236. *Amino-sugars and Related Compounds. Part VIII.* Some Properties of 2-Deoxy-2-sulphoamino-D-glucose, Heparin, and Related Substances.*

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A synthesis of the crystalline hydrated ammonium salt of 2-deoxy-2-sulphoamino-D-glucose (D-glucosamine *N*-sulphate) is described. The acid lability of its *N*-sulphate residue is compared with that of the similar group in heparin. The infrared spectra of heparin and a series of sulphated polysaccharides have been compared, but it has not been possible to detect an absorption characteristic of the *N*-sulphate group.

Recognition [1] of the presence of *N*-sulphated amino-sugar groups in heparin, the polysaccharide blood-anticoagulant present in mammalian circulatory tissue, has prompted attempts to synthesise model compounds. Meyer and Schwartz [2] obtained a mixture of *O*- and *N*-sulphated derivatives on treatment of 2-amino-2-deoxy-D-glucose [3] with a sulphur dioxide–trioxide mixture. Wolfrom, Gibbons, and Huggard,[4] using suitably blocked derivatives of 2-amino-2-deoxy-D-glucose with sulphur trioxide in pyridine, later prepared, *inter alia*, the *N*-sulphates of 2-amino-2-deoxy-D-glucose and methyl 2-amino-2-deoxy-α-D-glucopyranoside and isolated them as their hydrated sodium salts, of which that of the latter was crystalline. Only a brief description of some of the properties of these salts has appeared.[5]

We now report the crystalline hydrated ammonium salt of 2-deoxy-2-sulphoamino-D-glucose.

Treatment [6] of aqueous 2-amino-2-deoxy-D-glucose hydrochloride at pH 9—10 with the pyridine–sulphur trioxide complex,[7] although effecting *N*-sulphation, gave a mixture of products that were difficult to purify. When 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucose [8] was treated with the pyridine–sulphur trioxide complex in a modification of Wolfrom, Gibbons, and Huggard's method,[5] *N*-sulphation occurred together with some de-*O*-acetylation and *O* ⟶ *N*-acetyl migration; this migration could not be prevented completely. The marked tendency of certain *O*-acetylated 2-amino-2-deoxy-D-glucose derivatives to undergo base-catalysed *O* ⟶ *N*-acetyl migration is well established.[9] De-*O*-acetylation of the crude 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-sulphoamino-β-D-glucose gave a mixture containing basic, neutral, and acidic components of which the last was 2-deoxy-2-sulphoamino-D-glucose subsequently isolated as the crystalline, hydrated ammonium salt. The salt appeared homogeneous on paper ionophoresis. 2-Deoxy-2-sulphoamino-D-glucose was obtained as an amorphous, hydrated sodium salt by Wolfrom *et al.*[4] from a crystalline, tetra-*O*-acetate precursor.

2-Deoxy-2-sulphoamino-D-glucose did not respond to the Morgan–Elson test [10] for

* Part VII, *J.*, 1960, 2587.

[1] For a review of heparin chemistry see Foster and Huggard, *Adv. Carbohydrate Chem.*, 1955, **10**, 335.
[2] Meyer and Schwartz, *Helv. Chim. Acta*, 1950, **33**, 1651; see also Wolfrom, Shen, and Summers, *J. Amer. Chem. Soc.*, 1953, **75**, 1519.
[3] Breuer, *Ber.*, 1898, **31**, 2193; Westphal and Holzmann, *ibid.*, 1942, **75**, 1274.
[4] Wolfrom, Gibbons, and Huggard, *J. Amer. Chem. Soc.*, 1957, **79**, 5043.
[5] Wolfrom, Gibbons, Huggard, and Neely, Abs. Papers, Amer. Chem. Soc. Meeting, Atlantic City, September, 1956, p. 21D.
[6] Warner and Coleman, *J. Org. Chem.*, 1958, **23**, 1133.
[7] Sisler and Audrieth, *Inorg. Synth.*, Vol. II, p. 173.
[8] Bergmann and Zervas, *Ber.*, 1931, **64**, 975; 1932, **65**, 1201.
[9] White, *J.*, 1938, 1498; Fodor and Ötvös, *Acta Chim. Acad. Sci. Hung.*, 1954, **5**, 205; *Chem. Ber.*, 1956, **89**, 701; van de Kamp and Micheel, *ibid.*, 1957, **90**, 2054.
[10] Morgan and Elson, *Biochem. J.*, 1934, **28**, 988; Aminoff, Morgan, and Watkins, *ibid.*, 1952, **51**, 379.

