424. *Amino-sugars and Related Compounds. Part IX.* Periodate Oxidation of Heparin and Some Related Substances.†*

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Methanolysis of heparin reduces the sulphate content from 5·2 to 1·2 groups per tetrasaccharide unit. Periodate cleaves *ca.* 44% of the uronic acid units in heparin, and more than 90% in the *N*-acetylated, desulphated derivative. Acidic hydrolysis of the reduced oxopolysaccharides gives, *inter alia*, a tetronic acid. Deaminative degradation of de-*N*-sulphated heparin gives a mixture of sulphated derivatives of D-glucopyranosyluronic acid-2,5-anhydro-D-mannose in which all the uronic acid is attacked by periodate. These facts accord with a fundamental structure for heparin involving 1→4-glucosaminidic linkages and with *ca.* 50% of the uronic acid units sulphated at position 2 and the remainder sulphate-free. Evidence is presented that the uronic acid has predominantly, if not exclusively, the *gluco*-configuration. A convenient method for sulphate determination in sulphated polysaccharides is described.

Although heparin, the dextrorotatory mucopolysaccharide present in mammalian circulatory tissue, has been extensively investigated [1] since its discovery in 1916, several features of the structure still remain to be established unequivocally. We now report

* Part VIII, *J.*, 1961, 1204.

† For a preliminary report see *Biochem. J.*, 1961, **80**, 12P.

[1] For a review see Foster and Huggard, *Adv. Carbohydrate Chem.*, 1955, **10**, 335.

the periodate oxidation of heparin and related compounds, and discuss the compatibility of the heparin structure tentatively postulated by Wolfrom and his co-workers [2] with these and other results.

Three successive treatments of ox-lung heparin with methanolic hydrogen chloride, in the conditions described by Kantor and Schubert [3] for chondroitin sulphate, reduced its sulphur content from 13·8% to 5·1%. A further small decrease to 4·9% resulted on treatment of the desulphated heparin with 0·04N-hydrochloric acid under conditions [4] known to hydrolyse all of the *N*- and some of the *O*-sulphate groups in heparin. A product with a similar sulphur content was obtained when a fourth methanolysis replaced the acid treatment. Adding acetic anhydride to a solution of the acid-treated, desulphated heparin at pH 10 (cf. Roseman and Ludowieg [5]) gave the *N*-acetyl derivative (*A*) with a sulphur content (4·3%) corresponding to *ca.* 1·2 sulphate groups per tetrasaccharide repeating unit (the parent heparin contained *ca.* 5·2 such groups) and infrared absorption bands at 1630 and 1550 cm.$^{-1}$ characteristic of the acetamide group. Absorption at 1738 cm.$^{-1}$ is attributed to methoxycarbonyl groups, although un-ionised carboxyl groups may also contribute since it is unlikely that all the ester groups would survive the hydrolysis and acetylation. The absence of absorption characteristic of carboxyl ions is not surprising since heparin itself is obtained as the acid salt from acetic acid solution.[6]

Various workers [7-9] have reported that the product obtained by methanolysis of heparin, under conditions which completely desulphate chondroitin sulphate, has a 1 : 1 S : N ratio. By extending the period of methanolysis, Helbert, Wang, and Brown [8] removed one of the two remaining sulphate groups per tetrasaccharide unit to give a product which was apparently similar to ours. When the material with 1 : 1 S : N ratio was subjected [7,9] to further methanolysis after selective *N*-acetylation, the product also had an S : N ratio of 0·5 : 1, but repetition of this stepwise procedure with more concentrated acid and fractionation of the product mixture gave [9] a heparin derivative with a sulphur content of only 0·2%.

The *N*-acetyl derivative (*A*) rapidly (*ca.* 3 hr.) consumed 2 mol. of periodate per tetrasaccharide unit and was thereafter slowly over-oxidised. The oxopolysaccharide isolated after consumption of *ca.* 2·4 mol. of periodate was reduced with sodium borohydride to give a polyalcohol having a uronic anhydride content (*ca.* 3·7%; cf. heparin, 28·7%) which indicated that more than 90% of the uronic acid units had been cleaved during oxidation. Acidic hydrolysis of the polyalcohol, followed by paper electrophoresis, showed that destruction of the uronic acid had been accompanied by formation of a tetronic acid (presumably L-erythronic acid arising by cleavage at positions 2 and 3), thereby indicating a hexosamine–uronic acid 1→4-linkage. Other components in the hydrolysate were 2-amino-2-deoxy-D-glucose, a trace of uronic acid, and neutral material, probably glycollaldehyde derived from C-1 and C-2 of the uronic acid units. After removal of mineral acid, successive treatments of the hydrolysate with a strongly acid resin did not completely remove the amino-sugar. Attempted lactonisation of the acidic components in the remaining mixture with a view to isolating erythronolactone resulted in appreciable decomposition.

