Dextran Sulphate—A Synthetic Analogue of Heparin

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The structure of the natural anticoagulant, heparin, is gradually being elucidated. As each step forward is made, attempts to synthesize molecules with the biological activity of heparin receive renewed impetus. It is now known (Jorpes, 1939) that heparin is composed of glucuronic acid and glucosamine though the mode of linkage of these two parts is not yet clear. Some of the hydroxyl groups of these components are esterified with sulphuric acid but the exact distribution of sulphate groups is uncertain. Recently, however, Jorpes, Bostrom & Mutt (1950) have shown that during the weak acid hydrolysis of heparin, sulphuric acid appears at the same time and with the same rate as amino groups, leading to the view that the amino group is combined with sulphuric acid. This conclusion is confirmed by the work of Meyer & Schwartz (1950).

An important contribution to our knowledge of the size of heparin molecules was made by Grönwall, Ingleman & Mosiman (1945), who measured sedimentation and diffusion constants for a substantially pure preparation in the ultracentrifuge. Since heparin is not absolutely homogeneous as regards molecular size it was not possible to calculate an exact value but a molecular weight of 17 000 was obtained as an average for all the molecules in the preparation.

Heparin is also inhomogeneous in respects other than molecular weight. Several mild physical methods have been employed to divide seemingly pure samples of heparin into components differing in sulphur and nitrogen content and activity. Fractional recrystallization of the brucine salt by Jorpes & Bergstrom (1937) pointed to the association of sulphur content with activity. In the Craig countercurrent extractor O'Keef, Russo-Alesi, Dolliver & Stiller (1949) separated heparin into a component having 8·2% S and an activity of 59 units/mg. and one with 13·3% S and an activity of 215 units/mg. By the technique of frontal analysis, Jensen, Snellman & Sylvén (1948) detected three or four components in heparin.

From the facts outlined above, it appears that blood anticoagulant activity is not the property of a unique molecular configuration but is shared by a number of similar molecules occurring in the product ordinarily called heparin. An outstanding feature which has long been recognized is the necessity for the presence of acidic sulphate groups in molecules with anticoagulant activity. The literature contains many examples of sulphuric esters of polysaccharides exhibiting heparin-like activity *in vitro*. Sulphuric esters of dextran (Grönwall *et al.* 1945), cellulose (Astrup & Piper, 1945*a*), inulin (Ingelman, 1946) and alginic acid (Snyder), to mention but a few, have been prepared. Usually these esters have been reported to show one-tenth to one-half of the activity of heparin *in vitro*, but inulin sulphuric ester is claimed to be as active as heparin *in vitro*.

Compounds with blood anticoagulant activity are easily prepared, but when submitted to toxicity tests some active preparations have proved fatal in doses only a few times greater than the minimum dose effective in raising the blood clotting time. Astrup & Piper (1945*a*, *b*) found that some sulphuric esters of cellulose combined with fibrinogen to form an insoluble complex. They considered such an adverse effect on fibrinogen should not occur with an anticoagulant intended for clinical use.

There appeared to be sufficient scope for systematic variation in the structure of anticoagulant molecules to enable combination with fibrinogen to be minimized while retaining a useful degree of anticoagulant activity. It seemed essential to control two factors: (1) molecular weight; (2) number of sulphate groups.

Considering first the effect of molecular weight, it is desirable to start with a polysaccharide of which the molecular weight can be subjected to controlled variation. On account of its use as a blood plasma substitute (Ingelman, 1949; Bull *et al.* 1949), pure dextran is produced in large quantities and considerable attention has been given to molecular weight control (Ingelman, 1949; Ricketts, Lorenz & Maycock 1950). Grönwall *et al.* (1945) describe three preparations of dextran sulphuric acid ester. Their least toxic preparation was made from dextran of relative viscosity 2·4 in 6% solution. It may be inferred from the viscosity data given by Ingelman & Halling (1949) that this dextran had a molecular weight of about 32 000. The sodium salt of this dextran sulphate contained 12% sulphur, or about 1 sulphate group per glucose unit so the molecular weight of the sodium dextran sulphate would be about

50 000. This is about 3 times the molecular weight of heparin. However, in a subsequent paper, reporting the toxicity of an inulin sulphuric acid ester of molecular weight 14 000, Ingelman (1946) expresses the view that 'molecular weight is not a principal factor in determining toxicity'.

