Brit. J. Pharmacol. (1954), **9**, 224.

THE BLOOD ANTICOAGULANT EFFECT OF SHORT CHAIN-LENGTH DEXTRAN SULPHATES

BY

C. R. RICKETTS

From the M.R.C. Industrial Injuries and Burns Research Unit, Accident Hospital, Birmingham

(RECEIVED MARCH 9, 1954)

Several previous papers have shown that dextran sulphate of high molecular weight has toxic properties, but that of molecular weight about 7,000, corresponding to a chain-length of 20 glucose units, is sufficiently non-toxic for clinical use. The relation between the degree of sulphation and anticoagulant activity for molecules of this size has been explored (Ricketts, 1952a). In extending the work to compounds of shorter chain-length, some experiments were made with sulphuric esters of oligosaccharides in the maltose series, because they were the only oligosaccharides characterized at the time. Since then the separation and identification of dextran oligosaccharides, by chromatography of their benzyl glycosylamine derivatives (Bayly and Bourne, 1952), have enabled exploration to be continued.

Sulphuric esters of the lower members of the maltosaccharide series showed less anticoagulant effect in rabbits than was expected on the basis of activity measurements *in vitro* (Ricketts, 1952b). This paper describes the preparation of some dextran sulphates of short chain-length, their anticoagulant activity *in vitro*, and their effect upon the blood clotting time of the rabbit.

MATERIALS AND METHODS

Two series of compounds, U and O, were made. The U series was obtained by hydrolysing dextran to varying extents and sulphating the product; these compounds were extremely polydisperse in molecular composition. Saccharides for the O series were prepared from a single hydrolysate of dextran by chromatographic separation on a charcoal column. While it was not possible to isolate individual saccharides in sufficient amounts, mixtures of several components—identified by paper chromatography of their benzyl glycosylamines, and by determination of the mean chain-length from the reducing power of the single terminal aldehyde groups—were obtained. These saccharides were then sulphated. The detailed procedures were as follows:

Preparation of Short Chain-length Polydisperse Dextran Sulphates (U Series)

Hydrolysis.—Dextran (60 g.) of the type used as a plasma substitute (intrinsic viscosity 0.33) was partially hydrolysed by heating a thoroughly stirred solution 5.45% w/v in 0.82 N-sulphuric acid, on a boiling water bath for the times shown in Table I. These times were selected, on the basis of a preliminary experiment, to yield dextrans of mean chain-length 20, 10, 7, and 5 glucose units. After cooling to stop the hydrolysis the solution was dialysed and concentrated to 100 ml. Dextran was precipitated by addition of 500 ml. ethanol, ground to a powder in ethanol, washed with ether, and dried over phosphorus pentoxide *in vacuo*. Yields are quoted in Table I as a percentage weight of the initial dextran. They decrease as the chain-length decreases on account of the proportionately greater conversion to glucose.

Sulphation.—Ten grams of each of these dextrans were sulphated, using 15 ml. chlorosulphonic acid in 70 ml. pyridine, and the dextran sulphate was isolated as described in a previous paper (Ricketts, 1952a). The weight of dextran sulphate obtained and its sulphur content are recorded in Table I. Theoretical yield was calculated on the assumption that all the dextran could have been converted to dextran sulphate of the observed sulphur content. The percentage of the theoretical yield actually isolated is shown. Intrinsic viscosities were determined.

TABLE I

PREPARATION AND PROPERTIES OF POLYDISPERSE SHORT CHAIN DEXTRAN SULPHATES (U SERIES), SHOWING DECREASING ANTICOAGULANT ACTIVITY *IN VITRO* WITH DECREASING INTRINSIC VISCOSITY

Serial No.	Dextran		Dextran Sulphate			
	Hydrolysis Time (min.)	Yield %	Yield %	% S in Na Salt	Intrinsic Viscosity	Anticoagulant Activity *in vitro* (u./mg.)*
U 20	17	80	67	18·8	0·049	19·7
U 10	36	53	69	17·8	0·038	9·4
U 7	51	23	52	17·9	0·028	8·1
U 5	72	12	49	18·2	0·024	5·1

* Assayed against a dextran sulphate standard with a heparin activity of 15 u./mg.