reducing *N*-acetylated amino-sugars but gave a colour with $\lambda_{max.}$ 530 mμ (2-amino-2-deoxy-D-glucose gives a colour with $\lambda_{max.}$ 511 mμ) in the Elson–Morgan test [11] for reducing amino-sugars. The Elson–Morgan test was therefore deprived of convenience as a method for following the hydrolysis of the *N*-sulphate group. However, since 2-deoxy-2-sulphoamino-D-glucose gave a soluble salt with 4-amino-4′-chlorobiphenyl [12] and its presence did not interfere with the precipitation of sulphate by the reagent, it was possible to follow the hydrolysis of the *N*-sulphate group by using this base and Jones and Letham's spectrophotometric method.[13]

A 1% solution of ammonium 2-deoxy-2-sulphoamino-D-glucose in 0·1N-hydrochloric acid at 100° gave a hydrolysis constant k 6·24 × 10^{-2} min.$^{-1}$ for cleavage of the *N*-sulphate group. Wolfrom *et al.*[5] give k 5·6 × 10^{-2} min.$^{-1}$ for hydrolysis of the amorphous sodium salt carried out under similar conditions but followed by a different method.[14] Hydrolysis of a 1% solution of 2-deoxy-2-sulphoamino-D-glucose in 0·04N-hydrochloric acid at 100° gave k 1·84 × 10^{-2} min.$^{-1}$. The hydrolysis was essentially complete in 150 min. Various workers [15,16] have shown that, on treatment of heparin under similar conditions, hydrolysis of the *N*-sulphate group is essentially complete in 100—120 min. Thus, the acid-sensitivity of the polysaccharide *N*-sulphate moiety is not markedly influenced by its environment. Wolfrom and McNeely [16] found that a 2% solution of heparin (sodium or acid barium salt) in 11% acetic acid at 68° lost its anticoagulant activity in *ca.* 50 hr.; this treatment caused *ca.* 50% hydrolysis of the *N*-sulphate groups in the polysaccharide. After 50 hr. ammonium 2-deoxy-2-sulphoamino-D-glucose as a 1% solution in 11% acetic acid at 68° had undergone 31% hydrolysis.

Although hydrolysis of the *N*-sulphate group in heparin may be followed by 2,4-dinitrophenylation of the liberated amino-groups,[15] accurate determination of sulphate ion

Infrared spectral data ($\nu_{max.}$ *in* 797—855 *cm.*$^{-1}$ *region*) *for certain sulphated carbohydrates.*

Sulphated carbohydrate	S (%)	$\nu_{max.}$ [a] (cm.$^{-1}$)	Sulphated carbohydrate	S (%)	$\nu_{max.}$ [a] (cm.$^{-1}$)
Dextran sulphate	7·7	820	Chitin sulphate	11·45	810
Dextran sulphate	12·6	805	Chitosan sulphate	15·1	797
Dextran sulphate	18·6	810vb	Nigeran sulphate	14·7	805
1,6-Anhydro-β-D-glucose sulphate	16·1	812	Laminarin sulphate	14·9	803
Amylose sulphate	14·5	815	Chondroitin sulphate *A* [b]		852
Amylopectin sulphate	8·9	807	Chondroitin sulphate *B* [b]		855, 842
Glycogen sulphate	14·0	807	Chondroitin sulphate *C*		820 [c]
Cellulose sulphate	17·0	805b	Heparin	13·2	812
			De-*N*-sulphated heparin	8·05	817

[a] The spectra were determined for KCl or KBr discs. [b] Samples kindly provided by Prof. A. Dorfman. [c] Values taken from ref. 20.

in the early stages of hydrolysis is complicated by the presence of polysaccharide.[17] However, if acidic polysaccharide material in the hydrolysate is precipitated as the cetyltrimethylammonium salt,[18] the remaining sulphate ion may be determined by using 4-amino-4′-chlorobiphenyl. In this way a smooth release of sulphate ion was observed during the hydrolysis of heparin; a full account of these results is reserved for a future publication.