To resolve this point a series of sulphuric esters of dextran differing widely in molecular weight and sulphur content was prepared. The cause of their toxicity was carefully investigated and will be reported separately by Dr K. Walton.

Dextran sulphate with molecules of a certain size was found to be free from toxic effects. The relation between blood anticoagulant activity and number of sulphate groups in molecules of this size was explored.

On the basis of these experiments the preparation of dextran sulphate in a form suitable for clinical trial is reported.

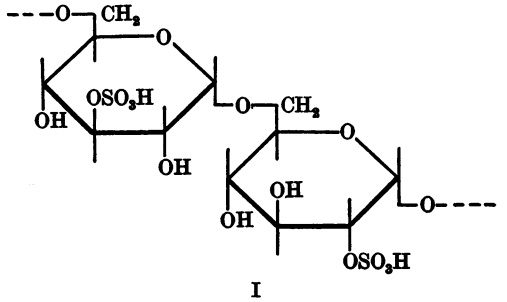
EXPERIMENTAL

Material

The starting material was dextran prepared for use as a plasma substitute (Bull *et al.* 1949). In the course of preparation the dextran of *Leuconostoc mesenteroides* was partially hydrolysed by boiling with 0·075 N-H_2SO_4 until its intrinsic viscosity was between 0·21 and 0·36. Tests to ensure freedom from toxicity and antigenicity were carried out as described by Bull *et al.* (1949).

Methods

Sulphate groups were introduced by treating the finely powdered dry dextran with chlorosulphonic acid and pyridine using the proportions of reagents and conditions of Grönwall *et al.* (1945), except when variations are specifically mentioned, for example in Table 2. The dextran sulphate was isolated as the sodium salt in the way described by these authors.



I

Sulphur was determined by oxidation of organic matter with Benedict's sulphur reagent, precipitation of inorganic sulphate as benzidine sulphate (Owen, 1936) and titration with standard alkali. A dialysate was carefully tested to ensure the absence of inorganic sulphate from each preparation, so that the sulphur may be regarded as ester sulphate. The sulphur thus determined was assumed to be combined in the way illustrated in the formula I. On this basis Fig. 1 shows the calculated relationship between percentage sulphur in the sodium salt and the number of sulphate groups per glucose unit.

Intrinsic viscosity was obtained from measurements of relative viscosity at several concentrations thus:

$$\eta_{sp.} = \eta_{r.} - 1,$$

$$[\eta] = \text{limit}_{C \to 0} \frac{\eta_{sp.}}{C},$$

where $\eta_{r.}$ = relative viscosity, $\eta_{sp.}$ = specific viscosity, $[\eta]$ = instrinsic viscosity.

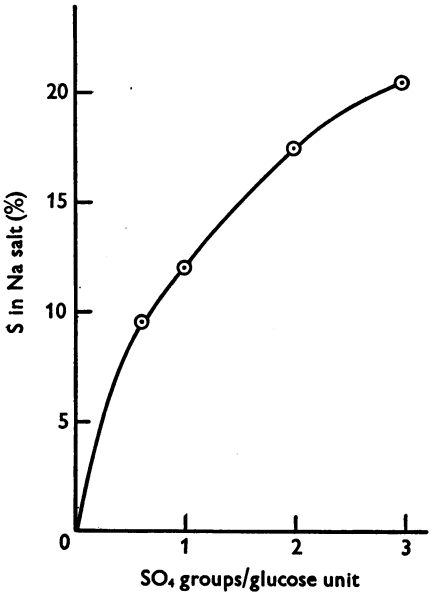


Fig. 1. Showing the calculated relation between sulphur content of sodium dextran sulphate and average number of sulphate groups per glucose unit.

Osmotic pressure was measured in an Adair (1949) type of osmometer and recorded in cm. of solution after correction for capillarity. All measurements were made at 3–4°.

Anticoagulant activity was measured by the method of Kuizenga, Nelson & Cartland (1943) using sheep plasma.

RESULTS

Variation of molecular weight

Three main types of dextran sulphate, designated by the letters *D*, *E* and *I*, were prepared. The *D* and *I* series were from unfractionated hydrolysates of the intrinsic viscosity quoted in Table 1. The *E* series was prepared from the low molecular 50 % of a hydrolysate with intrinsic viscosity 0·24; this fractionation was made to minimize any overlap in molecular composition between the *D* and *E* series.

