

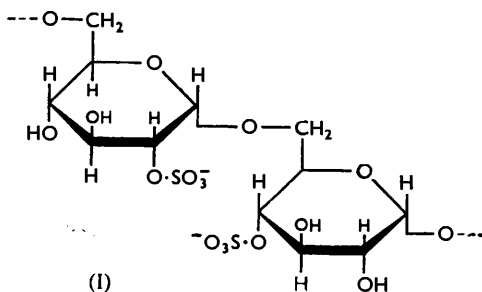
730. *The Distribution of Ester Groups in Dextran Sulphate and their Stability towards Hydrolytic Reagents.*

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Dextran sulphate having, on average, one sulphate group per glucose unit, was oxidised with sodium metaperiodate. Formic acid (0.2 mol.) was produced, indicating the presence of glucose units without sulphate groups in the molecule. Alkaline hydrolysis removed sulphate groups from dextran sulphate and was slower the higher the degree of sulphation. By ionophoresis in borate buffer, sugars with M_g values corresponding to glucose, gulose, altrose, and mannose were found in the hydrolysate. Acidic hydrolysis of dextran sulphate rapidly removed sulphate groups without formation of new hexoses.

DEXTRAN SULPHATE is prepared by the action of chlorosulphonic acid in pyridine on the dextran produced by *L. mesenteroides*. Preparations have been made with widely differing mean molecular weights, from the esters of oligosaccharides (M ca. 10^3) through the intermediate ranges to esters of native dextran (M ca. 10^6), and with varying degrees of sulphation almost up to the theoretical maximum. A blood-anticoagulant drug has been developed using dextran sulphate of low molecular weight,¹ and some novel procedures for the separation of plasma proteins have been developed which use preparations of high molecular weight.² Little is known about the distribution of sulphate groups in the dextran sulphate molecule and their stability toward hydrolytic reagents.

To simplify discussion of the distribution of sulphate groups in the dextran sulphate molecule, consider the case of an unbranched dextran having an average of one sulphate group per glucose unit. In a uniform distribution each unit would carry a sulphate group.



A departure from the uniform distribution would result in some glucose units having more than one sulphate group while others had none. On oxidation with sodium metaperiodate, only those glucose units having no sulphate group have the three adjacent hydroxyl groups necessary to yield formic acid. In the experiments described an unbranched dextran having, on average, 1.08 sulphate groups per glucose unit yielded 0.20 mol. of formic acid per glucose sulphate unit with the consumption of 0.43 mol. of sodium metaperiodate.

¹ Ricketts and Walton, *Chem. and Ind.*, 1952, 869; British Pharmacopoeia Addendum, 1955, p. 16.

² Walton and Oncley, *J. Amer. Chem. Soc.*, in the press.

These results indicate the presence of glucose units without sulphate groups in the dextran sulphate molecule.

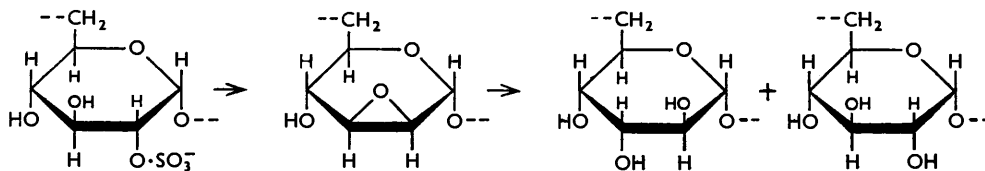
Percival,³ discussing the stability of carbohydrate sulphates under alkaline conditions, pointed out that a sulphate group is susceptible to hydrolysis only if there is an adjacent hydroxyl group in the *trans*-position so that an ethylene oxide type of anhydro-ring is readily formed. A well-defined example is due to Duff⁴ who converted 1 : 6-anhydro- β -D-galactopyranose 2-sulphate into 1 : 6-2 : 3-dianhydro- β -D-talopyranose by treatment with sodium methoxide. The analogy between sulphuric esters and the much studied toluene-*p*-sulphonic esters of sugars is evident. Dextran sulphate (I; SO₃⁻ at arbitrary positions) presents a favourable case for examining the application of these reactions to polysaccharides because, with the exception of terminal glucose units, the reducing groups are blocked, there are no sulphates of primary alcohol groups and adjacent hydroxyl groups are always in the *trans*-position.