Heparin was oxidised initially more slowly by periodate than was the *N*-acetyl derivative (*A*), but subsequent overoxidation progressed more rapidly to give a consumption of 6 mol. of oxidant per tetrasaccharide unit during 371 hr.; oxidation was not complete at

[2] Wolfrom, Montgomery, Karabinos, and Rathgeb, *J. Amer. Chem. Soc.*, 1950, **72**, 5796.
[3] Kantor and Schubert, *J. Amer. Chem. Soc.*, 1957, **79**, 152.
[4] Foster, Martlew, Stacey, Taylor, and Webber, *J.*, 1961, 1204.
[5] Roseman and Ludowieg, *J. Amer. Chem. Soc.*, 1954, **76**, 301.
[6] Wolfrom, Weisblat, Karabinos, McNeely, and McLean, *J. Amer. Chem. Soc.*, 1943, **65**, 2077.
[7] Danishefsky, Eiber, and Carr, *Arch. Biochem. Biophys.*, 1960, **90**, 114.
[8] Helbert, Wang, and Brown, Amer. Chem. Soc. Meeting, Chicago, September 1961, Abs. Papers, p. 8D.
[9] Wolfrom, Vercellotti, and Thomas, *J. Org. Chem.*, 1961, **26**, 2160.

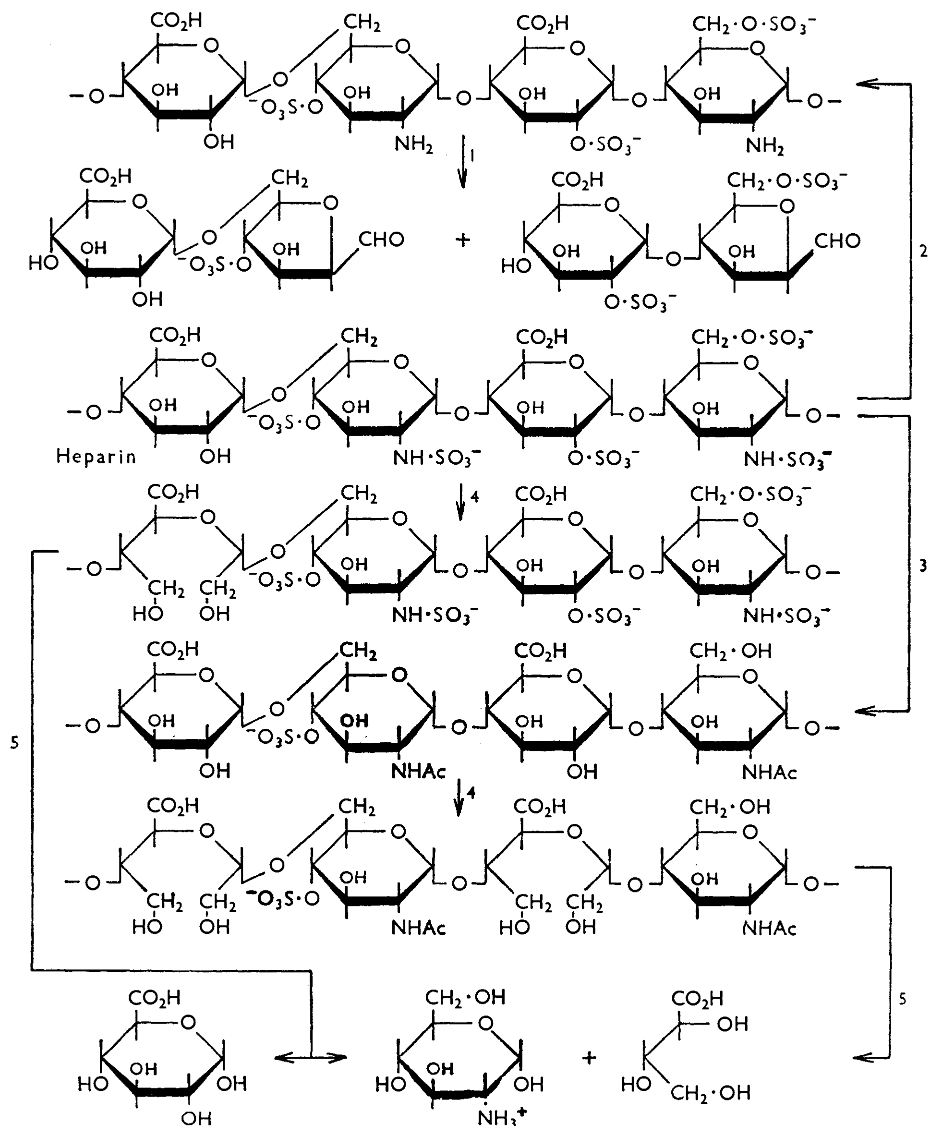
this stage. The progressively diminishing recovery of oxidised polysaccharide (isolated by dialysis) indicated fragmentation during the overoxidation. For example, after consumption of 3·5 mol. of oxidant (121 hr.) the recovery of non-dialysable product was only 71%, whereas the original heparin was essentially non-dialysable. After reduction with sodium borohydride of aldehyde groups in the periodate-oxidised product, decarboxylation showed that the uronic acid content had fallen by about 44% after 97 hr.; thereafter the uronic acid content of the non-dialysable products remained essentially constant. The amino-sugar content of the products, as determined colorimetrically [10] after acid hydrolysis, was not significantly changed as oxidation proceeded.