Treatment of heparin with 0·04N-hydrochloric acid at 95—100° for 2 hr. resulted in hydrolysis of all the *N*- and some of the *O*-sulphate groups,[15] to yield de-*N*-sulphated

[11] Elson and Morgan, *Biochem. J.*, 1933, **27**, 1824; see also Belcher, Nutten, and Sambrook, *Analyst*, 1954, **79**, 201; Rondle and Morgan, *Biochem. J.*, 1955, **61**, 586.
[12] Belcher, Nutten, and Stephen, *J.*, 1953, 1334.
[13] Jones and Letham, *Analyst*, 1956, **81**, 15.
[14] Professor M. L. Wolfrom, personal communication; Wolfrom, Gibbons, Huggard, and Neely, *J. Amer. Chem. Soc.*, in the press.
[15] Foster, Martlew, and Stacey, *Chem. and Ind.*, 1953, 899; Korn, *Biochem. J.*, 1959, **234**, 1321.
[16] Wolfrom and McNeely, *J. Amer. Chem. Soc.*, 1945, **67**, 748; Jorpes, Boström, and Mutt, *J. Biol. Chem.*, 1950, **183**, 607.
[17] Briscoe, Challenger, and Duckworth, *J.*, 1956, 1755.
[18] Bera, Foster, and Stacey, *J.*, 1955, 3788.

heparin (*ψ*-heparin,[15] heparamine [19]). The S : N ratio of 1·33 for this polysaccharide corresponds to 2·66 *O*-sulphate groups per tetrasaccharide unit; normal heparin preparations contain two *N*- and three *O*-sulphate groups per tetrasaccharide unit. A comparison of the infrared spectra (KCl discs) of heparin and de-*N*-sulphated heparin revealed an overall similarity with only slight differences in the 925—975, 1100—1200, and 1490—1575 cm.$^{-1}$ regions and did not permit the identification of absorption characteristic of the *N*-sulphate group in the heparin spectrum. The location of absorption associated with N–H deformation in the *N*-sulphate group is unknown. Ammonium 2-deoxy-2-sulphoamino-D-glucose shows no absorption and *N*-isopropyltoluene-*p*-sulphonamide [17] only extremely weak absorption in the 1500—1600 cm.$^{-1}$ region where normal amides show strong absorption associated with N–H deformation. Weak, broad absorption at 1525 cm.$^{-1}$ shown by de-*N*-sulphated heparin, but not by heparin, may possibly be associated with the $-NH_3^+$ grouping; 2-amino-2-deoxy-D-glucose hydrochloride shows a strong absorption for this group at 1537 cm.$^{-1}$.

Axial sulphate groups (*e.g.*, as in galactopyranose 4-*O*-sulphate) in certain mono- and poly-saccharides show [20] absorption for C–O–S at *ca.* 850 cm.$^{-1}$ whereas hexopyranose 6-*O*-sulphates absorb at *ca.* 820 cm.$^{-1}$; the sulphated hydroxymethyl groups in the latter compounds are probably equatorial. However, from the absorptions recorded in the Table for a range of sulphated glucans, it seems unlikely that sulphated hydroxymethyl and equatorial secondary hydroxyl groups can be distinguished on the basis of absorption in the 800—820 cm.$^{-1}$ region. Absorptions within this region were shown by polysaccharides effectively containing solely sulphated hydroxymethyl groups (chondroitin sulphate *C*), sulphated equatorial secondary hydroxyl groups (dextran sulphates), or probably both (*e.g.*, amylose sulphate, nigeran sulphate, etc.). In addition to absorbing at 812—817 cm.$^{-1}$ heparin and de-*N*-sulphated heparin absorbed at 795 cm.$^{-1}$. It seems unlikely that the latter absorption is associated with the sulphate groups since, although it was also shown by amylose sulphate (792 cm.$^{-1}$) and amylopectin sulphate (787 cm.$^{-1}$), it was not shown by any other sulphated glucan examined.

