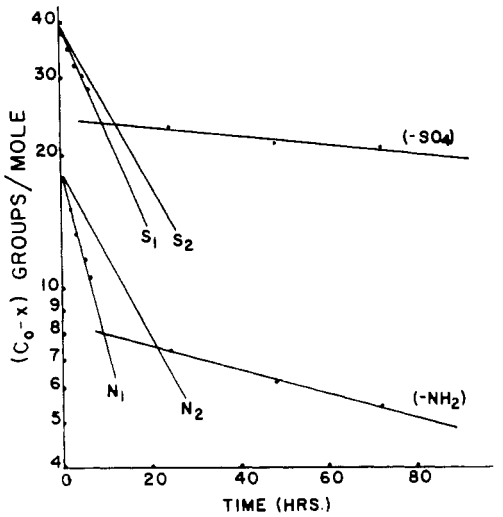

FIG. 4.—Semilog (first-order) plot of total sulfate groups (—SO_4) and of *N*-sulfate groups as indicated by appearance of free amino groups (—NH_2). Only linear components of the curves which fit the experimental points are shown. S_2 and N_2 represent S_1 and N_1 corrected for the respective slower rates extrapolated to zero time.

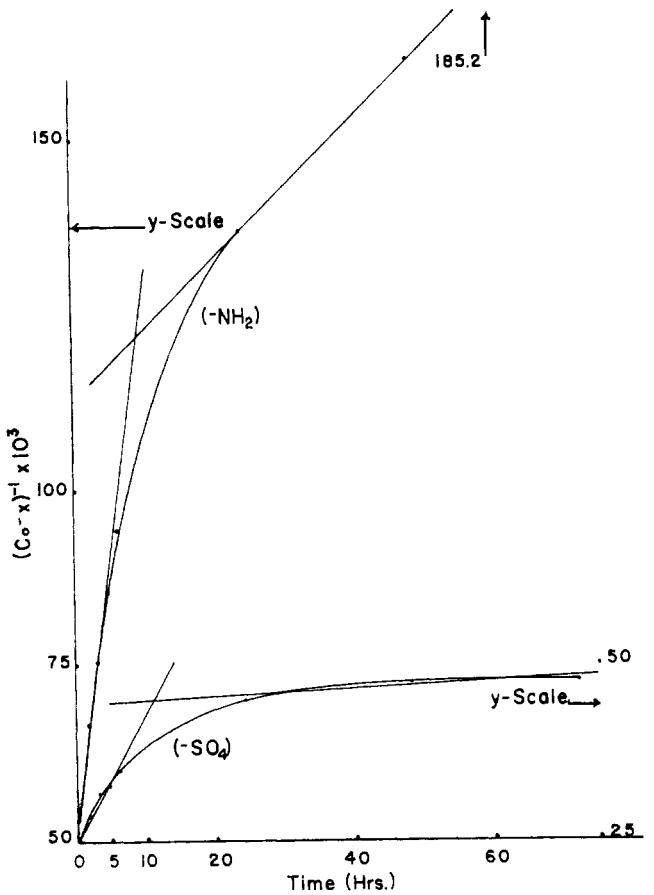

Fig. 5.—Second-order plot of total sulfate groups (—SO_4) and of free amino groups (—NH_2). Line passes through the off-scale point. Initial quantities, C_0, assumed equal.

Wolfrom (1962). The significance of this observation in terms of structure or biological function is not known to us at this time.

A first-order plot of the loss of all sulfate groups and of the loss of *N*-sulfate groups (based on the appearance of free amino groups) is shown in Figure 4. In each case the experimental points lie on a curve which can be resolved into two linear components (Koshland *et al.*, 1958). The initial velocities, N_1 and S_1, are not equal; but, after adjustment for the corresponding slower velocities, the corrected initial velocities, N_2 and S_2, are equal. These results may be interpreted as evidence for two types of *N*-sulfate groups in heparin. The change in rate is abrupt after about one-half the *N*-sulfate groups have been released. The residual *N*-sulfate groups hydrolyze at a slower rate, which may reflect a difference in local charge or a steric effect. This suggestion must be viewed with caution, however, since the rate of self-hydrolysis may be complex. Kinetic analysis (Pessen, 1961; Mullet and Noddings, 1962) indicated, with some ambiguity, that the reaction was second order. It is apparent from Figure 5 that the experimental data may also be described by a second-order plot; however, this plot also shows a rate change when about one-half the *N*-sulfate groups have been hydrolyzed. In spite of the uncertainty about the order of the reaction, it seems reasonable to assume that the abrupt rate change was due to steric or electrostatic factors, since there was no evidence for any untitratable sulfate groups.