This oxidation pattern indicates that the primary attack on heparin involves half of the uronic acid residues (arbitrarily expressed as one uronic acid residue per tetrasaccharide repeating unit), and that the overoxidation probably involves further attack on some of the cleaved residues, either before or after hydrolysis of the oxidised heparin; in either case there is slow fragmentation of the polysaccharide. Supporting evidence for this view was provided by the evolution of 0·13 mol. of carbon dioxide during the consumption of 1·8 mol. of oxidant by the polysaccharide. Moreover, sodium (methyl 4-*O*-methyl-α-D-glucosid)-uronate, a model compound similar to the oxidisable uronic acid unit in heparin, consumed 1 mol. of periodate in *ca.* 10 hr. and was then slowly overoxidised. By contrast, methyl (methyl 4-*O*-methyl-α-D-glucosid)uronate did not undergo overoxidation after its initial consumption of 1 mol. of oxidant. The presence of esterified carboxyl groups in the *N*-acetyl derivative (*A*) may therefore be a contributory factor to its slower overoxidation, as compared with that of heparin. As with the *N*-acetyl derivative (*A*), acidic hydrolysis of the reduced oxidation products of heparin gave a tetronic acid (chromatographic detection), thereby confirming the hexosamine–uronic acid 1→4-linkage. Other products detected chromatographically were 2-amino-2-deoxy-D-glucose, unoxidised uronic acid, and (probably) a disaccharide. In the products of longer oxidation, smaller acid fragments, which presumably resulted from the overoxidation of cleaved uronic acid units, were also detected.

Since periodate cleaves both uronic acid residues in the tetrasaccharide unit of the *N*-acetylated, desulphated heparin but only one uronic acid residue of heparin, and since the uronic acid units are linked through position 4, the location of a sulphate ester grouping at positions 2 or 3 of the unoxidised uronic acid residue of heparin is indicated. (The reactions involved are included in the annexed scheme.) Evidence for the precise location of this sulphate group is as follows. Treatment of heparin with 0·04N-sulphuric acid at 95—100° for 2 hr. yielded a de-*N*-sulphated heparin [4] still containing 3 sulphate groups per tetrasaccharide unit. This material was specifically degraded [11] by deamination with nitrous acid, and the products were conveniently isolated as the insoluble cetyltrimethylammonium salts. Cetyltrimethylammonium salts of simple sugar sulphates are known [12] to be insoluble, whereas the salts of simple uronic acids are soluble. Elemental analyses of the cetyltrimethylammonium salts supported their formulation as an equimolar mixture of the mono- and di-sulphate esters of D-glucopyranosyluronic acid-2,5-anhydro-D-mannose. These are the expected products from de *N*-sulphated heparin containing alternate sulphate-free and monosulphated uronic acid units, and undergoing deaminative degradation (see reaction scheme) in a manner analogous to that established for the methyl 2-amino-2-deoxy-D-glucopyranosides.[11] Since cetyltrimethylammonium ions interfered in the iodine titration, periodate oxidation of the sodium salts was examined. Although the consumption of oxidant did not reach a steady value, allowance for overoxidation (graphical extrapolation) gave satisfactory agreement with the 1·5 mol. per disaccharide unit calculated for the equimolar mixture. Moreover, examination of the oxidised products by the

[10] Belcher, Nutten, and Sambrook, *Analyst*, 1954, **79**, 201.
[11] Foster, Martlew, and Stacey, *Chem. and Ind.*, **1953**, 825.
[12] Bera, Foster, and Stacey, *J.*, 1955, 3788.

carbazole test [13] showed that all the uronic acid had been destroyed. Since the sulphate group in the sulphated uronic acid unit has already been located at positions 2 or 3, destruction of all the glycosidically bound uronic acid means that position 3 is not sulphated.