Hydrolysis of dextran sulphate is conveniently followed by means of the metachromatic reaction with the dye toluidine-blue,⁵ which forms a purple salt with anions of high molecular weight, one molecule of dye combining with each sulphate group in dextran sulphate.⁶ When shaken with light petroleum the purple salt separates at the interface,⁷ leaving an aqueous solution from which dye has been removed stoichiometrically. A colorimetric measurement thus enables the ester sulphate groups of dextran sulphate to be estimated.

Samples of three dextran sulphates, containing severally 0.17, 1.08, and 2.1 sulphate groups (average) per glucose unit, were submitted to alkaline hydrolysis. The quantity of dextran sulphate was varied so that the same amount of ester sulphate was available for hydrolysis in each experiment. Loss of sulphate groups was followed colorimetrically. The figure shows that the lower the degree of sulphation the more rapidly were ester sulphate groups removed by hydrolysis. It appears that at the higher degrees of sulphation hydrolysis is limited by the availability of free hydroxyl groups adjacent to sulphate groups.

From these considerations it seemed likely that the product obtained by alkaline hydrolysis of dextran sulphate would contain hexoses other than glucose. Dextran sulphate containing 1.08 sulphate groups per glucose unit was therefore submitted to alkaline hydrolysis under conditions which had been found to remove about half of the ester sulphate groups. After removal of sodium ions by means of an exchange resin the polymeric product was submitted to acidic hydrolysis under conditions known to hydrolyse dextran completely. The neutralised hydrolysate was submitted to paper chromatography. Though no clear resolution could be obtained there was an indication of the presence of more than one component. Ionophoresis in borate buffer on paper, followed by spraying with aniline hydrogen phthalate or silver nitrate in acetone, revealed four components : that migrating most rapidly had an M_r value corresponding with glucose and it seemed likely that the other components were also hexoses.

The principles, (1) formation of an ethylene oxide ring with Walden inversion at the carbon atom losing an ester group and (2) opening of the ring in two possible ways with Walden inversion at the carbon atom receiving a hydroxyl group, indicate that the hexoses formed during the alkaline and subsequent acid hydrolysis of dextran sulphate should be gulose and altrose. For example, a glucose unit of the dextran molecule having a 2-sulphate group on the pyranose ring could, on alkaline hydrolysis, give rise to the altrose configuration (see annexed scheme). Ionophoresis with control mixtures showed that two



³ Percival, *Quart. Rev.*, 1949, **3**, 369.

⁴ Duff, *J.*, 1949, 1597.

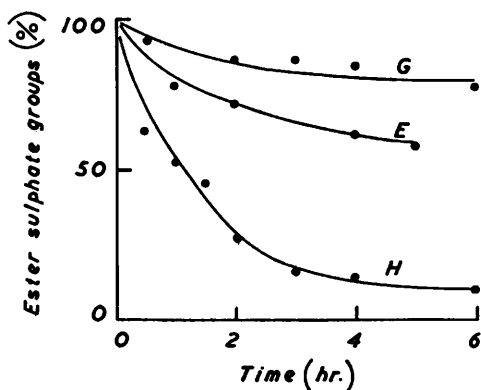
⁵ Michaelis, *J. phys. Chem.*, 1950, **54**, 1.

⁶ Ricketts and Walton, *Brit. J. Exp. Path.*, 1954, **35**, 227.

⁷ MacIntosh, *Biochem. J.*, 1941, **35**, 776.

of the components in the hydrolysate migrated with M_G values corresponding to gulose and altrose. The fourth and slowest component had an M_G value corresponding with that of mannose; no satisfactory explanation of this is available: in a control experiment with dextran no mannose was detected. There was no sign of hexose sulphates, probably because the sulphate groups were split off in the subsequent acid hydrolysis yielding the corresponding hexoses (see below).