Since heparin inhibits the growth of certain bacteria *e.g.*, *Micrococcus pyogenes*,[21] a series of heparin derivatives was prepared and tested for antibacterial activity. The free amino-group in de-*N*-sulphated heparin reacted readily with acid chlorides in water, acetone, or aqueous ethanol and in this way the following *N*-substituents were introduced: [22] benzoyl, benzyloxycarbonyl, *m*-trifluoromethylbenzoyl, nicotinoyl, isonicotinoyl, toluene-*p*-sulphonyl, *p*-acetamidobenzenesulphonyl, 2,4-dinitrophenyl, and di-*O*-phenylphosphoryl. Neither de-*N*-sulphated heparin nor any of the *N*-substitued derivatives antagonized the growth of selected strains of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. A parallel series of derivatives of isopropylamine was also prepared, some of which were hitherto unknown (see Experimental).

Since this work was completed,[23] a variety of *N*-substituted derivatives of de-*N*-sulphated heparin have been described [18,24] which paralleled heparin in clearing alimentary lipemia.[25]

Experimental

Ammonium 2-Deoxy-2-sulphoamino-D-glucose Hydrate.—A solution of 1,3,4,6-tetra-*O*-acetyl-β-D-glucose (10 g.; prepared according to Bergmann and Zervas's method [8]) in dry pyridine

[19] Velluz, Nomine, and Pierdet, *Compt. rend.*, 1958, **247**, 1521.
[20] Lloyd and Dodgson, *Nature*, 1959, **184**, 548, and references cited therein.
[21] Warren and Graham, *J. Bacteriol.*, 1950, **60**, 171; cf. Heilbrunn and Wilson, *Proc. Soc. Expt. Biol. Med.*, 1949, **70**, 179; Roth, *Arch. Biochem. Biophys.*, 1953, **44**, 265.
[22] A preliminary report of some of these results has been given by Foster, Martlew, and Stacey, Abs. Papers Amer. Chem. Soc. Meeting, New York, September 1954, p. 6D.
[23] Martlew, Ph.D. Thesis, University of Birmingham, 1954.
[24] Velluz, Plotka, and Nomine, *Compt. rend.*, 1958, **247**, 2203; Velluz, *Bull. Soc. Chim. biol.*, 1959, **41**, 415.
[25] Nikkila and Haahti, *Acta Chem. Scand.*, 1954, **8**, 363.

(170 ml.) was treated with pyridine–sulphur trioxide complex [7] (21 g.) at room temperature for 24 hr. The mixture was then added to a solution of sodium hydrogen carbonate (22 g.) in water (2·25 l.) at 0° and the resultant solution was freeze-dried. The residue was extracted thrice with methanol (total volume 300 ml.), and the combined extracts were evaporated yielding a glassy residue (12 g.). Examination of the residue by paper ionophoresis using the enclosed strip technique,[26] an acetate buffer (pH 5), and detection with alkaline silver nitrate [27] revealed a neutral component (probably arising by *O* ⟶ *N*-acetyl migration) and four acidic components with M_{GA} values (GA = D-gluconic acid) 1·00, 0·94, 0·875, and 0·815, the third predominating. The acidic components probably differ in the number of acetyl groups they contain since in the subsequent de-*O*-acetylation stage a single component of M_{GA} 1·00 was formed.

A small piece of clean sodium was added to a solution of the above product (11 g.) in dry methanol (300 ml.) at 0°, and the mixture was stored at 0° overnight. A precipitate (1·2 g.) separated which was collected and washed with cold methanol. Concentration of the mother-liquor (to *ca.* 60 ml.) gave a further yield (2·8 g.) of solid. Paper-ionophoretic examination (acetate buffer) of the two solid fractions and the remaining solution revealed, in each case, acidic (M_{GA} 1·00), neutral, and basic (trace) components in different proportions. The proportion of neutral component was greatest in the solution.