Reactions: 1, Deamination with nitrous acid. 2, De-*N*-sulphation by mild acidic hydrolysis. 3, Methanolysis, then *N*-acetylation. 4, Oxidation by IO_4^-, then reduction by $NaBH_4$. 5, Acid hydrolysis.

* In the formula for heparin, arbitrary allocation of configuration and position has been made for the glucuronidic linkages.

Hence approximately half the uronic acid units in heparin must be sulphated at position 2.

The structural features of heparin indicated by the preceding results accord with the relevant parts of the structure tentatively postulated by Wolfrom and his co-workers.[2]

[13] Dische, *J. Biol. Chem.*, 1947, **167**, 189.

They conflict, however, with the brief report [14] of the behaviour of a sodium heparinate [6] in which only one uronic acid residue (as well as one hexosamine residue) was destroyed per hexadecasaccharide unit during oxidation with periodate at 5°. During the deaminative degradation of the de-*N*-sulphated heparin, $[\alpha]_D$, initially +52°, fell to about zero for the disaccharide products. This rotational change is consistent with cleavage of α-glucosaminidic linkages, but it is difficult to reconcile the low final rotation with the proposed [2] exclusive α-configuration for the glucuronidic linkages; the deamination procedure would not be expected to affect the glucuronidic linkages. Nevertheless, the strongly dextrorotatory character of the glucopyranosyl-2-amino-2-deoxy-D-glucose (and of its derivatives) isolated [15] in moderate yield after acidic hydrolysis of a reduced heparin derivative indicates that some, although not necessarily all, of the glucuronidic linkages do have the α-configuration.

Unequivocal evidence that the principal uronic acid component of heparin is D-glucuronic acid has recently been provided,[9,16] despite counter-claims [17] based mainly on colorimetric tests with anthrone. Further, qualitative evidence that the uronic acid in heparin has predominantly, if not exclusively, the *gluco*-configuration has now been obtained. Thus, reduction with lithium aluminium hydride of the cetyltrimethylammonium salts described above, and acidic hydrolysis of the product (presumably a disaccharide), gave a single hexose with paper-chromatographic and electrophoretic properties indistinguishable from those of D-glucose and significantly different from those of D-gulose. The only other detectable product had zero electrophoretic mobility in borate buffer, in agreement with its expected structure as 2,5-anhydro-D-mannitol. The isolation of D-glucaric acid after oxidative hydrolysis [18] of heparin had shown that the precursor uronic acid had the D-*gluco*- or L-*gulo*-configuration. Attempted isolation of the acidic disaccharide, after methanolysis to remove the sulphate groups, was unsuccessful since the glycosidic linkage was also cleaved. After reduction of the methanolysis product with lithium aluminium hydride and removal of glycosidic and acetal [19] methyl groups by acidic hydrolysis, paper chromatography again revealed glucose as the only reducing product.

As a result of recent findings, the position of the glucuronidic linkage in the heparin structure proposed by Wolfrom and his co-workers [2] must be reallocated. Thus both periodate oxidation and methylation studies [20] of a de-*N*-sulphated heparin showed that the hexosamine unit was unsubstituted at position 3, thereby ruling out the proposed 1→3-linkage. Moreover, colorimetric tests indicated that the crystalline *O*-D-glucopyranosyl-2-amino-2-deoxy-D-glucose [15] obtained by acidic hydrolysis of carboxyl-reduced heparin was 1→4-linked and an *O*-(D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucose [21] isolated after degradation of *N*-acetylated, de-*N*-sulphated heparin was apparently 1→6-linked. However, although the periodate-oxidation data for heparosinsulphuric acid [2] permit the presence of a 1→6-glucuronidic linkage in heparin, such a linkage is not compatible with the behaviour [2] of Wolfrom and Montgomery's [22] *N*-acetylated, desulphated heparin on oxidation. It must therefore be recognised that heparin may contain both 1→4- and 1→6-glucuronidic linkages. These features have been included arbitrarily in the formulæ of our reaction scheme.