Preparation of Short Chain-length Slightly Disperse Dextran Sulphates (O Series)

Hydrolysis.—Dextran (30 g.) of the type used as a plasma substitute, intrinsic viscosity 0.33, but prepared from the B 512 strain of *Leuconostoc mesenteroides* to

minimize the branching in the product, was partially hydrolysed by heating a thoroughly stirred solution, 5.45% w/v in N-sulphuric acid, on a boiling water bath for 90 min. This time was calculated on the basis of a preliminary experiment to yield a product having one quarter of the reducing power of glucose, i.e., of mean chain-length 4 glucose units. After cooling to stop the hydrolysis the solution was neutralized with sodium hydroxide and brought to a final volume of 500 ml.

Chromatography.—The methods of Whistler and Durso (1950) and Bailey, Whelan, and Peat (1950) were modified slightly. A column 60 cm. long by 4.5 cm. diameter was packed for a length of 30 cm. with a mixture of equal weights of Hyflo Super-Cel (Johns Manville Co.) and washed activated charcoal (B.D.H.). The column was wet with water, and the dextran hydrolysate (500 ml., optical rotation +17.41° in 20 cm. tube) was run slowly on to the adsorbent. The adsorption and subsequent elution were followed by optical rotation measurements in a 20 cm. polarimeter tube. The column was eluted successively with water, 5, 15, 25, and 35% v/v ethanol. Fig. 1 summarizes these operations. Appropriate portions of the eluate were combined as illustrated to form five fractions, A to E.

Solvents were removed by distillation under reduced pressure and the saccharides were recovered by precipitation from syrupy solution with excess ethanol. Fraction A, 0.04 g., was mainly glucose with a trace of sodium sulphate and was discarded. The other fractions, B, 1.1 g.; C, 3.1 g.; D, 6.0 g.; and E, 0.67 g., were sulphate free.

Characterization of Fractions.—Reducing power was determined (Schaffer and Hartman, 1921) on materials dried to constant weight *in vacuo* at 60° C. This provided the estimate of mean chain-length recorded in Table II. The individual saccharides present in each fraction were revealed by chromatography of their benzyl glycosylamines using the method developed by Bayly and Bourne (1952). Fig. 2 shows one of the chromatograms obtained. This chromatogram includes as a reference spot pure 1:6 penta-saccharide. The penta-saccharide mixed with a little glucose was applied to the paper at R, the other fractions being applied as indicated. The solvent was butanol 40, ethanol 12, water 20, 0.88 ammonia 1, parts by volume. From the chromatogram it may be inferred that fraction B contains tetra- and penta-saccharides, fraction C penta-, hexa-, and probably hepta-saccharides, fraction D hepta- and higher saccharides. Fraction E is composed of saccharides too large to separate by chromatography.

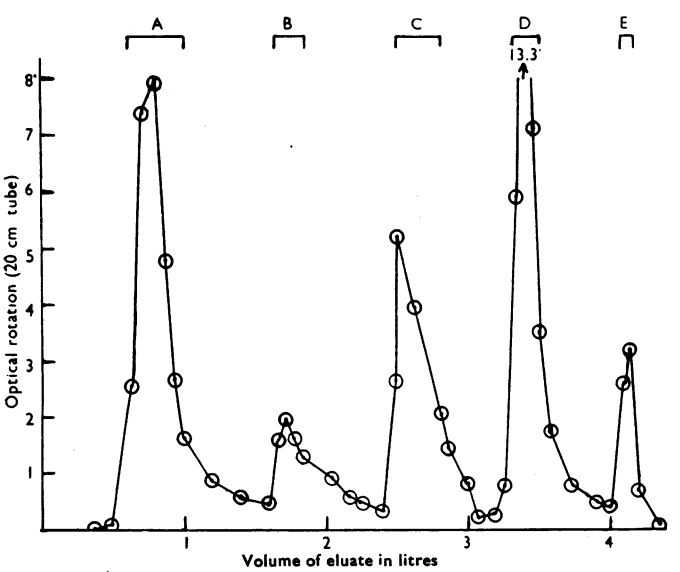

FIG. 1.—Showing changes in optical rotation during the elution of dextran fractions from a charcoal column. Portions of the eluate were combined as indicated to provide fractions A to E.