Thus alkaline hydrolysis of dextran sulphate generates four of the eight isomeric hexoses in the D-series. The optimum yield of hexoses other than glucose would be expected from a dextran sulphate having an average of one sulphate group per glucose unit. Actually other hexoses were not detectable in hydrolysates of dextran sulphates having 2.1 and 0.17 sulphate groups per glucose unit. In the former case the extent of alkaline



Showing hydrolysis of ester groups from three preparations of dextran sulphate by 2.5N-sodium hydroxide at 100°. Preparation G has 2.1, E 1.08, and H 0.17 ester sulphate groups per glucose unit.

hydrolysis is small (see Figure). In the latter there is a preponderance of unsulphated glucose units.

By contrast with alkaline hydrolysis, acidic hydrolysis of ester sulphate groups occurs readily. From a dextran sulphate of high molecular weight (*ca.* 10^6) containing 2.1 ester sulphate groups per glucose unit, 98% of the ester sulphate groups were removed by 0.1N-sulphuric acid at 100° in 30 minutes. The product appeared to be a partially hydrolysed dextran. This was confirmed by completing the hydrolysis and demonstrating by ionophoresis that only one hexose, corresponding in M_G value with glucose, was present.

To provide a direct comparison with alkaline hydrolysis, the dextran sulphate containing 1.08 sulphate groups per glucose unit, was completely hydrolysed in acid solution and yielded only one hexose (corresponding in M_G to glucose).

The four preparations of dextran sulphate were selected for their suitability for the experiments described. All unbranched dextran sulphates are composed of a selection of the nine possible types of sulphated glucose unit and therefore it is to be expected that the reactions described will apply in varying degrees to all samples of dextran sulphate.

EXPERIMENTAL

Sulphation of Dextran.—Four preparations of sodium dextran sulphate were made by treating dry powdered dextran with chlorosulphonic acid in pyridine as previously described⁸ (see Table).

Preparation	E	F	G	H
Intrinsic viscosity of dextran	0.14	0.03	0.67	0.32
Cl·SO ₃ H (ml. per 10 g. of dextran)	16.6	7.0	15.0	4.7
Pyridine (ml. per 10 g. of dextran)	133	74	61	74
S (%) in Na salt	12.6	12.0	17.6	2.87
SO ₄ groups per glucose unit	1.08	1.00	2.1	0.17

Oxidation of Dextran Sulphate with Metaperiodate.—Sodium dextran sulphate (E) (170.5 mg.) which had been freeze-dried and dried to constant weight *in vacuo* (P₂O₅ at 100°) was oxidised

⁸ Ricketts, *Biochem. J.*, 1952, **55**, 129.

in 0.0342M-sodium metaperiodate (100 ml.). The formic acid produced⁹ and the metaperiodate consumed¹⁰ were determined at intervals. The results were calculated as mols. of formic acid produced and metaperiodate consumed per glucose sulphate unit :

Time (hr.)	48	72	96	Mean of 72 and 96 hr.
H·CO ₂ H (mol.)	0.195	0.199	0.203	0.201
IO ₄ ⁻ consumed (mol.)	0.427	0.428	0.434	0.431

Alkaline Hydrolysis of Dextran Sulphate.—The three dextran sulphates (H 40 mg. ; E 10.1 mg. ; G 7.2 mg. ; *i.e.*, containing the same amount of ester sulphate) were dissolved in 2.5N-sodium hydroxide (20 ml.). Portions (2 ml.) were heated in sealed ampoules in a boiling-water bath. At intervals ampoules were withdrawn, cooled, neutralised, diluted, and analysed for ester sulphate.⁷ A calibration curve was constructed, with preparation E as standard, and results were calculated as percentage loss of ester sulphate groups (see Figure).