[14] Chatterjee, Durant, Hendrickson, Lee, and Montgomery, *Biochem. Biophys. Res. Comm.*, 1961, **4**, 425.
[15] Wolfrom, Vercellotti, and Horton, *J. Org. Chem.*, 1962, **27**, 705.
[16] Foster, Olavesen, Stacey, and Webber, *Chem. and Ind.*, 1961, 143; Cifonelli and Dorfman, *Biochem. Biophys. Res. Comm.*, 1961, **4**, 328; 1962, **7**, 41; Danishefsky, Eiber, and Langholtz, *J. Biol. Chem.*, 1962, **237**, 1413.
[17] Helbert and Brown, Amer. Chem. Soc. Meeting, St. Louis, March 1961, Abs. Papers, p. 13D; Brown, Rosenthal, and Helbert, *ibid.*, Chicago, September 1961, p. 8D.
[18] Wolfrom and Rice, *J. Amer. Chem. Soc.*, 1946, **68**, 532.
[19] Grant, *New Zealand J. Sci. Technol.*, 1956, **37**, 509.
[20] Velluz, Nominé, and Mathieu, *Bull. Soc. Chim. biol.*, 1959, **41**, 415; Nominé, Bucourt, and Bertin, *Bull. Soc. chim. France*, 1961, 561.
[21] Danishefsky, Eiber, and Langholtz, *Biochem. Biophys. Res. Comm.*, 1960, **3**, 571.
[22] Wolfrom and Montgomery, *J. Amer. Chem. Soc.*, 1950, **72**, 2859.

During this work a convenient method for determination of the sulphur content of sulphated polysaccharides was developed. Although applied only to heparin and certain of its derivatives the method is of potential, general application. A previous method [23] which involved release of sulphate ions under oxidative conditions of hydrolysis with nitric acid, followed by determination of barium sulphate with ethylenediaminetetra-acetic acid gave results of $\pm 1\%$ accuracy with *ca.* 50 mg. samples. Our method requires digestion of only *ca.* 5 mg. samples with nitric acid and hydrogen peroxide (nitric acid alone did not always prevent the formation of carbonaceous material), followed by determination of sulphate with 4'-chlorobiphenyl-4-ylamine and Jones and Letham's spectrophotometric method.[24] Because of the hygroscopicity of anhydrous heparin it was more convenient to allow samples to equilibrate with the atmosphere and to determine the moisture content, than to work with anhydrous material. Heparin samples equilibrated with the atmosphere may contain 15—20% of water, the amount varying with the humidity.

EXPERIMENTAL

The sodium heparinate was an ox-lung preparation after being dried at 111°/12 mm. for 1 hr. over phosphoric oxide, it had $[\alpha]_D$ +48° (*c* 1·0 in H_2O) and anticoagulant activity 124·5 I.U. per mg. [Found: S, 13·8. Calc. for $(C_{24}H_{31}N_2Na_7O_{35}S_5)_n$: S, 13·0%].

Periodate oxidations were performed at 25° and the consumption of oxidant was determined essentially by the standard arsenite method.[25] Paper electrophoresis was performed on Whatman No. 3 paper by the enclosed strip technique [26] with a borate (pH 10) [27] or acetate (pH 5) buffer. Paper chromatography was performed on Whatman No. 1 paper by downward irrigation with the organic phase of butanol–ethanol–water (4 : 1 : 5); detection was by alkaline silver nitrate,[28] aniline hydrogen phthalate,[29] and ninhydrin, in the appropriate cases.

Sulphate Analysis of Heparin and Related Compounds.—The polysaccharides were first equilibrated with respect to moisture by exposure to the atmosphere for *ca.* 48 hr. The water content (*ca.* 15%) was then determined by drying a portion of the equilibrated polysaccharide over phosphoric oxide at 111°/12 mm. The following method was evolved in an extensive series of experiments.

A sample of the equilibrated polysaccharide (2—6 mg. depending on the expected sulphate content) was treated with 8N-nitric acid (1 ml.) and 100-volume hydrogen peroxide (1 drop) in a digestion flask (5 ml.) fitted with a water-condenser through a B10 ground-glass joint. This solution was boiled under reflux on an oil-bath for 1 hr.; smooth boiling was essential in order to eliminate losses by splashing and was achieved by slowly passing air through the solution by means of a fine capillary inserted through the condenser. After the system had cooled, the condenser was carefully washed with a small volume of water, the washings being added to the digestion flask. The nitric acid was then removed by evaporation through a vertical, lagged air-condenser (*ca.* 7 cm.), the residue was dissolved in concentrated hydrochloric acid (B.D.H. micro-analytical; *ca.* 0·5 ml.), and the solution was again concentrated. This process was repeated twice more with hydrochloric acid, and finally with water. An aqueous solution of the residue was quantitatively transferred to a volumetric flask and the volume made up to 5 ml. The sulphate content of this solution (60—200 µg./ml.) was then determined by Jones and Letham's method.[24]

Losses which might arise from the transfer of small volumes were largely eliminated by using one flask, with interchangeable condensers, for the digestion and subsequent evaporations. The procedure was evaluated by using sodium sulphate decahydrate, for which 9·8, 9·8, 10·0, and 9·8% of sulphur were found (Calc., 9·9%).