TABLE II

PREPARATION AND PROPERTIES OF SLIGHTLY DISPERSE SHORT CHAIN DEXTRAN SULPHATES (O SERIES), SHOWING RELATION OF ANTICOAGULANT ACTIVITY *IN VITRO* WITH MEAN CHAIN-LENGTH

Serial No.	Dextran		Dextran Sulphate			
	Weight g.	Mean Chain-Length	Yield of Ester %	% S in Na Salt	Intrinsic Viscosity	Anticoagulant Activity *in vitro* (u./mg.)*
OE	0·67	17·8	54	18·2	0·038	14·3
OD	5·85	9·4	78	20·0	0.029	8·6
OC	3·1	5·7	75	18·8	0·020	8·5
OB	1·1	4·4	75	18·7	0·018	8·5

* Assayed against a dextran sulphate standard with a heparin activity of 15 u./mg.

Sulphation.—Some 0.8 to 5 g., according to the amount available, of each of these fractions was sulphated, using 2 ml. chlorosulphonic acid per gram of dextran and 20 to 50 ml. of pyridine. The yield, sulphur content, and intrinsic viscosity of each preparation is shown in Table II.

The Stability of the Dextran Sulphate Preparations

It was found that the U series preparations slowly decomposed, the powder form in the course of several months and solutions in several weeks. Preparations U 5 and U 7 decomposed first, followed by U 10; U 20 appears to be indefinitely stable. On the other hand the O series compounds all appeared to be indefinitely stable. This was attributed to the presence of glucose sulphate in preparations of the U series and its absence from preparations of the O series. Experimentally, the mixture obtained by sulphating glucose and isolating the glucose sulphates as sodium salts decomposed in a period of months. Decomposition always takes the form of increasing acidity, the production of reducing substances and, ultimately,

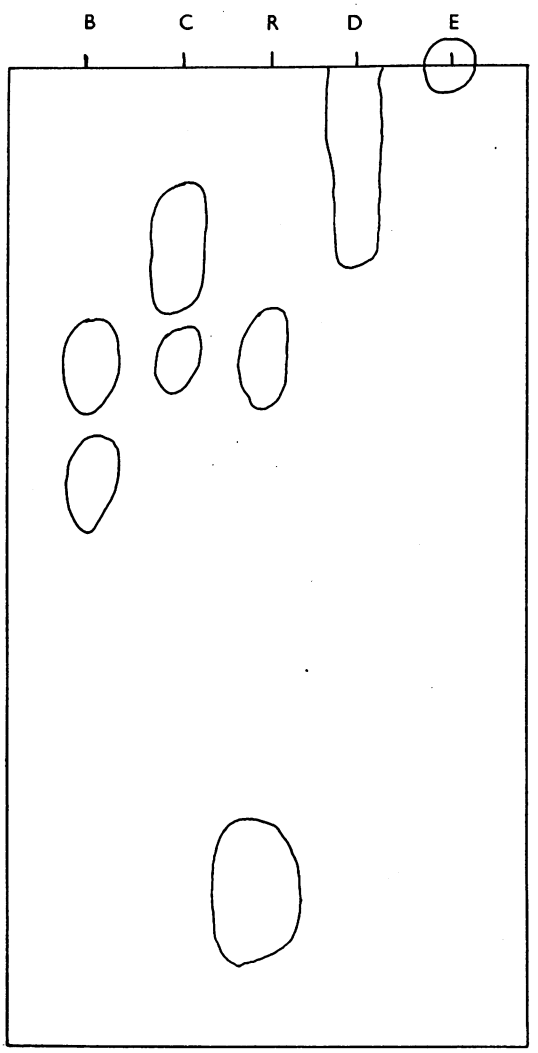

FIG. 2.—Chromatogram showing individual saccharides in the dextran fractions of Table II. The reference spot at R was a mixture of glucose with pure penta-saccharide.

charring of powders. It seems likely that decomposition is initiated by traces of acid and subsequently becomes autocatalytic.