The analytical and titrimetric data indicated that all the acidic groups of heparin were titratable prior to hydrolysis. This finding makes less tenable the view that heparin contains disulfate linkages such as —NH—SO_2—O—, —NH—SO_2—NH—, and —O—SO_2—O—. This possibility has been suggested by several investigators (Masamune *et al.*, 1940; Foster and Huggard, 1955). Such linkages could exist in the present case only if they were so labile as to be hydrolyzed during passage through Dowex-50 (hydrogen form) at room temperature.

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Mode of Action of a Cellulase Component from *Cellvibrio gilvus**

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The mode of action of one of four electrophoretically distinguishable β (1 → 4) glucan hydrolases from *Cellvibrio gilvus* has been investigated. Analysis of the intermediates and products during hydrolysis of cellulose-oligosaccharides and chemically reduced cellulose-oligosaccharides indicated preferential attack at the second and third glucosyl bonds from the nonreducing end of the polysaccharide chain. Kinetic studies of the release of isotope from C^{14}-cellulose-oligosaccharides labeled exclusively in the nonreducing terminal glucosyl moiety confirmed this conclusion. No glycotransferase activity could be detected, and α-cellobiose was established as the primary initial reaction product during hydrolysis of reduced cellotetraose.

Kinetic data describing simultaneous changes in the degree of polymerization (D.P.) of carboxymethylcellulose and the production of soluble reducing sugars demonstrated conspicuous differences between the mode of action of purified β-1,4-glucan hydrolases from *Cellvibrio gilvus* and the random action of either phosphoric acid or a random-cleaving hydrolase from *Myrothecium verrucaria* (Storvick and King, 1960). An indication that attack by the enzymes from *C. gilvus* might remove successive cellobiosyl moieties was obtained by qualitative examination of reaction products on paper chromatograms.

Since then greatly improved procedures for isolation of gram-lots of the lower members of the cellulose-oligosaccharide series in high purity and for quantitative microanalysis of mixtures of the cellulose-oligosaccharides have been described. With these procedures the mode of action of one of the β-1,4-glucan hydrolases of *C. gilvus* has been examined in greater detail with end-labeled substrates prepared by chemical reduction of the reducing glucose group and by addition of the C^{14}-glucose moiety to the nonreducing end of β-1,4-glucans of known structure.

With these substrates it has been possible to determine the relative rates of attack on the lower members of the cellulose-oligosaccharide series, the susceptibility of each glucosyl bond to enzymic attack, and the configuration of the reducing group as it is released from the enzyme after hydrolysis.

Methods and Results

Isolation and Characterization of Cellulose Oligosaccharides.—Cellotriose, cellotetraose, cellopentaose, and cellohexaose were isolated from fuming HCl hydrolysates of cellulose by the procedures of Miller *et al.* (1960). Heavily loaded preparative scale columns yielded sugars contaminated with the next lower member of the polymer series. The odd and even numbered members of the polymer series were therefore pooled and subjected to a second column chromatographic separation, yielding cellotriose, cellotetraose, cellopentaose, and cellohexaose samples which showed no mutual contamination when tested on paper chromatograms and which gave within 1.5% of the theoretical D.P. values when analyzed as described by Timell (1960). Complete acid hydrolysis of 10 μmoles of the pentasaccharide yielded 50.9 μmoles of glucose as determined by notatin (Saifer and Gerstenfeld, 1958). Infrared spectra, however, revealed heavy contamination with stearic acid, which was a component of the column packing. After repeated reprecipitation from water with ethanol the infrared spectra were indistinguishable from those of Higgins *et al.* (1961) for authentic compounds, and there was no indication of either carboxyl or aliphatic groups. The specific optical rotations were within 1–5% of those reported by Wolfrom and Dacons (1952) with the exception of the tetrasaccharide, which deviated by 9%. Since the solubilities decrease so rapidly as D.P. increases, deviations of this magnitude are to be anticipated, a problem to which Wolfrom and Dacons also refer.

In the solvent system ethylacetate-pyridine-water (5:2:5 v/v/v) the isolated sugars through cellopentaose migrated so that a plot of log $1/(1 - R_F)$ against D.P. was linear. The R_F of cellohexaose was too low to permit estimation.

Preparation of Reduced Substrates.—Cellotetraose, cellopentaose, and cellohexaose were converted to the corresponding nonreducing sugars by reduction with $NaBH_4$. Approximately 1 μmole of borohydride and 1 μmole of sugar in 10 ml of water were heated at 96° for 30 minutes. Then an additional 0.25 μmole of borohydride was added and heating was continued for another 30 minutes. After the mixture had cooled to

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