N-*Acetyl Derivative* (*A*) *of Partially Desulphated Heparin.*—Dry sodium heparinate (3 g.) was shaken for 24 hr. with anhydrous 0·06M-methanolic hydrogen chloride [prepared [3] from

[23] Belcher and Fildes, unpublished work cited in ref. 12.
[24] Jones and Letham, *Analyst*, 1956, **81**, 15.
[25] Jackson, *Org. Reactions*, 1944, **2**, 341.
[26] Foster, *Chem. and Ind.*, 1952, 1050.
[27] Foster, Newton-Hearn, and Stacey, *J.*, 1956, 30.
[28] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[29] Partridge, *Nature*, 1949, **164**, 443.

freshly distilled acetyl chloride (3 ml.) and dry methanol (600 ml.)]. Insoluble material was collected by centrifugation, and the desulphation repeated twice with fresh methanolic hydrogen chloride. After each desulphation, the solid material from a sample of the homogeneous suspension was collected by centrifugation, washed with methanol, and dissolved in water. An excess of sodium chloride was added to the solution which was then dialysed overnight against running water. The dialysate was freeze-dried and the product analysed for moisture and sulphate content. The sulphur content (13·8%) (all determinations were in duplicate) was reduced to 9·5%, 5·2%, and 5·1% by the three successive treatments. A portion of the partially desulphated heparin was treated for a fourth time with methanolic hydrogen chloride, and the product recovered as for the analytical samples (Found: S, 4·8%). A 2% solution of the remaining desulphated heparin in 0·04N-hydrochloric acid was kept at 95—100° for 2 hr., then cooled, neutralised with sodium hydrogen carbonate, dialysed against running water for several days, and freeze-dried (Found: S, 4·9%).

An aqueous solution of the acid-treated, desulphated heparin was cooled to 0°, adjusted to pH 10 by addition of 0·1N-sodium hydroxide, and immediately treated with acetic anhydride (2 mol.). After storage at 0° for 2 hr., the solution was dialysed against running water and freeze-dried to yield the *N*-acetyl derivative (*A*) of partially desulphated heparin, having $[\alpha]_D$ +77° (*c* 0·9 in H_2O) after drying [Found: S, 4·3. Calc. for $(C_{30}H_{45}N_2NaO_{25}S)_n$: S, 3·8. Calc. for $(C_{30}H_{44}N_2Na_2O_{28}S_2)_n$: S, 6·5%]. The infrared spectrum (KCl disc) of this product showed $\nu_{max.}$ at, *inter alia*, 1738 (ester C=O), 1630 and 1550 cm.$^{-1}$ (*N*-acetyl).

Periodate Oxidation of the N-*Acetyl Derivative* (*A*).—(*a*) The consumption (mol. per tetrasaccharide unit of oxidant by a solution of the derivative (*A*) (100·6 mg.) in 0·025M-sodium metaperiodate (50 ml.) was as follows:

Time (hr.)	0·25	0·75	1·5	3·25	5·0	7·75	24	48	96	144
Consumption	0·92	1·27	1·63	2·20	2·20	2·50	2·64	2·85	3·21	3·38

(*b*) A solution of the derivative (*A*) (228 mg.) in water (90 ml.) was treated with a solution of 0·25M-sodium metaperiodate (10 ml.) for 6·5 hr. at room temperature. After the excess of periodate had been destroyed by ethylene glycol (0·16 ml.), the solution was dialysed against running water for 24 hr. and freeze-dried. An aqueous solution of the residue (187·5 mg.; strongly reducing to Benedict's reagent) was then treated with a large excess of sodium borohydride for 24 hr. at room temperature. After the excess of reductant had been destroyed with acetic acid, the solution was dialysed against running water for 24 hr. and freeze-dried, to yield the polyalcohol product (*B*) which did not reduce Benedict's reagent [Found: uronic anhydride (by decarboxylation [30]), 3·7%].

Acidic Hydrolysis of Product (*B*).—(*a*) A 1% solution of product (*B*) in 2N-sulphuric acid was kept at 100° for 3 hr. and then neutralised with barium hydroxide. Paper electrophoresis (acetate buffer) of the hydrolysate, with detection by silver nitrate, revealed a spot of zero mobility, as well as three components having, severally, the same mobilities as sodium D-erythronate, sodium D-glucuronate (trace), and 2-amino-2-deoxy-D-glucose hydrochloride.

(*b*) In a similar hydrolysis of product *B* (500 mg.), the mineral acid was removed by using a (5% v/v) chloroform solution of methyldi-n-octylamine.[31] After passage down a column of Amberlite IR-120 resin (H^+ form; 200 ml.), the hydrolysate was re-treated with methyldi-n-octylamine and Amberlite IR-120. Paper electrophoresis revealed the same acidic and neutral components as in (*a*), and a small amount of residual 2-amino-2-deoxy-D-glucose. On freeze-drying, the neutralised solution yielded a dark oil (100 mg.); heating at 80° under reduced pressure to promote lactonisation of acidic components yielded a black tar.