A portion (3 g.) of the combined solid products, dissolved in water (2 ml.), was absorbed on Deacidite FF (CO_3^{2-} form, 60 ml.). Elution with water (5 l.) removed the neutral and basic components and elution with 4% aqueous ammonium carbonate (700 ml.) removed most of the acidic compound. Ammonium carbonate was separated from the eluate by using Ultrasorb charcoal.[28] To improve the flow-rate, an Ultrasorb–Celite mixture, prepared according to Lindberg and Wickberg's general method [29] for charcoal–Celite mixtures, was used. The ammonium carbonate eluate was passed through a column (18 cm.) of Ultrasorb–Celite (180 ml.) and subsequent washing with water (650 ml.) removed most of the ammonium carbonate but no reducing sugar. Elution with a further amount (4 l.) of water removed the *N*-sulphate (1·17 g.). Recrystallization of the product from aqueous acetone gave *ammonium 2-deoxy-2-sulphoamino-D-glucose hydrate* as needles, m. p. 150—153° (decomp.) after darkening at *ca.* 130°, $[\alpha]_D^{20}$ +70·5° (2 min.) ⟶ +62° (equilibrium after *ca.* 3 hr.) (*c* 1·0 in water), $[M]_D$ +182° (Found: C, 24·4; H, 6·1; N, 9·75; S, 11·0. $C_6H_{16}N_2O_8S,H_2O$ requires C, 24·5; H, 6·1; N, 9·5; S, 10·9%). The infrared spectrum (Nujol mull) had an absorption at 1640 cm.$^{-1}$ indicative [30] of water of crystallization.

The *N*-sulphate appeared homogeneous on paper ionophoresis (acetate buffer) and detection with alkaline silver nitrate [27] and aniline hydrogen phthalate; [31] it did not react with ninhydrin.

Under the conditions recorded by Belcher, Nutten, and Sambrook [11] for the Elson–Morgan test, 2-deoxy-2-sulphoamino-D-glucose gave a colour with $\lambda_{max.}$ 530 mμ. Under the conditions given by Aminoff, Morgan, and Watkins [10] for the Morgan–Elson test, 2-deoxy-2-sulphoamino-D-glucose gave a negative result.

Acidic Hydrolysis of 2-Deoxy-2-sulphoamino-D-glucose.—Aliquot parts (0·1 ml., measured by a micro-syringe) of 1% solutions of ammonium 2-deoxy-2-sulphoamino-D-glucose hydrate in (*a*) 0·04N- and 0·1N-hydrochloric acid and in (*b*) 11% acetic acid sealed in ampoules were severally maintained at (*a*) 100° and (*b*) 68°. At intervals, ampoules were cooled and opened and the contents diluted so that the concentration of sulphate ion was within the range 60—200 μg./ml. The sulphate ion was then determined by Jones and Letham's method.[13]

Derivatives of Heparin.—The sodium heparinate used for the preparation of heparin derivatives was a commercial sample (Lederle Laboratories), $[\alpha]_D$ +46° (*c* 1·0 in H_2O) after drying at 110° for 3 hr. over phosphoric oxide, anticoagulant activity 80 I.U. per mg. [Found: N, 2·3; S, 13·2%; S : N ratio, 2·51. Calc. for $(C_{24}H_{31}N_2Na_7O_{35}S_5)_n$: N, 2·3; S, 13·0%; S : N ratio, 2·5]. Because of the extremely hygroscopic nature of the dried polysaccharide it was more convenient to analyze the polysaccharide in its normal, hydrated state and then correct the values for

[26] Foster, *Chem. and Ind.*, 1952, 1050.
[27] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[28] Manufactured by British Carbo Norit Union Ltd., West Thurrock, Essex.
[29] Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569.
[30] Barker, Bourne, and Whiffen, in "Methods of Biochemical Analysis," Interscience Publ. Inc., New York, 1956, Vol. III, p. 224.
[31] Partridge, *Nature*, 1949, **164**, 443.

moisture content. The above analyses have been corrected for a 15% moisture content. The S : N ratio is independent of moisture content. A sample of a similar heparin preparation was dialysed against running water for 3 days and then isolated by freeze-drying; its infrared spectrum (KCl disc) showed the following absorptions (cm.$^{-1}$): 3460sb, 2980m, 2365w, 1637s, 1440s, 1230sb, 1155m, 1117m, 1030sb, 940msh, 885m, 817msh, 792m.