Anticoagulant Activity

Anticoagulant activity *in vivo* was observed after injection of the sodium salt of the dextran sulphate dissolved in 0.5 or 1 ml. saline into the marginal ear-vein of a rabbit. Clotting time was measured by the method of Dale and Laidlaw (1911) on blood taken from the opposite ear, the first drop of blood being discarded to minimize contamination with tissue thromboplastin.

Anticoagulant activity *in vitro* was measured using recalcified horse plasma (Ricketts and Walton, 1953). To avoid the uncertainties of comparing various dextran sulphates with heparin a dextran sulphate preparation was adopted as a standard. This standard had previously been compared with heparin in several types of assay and its activity found to be about 15 u./mg. This figure was used in calculating the activities of the different preparations.

RESULTS

The effect of the polydisperse (U series) preparations upon the blood clotting time of a rabbit is shown in Fig. 3. Their efficacy, on a weight basis, in raising the clotting time diminishes with decreasing molecular size. This is well illustrated by the effect of 120 mg./kg. of the short chain preparation U 7 being less than the effect of 15 mg./kg. of the preparation U 20, which has a longer molecular chain. Another useful comparison is the result obtained with the dextran sulphate fraction I 7.6 which has the biological properties advocated for a clinical anticoagulant (Ricketts and Walton, 1953). The effect with this fraction at 10 mg./kg. is greater than the effect of U 10 at 15 mg./kg.

The effect of the less disperse preparations (O series) upon the blood clotting time of a rabbit is shown in Fig. 4. Considering the effects at a dose of 10 mg./kg. it is clear that there is a decreasing effect on clotting time with decreasing molecular weight. This is confirmed by the result with the long chain preparation OE, which, in a dose of 6 mg./kg., has a greater effect than 10 mg./kg. of the shorter chain preparations OC and OB.

The *in vitro* anticoagulant activities of members of the U and the O series of preparations are given in the last column of Tables I and II.

DISCUSSION

Dextran sulphate shows anticoagulant activity over a very wide range of molecular weight. Large molecules have the undesirable effects of precipitating proteins and aggregating blood platelets. Dextran sulphate with molecules composed of about 20 glucose units has given satisfactory results as a clinical anticoagulant (Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy, and Burt, 1953). The present study has explored the anticoagulant action of molecules with fewer than 20 glucose units.

Preparations in both the U series and the O series have successively lower molecular weights; the main difference is that the preparations in the U series are considerably more polydisperse than those in the O series.

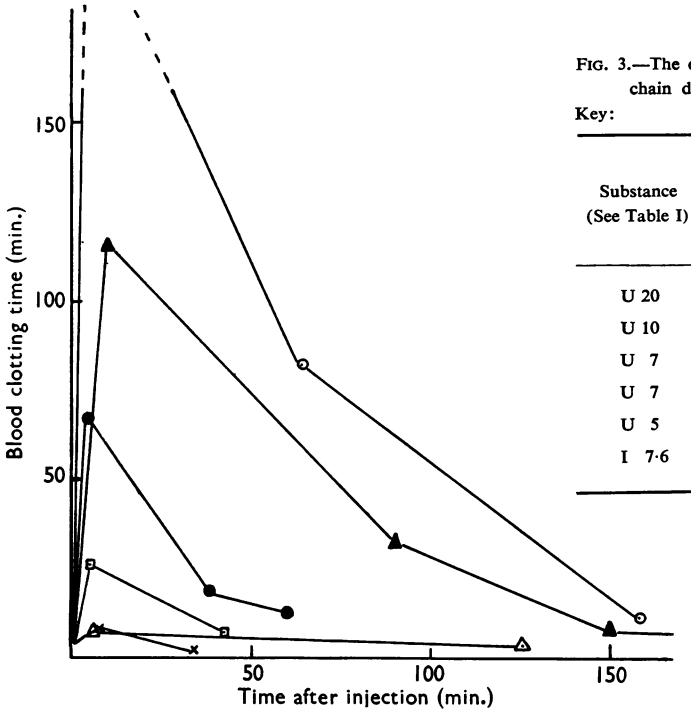

FIG. 3.—The elevation of blood clotting time in a rabbit by short chain dextran sulphate preparations of the U series.