Periodate Oxidation of Heparin.—(*a*) The consumption (mol. per tetrasaccharide unit) of oxidant by a solution of heparin (2·34 g.) in 0·025M-sodium metaperiodate (500 ml.) is shown below. At suitable intervals, aliquot portions (50—75 ml.) of the reaction solution were treated with an excess of ethylene glycol, dialysed, and freeze-dried, to yield samples of oxopolysaccharide.

Time (hr.)	19	41	97	121	155	179	203	227	275	299	323	347	371
Consumption	0·68	2·13	3·30	3·51	4·11	4·34	4·56	4·95	5·22	5·45	5·66	5·91	6·04
Recovery (%)	—	83	74	71	—	65	—	65	—	—	—	—	59

[30] Barker, Foster, Siddiqui, and Stacey, *Talanta*, 1958, **1**, 216.
[31] Smith and Page, *J. Soc. Chem. Ind.*, 1948, **67**, 48.

The oxidised products were treated with sodium borohydride, as described previously, with recovery (average yield *ca.* 80%) of reduced material by dialysis and freeze-drying. The uronic acid content of the reduced samples was determined by decarboxylation with 19% hydrochloric acid, under conditions [30] where D-erythronolactone did not undergo decarboxylation and heparin gave a 99% yield of carbon dioxide.

Oxidation time (hr.)	97	121	179	227
Uronic acid (% based on heparin)	55·8	56·8	57·3	57·4

After hydrolysis with N-sulphuric acid at 100° for 3 hr., all the reduced samples gave mixtures for which paper electrophoresis in acetate buffer showed a similar content of 2-amino-2-deoxy-D-glucose, D-glucuronic acid, and tetronic acid, as well as a ninhydrin-positive spot of zero mobility. The products of longer oxidation also yielded smaller acidic fragments.

(*b*) In a separate oxidation of heparin (0·47 g.), the carbon dioxide evolved during consumption of 1·8 mol. of oxidant per tetrasaccharide unit was swept by a stream of nitrogen into standard sodium hydroxide. Titration with standard acid revealed the release of 2·2 mg. of carbon dioxide (0·13 mol. per tetrasaccharide unit).

Periodate Oxidation of Model Compounds.—A solution of methyl (methyl 4-*O*-methyl-α-D-glucosid)uronate (23·3 mg.) in water (10 ml.) was treated with 0·1N-sodium hydroxide (1 ml.) followed by 0·25M-sodium metaperiodate (5 ml.), and the volume rapidly adjusted to 50 ml. The consumption (mol.) of oxidant was as follows:

Time (hr.)	0·20	0·67	1·93	4·0	10	27	48	72
Consumption	0·32	0·53	0·76	0·87	0·95	1·05	1·21	1·39

A similar oxidation of the methyl ester without addition of sodium hydroxide gave the following results:

Time (hr.)	0·34	0·83	2·0	4·0	6·0	8·5	23	47	97
Consumption	0·41	0·71	0·80	0·89	0·92	0·94	0·96	0·96	0·98

*De-*N*-sulphated Heparin.*—A solution of heparin (10 g.) in 0·04N-sulphuric acid (500 ml.) was kept at 95—100° for 2 hr., then cooled, neutralised with sodium hydrogen carbonate, and dialysed against running water for several days. The dialysate was freeze-dried to yield the sodium salt of de-*N*-sulphated heparin (amine sulphate) (9 g.) having $[\alpha]_D$ +52° (*c* 4·5 in H_2O) after drying (Found: S, 12·1%). A solution containing a portion of this product (0·56 g.) and sodium chloride (11 g.) in water (50 ml.) was dialysed against running water for several days, and freeze-dried, to yield the hydrochloride of the sodium salt of de-*N*-sulphated heparin (0·42 g.) [Found: S, 8·9. Calc. for $(C_{24}H_{35}Cl_2N_2Na_5O_{29}S_3)_n$: S, 8·8%].