If the relation between intrinsic viscosity and molecular weight determined by Ingelman & Halling (1945) be applied to the dextran used in these experiments the average molecular weight of the

dextran used for the *D* and *E* types of dextran sulphate is about 200 000 and 20 000 respectively. Allowing for the introduction of, say, 1·3 sulphate groups per glucose unit, the average molecular weight of the *D* and *E* types of dextran sulphate would be very approximately 300 000 and 30 000 respectively. The only estimate for the *I* type of dextran sulphate that can be obtained from the data given by Grönwall *et al.* (1945) is a figure of less than 20 000, allowing for the introduction of 1·3 sulphate groups per glucose unit.

Table 1. *Properties of dextran sulphates prepared from three dextrans differing widely in molecular weight*

Intrinsic viscosity of dextran	Serial	S in Na salt (%)	SO_4 groups/ glucose unit	Activity units/mg.
0·36	*D*	14·6	1·4	—
0·36	*D*2	17·8	2·1	—
0·36	*D*3	15·4	1·6	15
0·12	*E*	21·5	3·0	—
0·12	*E*1	13·7	1·3	15
0·02	*I*	13·5	1·2	—
0·02	*I*2	15·2	1·5	15

Evidence that the three types of dextran sulphate differ in average molecular weight was obtained from viscosity and osmotic pressure measurements. Viscosity was measured in 0·066 M-phosphate buffer at pH 7. Fig. 2 shows specific viscosity divided by concentration plotted against concentration for preparations *D*3, *E*1 and *I*4 (see Tables 1, 3). Extrapolation directly and using the logarithmic method mentioned by Bawn (1948) gave identical values for the intrinsic viscosity. From this graph the intrinsic viscosity of *D*3 may be read as 0·69, *E*1 as 0·20 and *I*4 as 0·04. Similar measurements in 0·9% (w/v) sodium chloride showed: *E*1, 0·18; *I*4, 0·03; and *I*3, 0·03; heparin sample *A*, 0·21; heparin sample *B*, 0·12.

The osmotic pressure of 2% (w/v) *E*1 and 1% (w/v) *I*3 in 0·9% sodium chloride solution was measured. The solution outside the membrane was tested with toluidine blue but no metachromatic reaction was obtained in a test which was known to be sensitive to less than 10 μg./ml. The molecular weights calculated from the equilibria were *E*1, 35 000 and *I*3, 8000, but several such osmometers are necessary to provide accurate estimates of number average molecular weight. It may be inferred from these data that the molecular weight of *I*3 is considerably less than that of *E*1. Using two osmometers and a more refined technique, Mr D. Sutcliffe has obtained a figure of 7300 ± 500 for the preparation *I*4.

Biological testing of the preparations showed that the *I* series of dextran sulphates was free from toxic effects and might be therapeutically useful. Dextran sulphates of the *E* and the *D* series were found to be toxic.

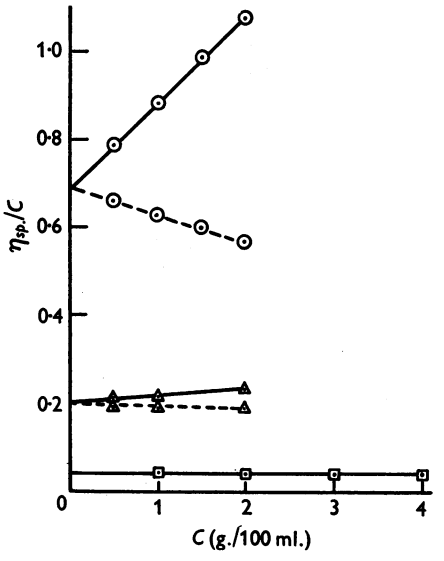


Fig. 2. Showing direct (——) and logarithmic (– – –) extrapolation to obtain intrinsic viscosity of dextran sulphate preparations *D*3, ⊙; *E*1, △ and *I*4, ⊡.

Variation in number of sulphate groups

Several dextran sulphates differing in sulphur content were prepared from a single batch of dextran which had an intrinsic viscosity of 0·04.