Identification of Products of Alkaline Hydrolysis.—Dextran sulphate E (100 mg.) was heated in 2.5N-sodium hydroxide (1 ml.) as above for 5 hr. After cooling, the solution was diluted and passed through a column (70 × 10 mm. diameter) of cation-exchange resin (Zeokarb 215), then concentrated under reduced pressure to 1 ml., mixed with 2N-sulphuric acid (1 ml.), and heated in a sealed ampoule in a boiling-water bath for 5 hr. After cooling the solution was neutralised with barium carbonate. Barium salts were separated on the centrifuge and washed with water. The solution and washings were combined and concentrated under reduced pressure to 1 ml. Partition chromatography on Whatman No. 1 paper with butanol-ethanol-water (5 : 1 : 4 by vol.) at 37° showed, after spraying with aniline hydrogen phthalate and baking at 110°, only one spot whose R_F value corresponded with that of glucose. Similarly with butanol-pyridine-water (6 : 4 : 3 by vol.), at room temperature only one spot (R_F = that of glucose control) was detected but elongation of the spot suggested the presence of more than one component. Ionophoresis¹¹ in borate buffer of pH 10, followed by spraying with aniline hydrogen phthalate,¹² or silver nitrate in acetone (2.5 ml. of saturated aqueous silver nitrate in 500 ml. of acetone), revealed 4 components. Mixtures of glucose with gulose, with altrose, and with mannose were submitted to ionophoresis alongside the hydrolysate. A control spot of 2 : 3 : 4 : 6-tetra-*O*-methyl glucose enabled a correction for endosmotic flow to be applied in the calculation of M_G values, which were tabulated. In the hydrolysate the mannose and gulose spots were similar and less intense than the glucose spot; the altrose spot was faint.

	M_G values			
Hydrolysate	0.716	0.831	0.916	0.987
Gulose + glucose	—	0.838	—	1.000
Hydrolysate	0.720	0.807	0.907	0.980
Altrose + glucose	—	—	0.920	1.000
Hydrolysate	0.724	0.836	0.912	0.990
Mannose + glucose	0.724	—	—	1.000

In a confirmatory experiment, dextran sulphate F (100 mg. in 1 ml. of 2.5N-sodium hydroxide) was submitted to alkaline and then acidic hydrolysis, as described; electrophoresis again revealed four components with M_G values close to those recorded above. However, when dextran sulphate G (72 mg. in 1 ml.) and dextran sulphate H (400 mg. in 1 ml.) were submitted to the same procedure, only one component could be detected, its M_G value corresponding to that of glucose.

Acidic Hydrolysis of Dextran Sulphate.—Portions (2 ml.) of a solution of dextran sulphate (G; 36 mg.) in 0.1N-sulphuric acid (100 ml.) were heated in sealed ampoules in a boiling-water bath. At intervals samples were analysed for ester sulphate as previously described: 98.1% of the ester sulphate groups were removed in 30 min. under these conditions; ampoules heated for longer periods showed complete hydrolysis.

Identification of the Products of Acidic Hydrolysis.—Dextran sulphate (G; 100 mg.) was heated in 0.1N-sulphuric acid (10 ml.) in a boiling-water bath. After 30 min. the solution was cooled quickly and neutralised with barium carbonate. Barium salts were separated in the centrifuge and washed with water. The solution and washings were combined and concentrated under reduced pressure to about 1 ml. The solution was submitted to paper chromatography

⁹ Jeanes and Wilham, *J. Amer. Chem. Soc.*, 1950, **72**, 2655.

¹⁰ Fleury and Large, *J. Pharm. Chim.*, 1933, **17**, 107, 196.

¹¹ Foster, *Chem. and Ind.*, 1952, 1050; *J.*, 1953, 982.

¹² Partridge, *Nature*, 1949, **164**, 443.

with butanol-pyridine-water (6 : 4 : 3 by vol.). Spraying with aniline hydrogen phthalate followed by baking at 110° revealed signs of higher saccharides near the origin but no glucose or oligosaccharides. Hydrolysis was completed by heating the solution with an equal volume of 2*N*-sulphuric acid in a sealed ampoule at 100° for 5 hr. After cooling, neutralisation with barium carbonate and evaporation under reduced pressure, paper chromatography, as above, showed the presence of a substance with the same R_F value as glucose and no indication of saccharides. Ionophoresis in borate buffer followed by spraying with aniline hydrogen phthalate or with silver nitrate in acetone identified glucose as the only hexose detectable after acidic hydrolysis of dextran sulphate.

To provide a direct comparison of the products of alkaline hydrolysis with those of acid hydrolysis, dextran sulphate E (200 mg.) was heated in *N*-sulphuric acid (5 ml.) for 5 hr. After cooling, the solution was neutralised with barium carbonate. After treatment as above, ionophoresis in borate buffer on paper revealed only one component; this had an M_G value corresponding with that of glucose.

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