*Deamination of De-*N*-sulphated Heparin.*—A solution of sodium nitrite (8 g.) in a mixture of water (200 ml.) and N-hydrochloric acid (64 ml.) was added to a solution of de-*N*-sulphated heparin (8 g.) in the minimum volume of water. After storage at 20° for 48 hr., the mixture was neutralised (pH 7) with N-sodium hydroxide, and treated dropwise with 2% aqueous Cetavlon (w/v) until precipitation of the white solid was complete. The precipitate was collected by centrifugation, washed with water, and freeze-dried in aqueous suspension to yield the disaccharide cetyltrimethylammonium salts (*C*) (9·5 g.) having $[\alpha]_D$ *ca.* 0° (*c* 2·5 in MeOH) [Found: C, 62·0; H, 10·4; N, 3·6; S, 3·8; uronic anhydride (by decarboxylation [30]), 15·0. Calc. for $C_{50}H_{100}N_2O_{14}S$: C, 61·0; H, 10·2; N, 2·8; S, 3·25; uronic anhydride, 17·9%. Calc. for $C_{69}H_{141}N_3O_{17}S_2$: C, 61·5; H, 10·5; N, 3·1; S, 4·75; uronic anhydride, 13·1%. Calc. for an equimolar mixture: C, 61·3; H, 10·3; N, 3·0; S, 4·1; uronic anhydride, 15·1%].

A solution of the cetyltrimethylammonium salts (*C*) (0·5 g.) in 60% aqueous methanol (1 l.) was passed down a column of Amberlite IR-120 (Na^+ form; 60 ml.) which was then washed with 60% aqueous methanol (500 ml.) and water (200 ml.). Concentration of the combined eluates gave the disaccharide sodium salts (*D*) (0·12 g.).

Periodate Oxidation of the Sodium Salts (*D*).—(*a*) The consumption (mol.) of oxidant by a solution of the sodium salts (*D*) (53 mg.) in 0·01M-sodium metaperiodate (50 ml.) was as follows:

Time (hr.)	0·32	0·75	3·0	8·8	23	73	95
Consumption	0·23	0·28	0·47	0·77	0·99	1·69	1·77

(*b*) After storage for 3 days, a solution of the sodium salts (*D*) (67 mg.) in 0·01M-sodium metaperiodate (50 ml.) was treated with ethylene glycol (0·1 ml.), followed by aqueous silver nitrate (85 mg. in 5 ml.). Precipitated silver salts were removed and the solution was freed

from cations and mineral acid by treatment with Amberlite IR-120 (H^+ form; 12 ml.) followed by a chloroform solution of methyldi-n-octylamine.[31] The final solution did not give a positive response in the carbazole test for uronic acids.[13] In a control oxidation of methyl α-D-glucopyranoside by this procedure, D-glucurone was readily detected in that portion to which it had been added after the addition of ethylene glycol.

Examination of the Cetyltrimethylammonium Salts (*C*).—(*a*) A solution of the cetyltrimethylammonium salts (*C*) (1 g.) in the minimum volume of tetrahydrofuran was added dropwise to a stirred suspension of lithium aluminium hydride (0·2 g.) in tetrahydrofuran (30 ml.). The mixture was heated under reflux for 36 hr. and the excess of reductant in the cooled solution was then decomposed with water. Precipitated material was collected and extracted with water, and the combined filtrates and extracts were freeze-dried. A solution of the residue in saturated aqueous sodium chloride (10 ml.) was introduced on to a charcoal–Celite column (250 ml.) and eluted with water (1 l.) followed by 30% aqueous ethanol (1 l.). The ethanolic eluate was concentrated (10 ml.), treated with 2N-sulphuric acid (2 ml.), and kept at 100° for 3 hr. Paper-electrophoresis of the neutralised (methyldi-n-octylamine) hydrolysate (80 mg.) in borate buffer revealed (silver nitrate) a spot of zero mobility and a component having the same mobility as D-glucose (D-gulose had M_G 0·83); paper chromatography similarly revealed D-glucose (D-gulose had R_G 1·25).

(*b*) A solution of the cetyltrimethylammonium salts (*C*) (1·3 g.) in 2% methanolic hydrogen chloride (40 ml.) was heated under reflux for 6 hr. and then evaporated to dryness at 20°/12 mm. Methanol (3 × 10 ml.) was evaporated from the residue which was then freed from last traces of acid by storage over potassium hydroxide *in vacuo*. A solution of this residue in tetrahydrofuran was added during 1 hr. to a stirred suspension of lithium aluminium hydride (9·5 g.) in tetrahydrofuran. The product obtained by heating this mixture under reflux for 2 hr. and then decomposing the excess of reductant as in (*a*), was deionised with Amberlite resins IR-120 (H^+ form; 70 ml.) and IR-4B (^-OH form; 150 ml.), and freeze-dried to yield product (*E*) (80 mg.). The IR-4B resin was then eluted with 10% aqueous sodium chloride (1 l.) and the eluate placed on a charcoal–Celite (1 : 1) column (300 ml.). After elution with water (3 l.), elution with 10% aqueous ethanol (2 l.) yielded product (*F*) (100 mg.). Paper electrophoretic and chromatographic analyses showed that acidic hydrolysis of products (*E*) and (*F*) gave the same components as in (*a*).

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