Table 2. *Properties of dextran sulphates prepared from the same batch of dextran but differing in sulphur content*

$ClSO_3H$ ml./10 g. dextran	Serial	S in Na salt (%)	SO_4 groups/ glucose unit	Activity units/mg.
14·6	*I*7	16·3	1·7	15
13·3	*BE*	15·9	1·6	15
12·0	*BD*	14·2	1·3	15
10·6	*BC*	14·3	1·3	15
9·3	*BB*	11·8	1·0	3·75
—	*AO*	9·2	0·6	<3

Preliminary experiments indicated that alteration in the sulphur content was not achieved by decreasing the proportion of chlorosulphonic acid from 44 ml./10 g. dextran as quoted by Grönwall *et al.* (1945) to 14·6 ml./10 g. dextran, but was obtained between this value and 9·3 ml./10 g. dextran. Table 2 shows this effect, together with the sulphur content and activity of the resultant dextran sulphate. Preparation *AO*, also shown in Table 2, was made by adding powdered dextran to the melted crystalline complex of chlorosulphonic acid and pyridine (Sobel, Drechter & Natelson, 1936) and

Table 3. *Data for large-scale preparations of dextran sulphate*

Intrinsic viscosity dextran	Weight dextran (g.)	Serial	Yield Na salt (g.)	S in Na salt (%)	SO_4 groups/ glucose unit	Activity units/mg.
0·02	60	*I* 3	88	17·1	1·9	15
0·03	60	*I* 4	115	17·1	1·9	15
0·03	30	*I* 5	41	17·1	1·9	—
0·05	30	*I* 6	41	16·5	1·8	—
0·04	60	*I* 7	80	16·3	1·7	15

isolating the product in the usual way. The relation between the number of sulphate groups per glucose unit and activity reveals a sharp increase to the maximum activity between 1 and 1·3 sulphate groups per glucose unit.

For comparison, the provisional International Standard for heparin contains 12·45 % sulphur in the sodium salt and has an activity of 130 units/mg. Heparin sample *A* contained 9·3 % S and sample *B* contained 8·6 % S.

Preparation of a non-toxic dextran sulphate

The foregoing experiments established the conditions for the preparation of an active non-toxic dextran sulphate. In view of the association of toxicity with increasing molecular weight the larger molecules were removed by fractional precipitation before introducing the sulphate groups. This precaution was intended to prevent significant amounts of toxic dextran sulphate accumulating when very large doses were given over a long period of time. A full description of the preparation of the dextran sulphate submitted to clinical trial will now be given.

To 3 l. of a 6·2 % (w/v) solution of a partial hydrolysate of *L. mesenteroides* dextran with intrinsic viscosity 0·25, 300 ml. N-H_2SO_4 were added and the solution boiled under reflux for 4 hr. After cooling, the solution was neutralized with N-NaOH, the final volume being 3·6 l. Acetone (3 l.) was added, and after standing overnight the syrupy precipitate containing the larger molecules was separated. A further 6 l. of acetone were added, the syrup was separated, poured into ethanol, ground to a powder, washed with dry ether and dried *in vacuo* over P_2O_5. About 100 g. were obtained; the intrinsic viscosity was 0·02.

To 400 ml. dry pyridine, 88 ml. chlorosulphonic acid were added drop by drop with vigorous stirring. During the addition the flask was cooled in a mixture of solid CO_2 and ethanol. The temperature was then raised to 65° when most of the pyridinium salts dissolved. Finely powdered dextran, 60 g., was added and dispersed in the reaction mixture by vigorous stirring. The temperature was maintained at 65–70° for 4 hr.

After cooling, 1·5 l. of crushed ice and sufficient 40 % (w/v) NaOH were added to turn the mixture dark red in colour and cause separation of the pyridine as an upper layer. The lower layer was diluted to 2 l. and brought to 37° when 2 l. of ethanol were added. The precipitated syrup was allowed to settle for 10 min. only at 37° to avoid crystallization of Na_2SO_4. The syrup was then separated, redissolved in 850 ml. water and similarly precipitated with 850 ml. of ethanol. The precipitation was repeated again from 640 ml. of solution and an equal volume of ethanol. The final syrup was dissolved in 800 ml. of water, neutralized with HCl, dialysed against running water and treated with charcoal at 50°. The pH of the filtrate was adjusted to 7–7·5 with NaOH and the solution concentrated under reduced pressure to 500 ml. Acetone (750 ml.) was added, and the precipitated syrup was poured into ethanol, ground to a powder, washed with ether and dried *in vacuo* over P_2O_5. This preparation was designated *I* 3. Details of this and a number of similar preparations are summarized in Table 3.