A 1% solution of sodium heparinate in 0·04N-hydrochloric acid was kept at 95—100° for 2 hr., then cooled, neutralized 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 having $[\alpha]_D$ +57° (*c* 1·0 in H_2O) after drying [Found: N, 2·65; S, 8·05%; S : N ratio, 1·33. Calc. for $(C_{24}H_{35}N_2Na_3O_{29}S_3)_n$: N, 2·85; S, 9·8%; S : N ratio 1·5. Calc. for $(C_{24}H_{36}N_2Na_2O_{26}S_2)_n$: N, 3·2; S, 7·3%; S : N ratio, 1·0]. The infrared spectrum (KCl disc) showed the following absorptions (cm.$^{-1}$): 3420sb, 2980m, 2370w, 1630s, 1527 wsh, 1430s, 1240sb, 1150m, 1030sb, 940m, 890m, 814m, 795m.

Biological Activity of Heparin Derivatives.—Serial dilutions of the *N*-substituted derivatives of de-*N*-sulphated heparin were prepared in nutrient broth (papain digest of beef) with a highest concentration of 1 mg./ml. Different tubes were inoculated with 24 hr. suspensions of *Staph. aureus* 663, *E. coli* 741, and *B. subtilis* 750 and incubated at 37°. After 24 hr., visual estimation of growth, as compared with controls, showed the absence of inhibitory activity.

Isopropylamine Derivatives.—Isopropylamine (7·5 g.) was added dropwise to a solution of acid chloride (7—10 g.) in carbon tetrachloride or benzene (100 ml.) with shaking and cooling. Isopropylamine hydrochloride was removed and the filtrate was washed successively with dilute hydrochloric acid, water, and aqueous sodium carbonate. Concentration of the dried ($MgSO_4$) solution gave the amide. The following derivatives were thus obtained: *benzyl* N-*isopropylcarbamate* (76%), m. p. 51—52°, b. p. 120° (bath)/0·05 mm. (Found: C, 68·1; H, 7·6; N, 7·0. $C_{11}H_{15}NO_2$ requires C, 68·4; H, 7·8; N, 7·25%); N-*isopropyl*-m-*trifluoromethylbenzamide* m. p. 88° (from aqueous ethanol) (Found: C, 57·7; H, 5·3; F, 24·2. $C_{11}H_{12}F_3NO$ requires C, 57·2; H, 5·2; F, 24·7%); p-*acetamido*-N-*isopropylbenzenesulphonamide* (81%), m. p. 163° (Found: C, 51·6; H, 6·4; N, 11·1; S, 12·5. $C_{11}H_{16}N_2O_3S$ requires C, 51·6; H, 6·25; N, 10·9; S, 12·5%); *diphenyl* N-*isopropylphosphoramidate* (91%), m. p. 73° (Found: C, 62·3; H, 6·5; N, 4·7; P, 10·5. $C_{15}H_{18}NO_3P$ requires C, 61·9; H, 6·2; N, 4·8; P, 10·7%); *phenyl* NN′-*di-isopropylphosphorodiamidate* (61%), m. p. 58—60° (Found: C, 56·2; H, 8·0; N, 10·9; P, 11·6. $C_{12}H_{21}N_2O_2P$ requires C, 56·3; H, 8·2; N, 10·9; P, 12·1%); NN′N″-*tri-isopropylphosphorotriamide* (77%), m. p. 124·5—125° (Found: C, 48·4; H, 10·7; N, 18·6; P, 13·95. $C_9H_{24}N_3OP$ requires C, 48·8; H, 10·9; N, 19·0; P, 14·0%).

m-Trifluoromethylbenzoyl chloride, prepared as was the *para*-isomer,[32] was an oil.

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[32] Burger and Hornbaker, *J. Org. Chem.*, 1953, **18**, 192.