Dextran sulphates of the *I* series decompose on boiling in aqueous solution with the formation of inorganic sulphate and reducing substances, a point of some importance in connexion with the sterilization of solutions for intravenous administration. For example, after autoclaving a neutral 20 % (w/v) solution of *I* 4 at 10 lb. steam pressure for 20 min., one-third of the sulphur was liberated as sulphuric acid and the solution was found to reduce Fehling's reagent strongly. Some of the dextran sulphate which had not decomposed was recovered and found to contain 13·5 % S and to be about as active as *I* 4.

It was found that this decomposition could be prevented by buffering the solution. The following mixture has proved satisfactory: dextran sulphate, 20 g.; sodium chloride, 0·72 g.; sodium bicarbonate, 0·24 g.; water, 100 ml. A solution of this composition had an initial pH of 8·3, and was autoclaved at 10 lb. for 10 min. without the appearance of inorganic sulphate or reducing substances, and without appreciable loss of activity. The final pH was 6·7 and the solution approximated to the isotonicity required for intravenous use.

DISCUSSION

With dextran as with other polysaccharides, blood anticoagulant activity is readily obtained by the introduction of sulphate groups. Interest therefore centres on whether any such compounds are sufficiently non-toxic for clinical use. A preliminary communication of the results of the biological investigation which proceeded in parallel with the experiments reported here has been given by Walton (1951) and a full account is in course of preparation. Toxicity was found to increase with molecular weight.

It is therefore important to consider the evidence relating to the molecular weight of the three types of dextran sulphate preparation used in the biological investigation. The intrinsic viscosity of the dextrans and of the dextran sulphates derived from them indicate corresponding differences in molecular weight. The critical distinction in toxicity is between the *E* type and the *I* type and here the osmotic pressures recorded support the contention that the *I* type is of considerably lower molecular weight than the *E* type, being of the order of 7000.

The anticoagulant activity expressed in units/mg. appears to be independent of the molecular weight but depends on a certain minimum number of sulphate groups per glucose unit. At 1·0–1·3 sulphate groups per glucose unit the sharp increase of activity to its maximum value is indicative of the appearance of some critical pattern of groups in the molecular structure. Heparin has greater activity with considerably fewer sulphate groups in the molecule. From the biological investigations evidence was obtained that the activity of heparin and dextran sulphate was qualitatively identical, in spite of the sevenfold difference in the weight corresponding with a given amount of anticoagulant activity.

SUMMARY

1. Molecular weight determines the toxicity of dextran sulphate. Molecules smaller than a critical size are not toxic.

2. Maximum blood anticoagulant activity is attained when the number of sulphate groups exceeds an average of 1·3 per glucose unit.

3. Preparation of a potentially useful anticoagulant drug is described.

It is a pleasure to acknowledge here the close and fruitful collaboration of my colleague, Dr Kenneth Walton, whose account of the biological aspects of our investigation will appear elsewhere, and to thank Prof. J. R. Squire for his help and encouragement. Thanks are also due to Prof. M. Stacey, F.R.S., for placing at my disposal the facilities of his Department for the larger scale preparations of dextran sulphate.

I am grateful to Mr M. Hall, for his invaluable help in the laboratory, and to Messrs Dextran Ltd. for gifts of dextran.

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The Occurrence of Substituted Uric Acids in Human Urine

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In the course of work on the separation of purines and pyrimidines from biological materials, purine fractions obtained from human urine have been examined. This work is not complete, but as it is being interrupted the results so far obtained are given here.

The purine fractions employed were obtained by ammoniacal silver precipitation of the urine after phosphate removal using the reagents described by Snell & Snell (1937), and separation was effected on columns of the ion-exchange resin Zeo-Karb 215 by methods similar to those used by Cohn (1949) for the separation of bases from nucleic acids. Uric acid was readily separated from the xanthines, but between these, other well-defined fractions were obtained having ultraviolet absorption spectra scarcely