Key:

Substance (See Table I)	Intravenous Dose		Symbol
	mg./kg.	u./kg.*	
U 20	15	270	○——○
U 10	15	130	⊡——⊡
U 7	15	112	△——△
U 7	120	900	▲——▲
U 5	15	70	×——×
I 7·6	10	120	●——●

* As determined by *in vitro* assay.

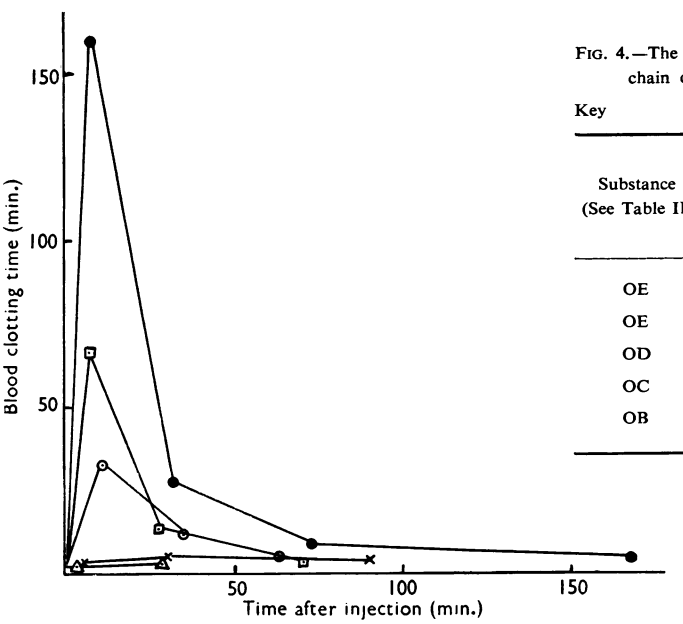

FIG. 4.—The elevation of blood clotting time in a rabbit by short chain dextran sulphate preparations of the O series.

Key

Substance (See Table II)	Intravenous Dose		Symbol
	mg./kg.	u./kg.*	
OE	10	140	●——●
OE	6	80	○——○
OD	10	80	⊡——⊡
OC	10	80	△——△
OB	10	80	×——×

* As determined by *in vitro* assay.

When anticoagulant activity is measured *in vitro*, the activity of the polydisperse preparations of the U series falls as the average size of the dextran sulphate molecules decreases—as indicated by the intrinsic viscosity (Table I). As might be expected, this fall in activity is not so marked in the O series where the lower members, OC and OB (Table II), do not contain inactive esters such as those of glucose and isomaltose.

The data of Figs. 3 and 4 show that the shorter chain preparations are less effective, on a weight basis, in raising the blood clotting time of rabbits. Thus the fall of anticoagulant activity with chain-length below about 20 glucose units is confirmed.

An alternative comparison of anticoagulant effect *in vitro* and *in vivo* may be made on a potency basis. Comparing the effect of equipotent doses of the O series preparations, shown in Fig. 4, it is clear that the effect on blood clotting time is less with preparations OB and OC, of shorter chain-length, than it is with the longer chain preparations OD and OE. A partial explanation of this may be that whereas potency is measured using plasma the effect *in vivo* is measured on whole blood, so that the inhibition of a different selection of clotting factors is involved. It is probable that these short chain preparations rapidly escape from the blood stream; this would also explain their slight effect upon blood clotting time in the doses used.

Thus molecules of dextran sulphate with fewer than 6 glucose units, though active *in vitro*, do not appreciably contribute to the elevation of blood clotting time *in vivo*.

SUMMARY

1. Investigation of the blood anticoagulant effect of dextran sulphate has been extended into the oligosaccharide region.

2. Anticoagulant activity assayed *in vitro* decreases with decreasing molecular chain-length.

3. The effect upon the blood clotting time of the rabbit decreases more markedly with decreasing size than would be predicted from assay *in vitro*. This may be due to rapid disappearance of the smaller molecules from the blood stream.

It is a pleasure to acknowledge many helpful discussions with Dr. J. P. Bull and Dr. K. W. Walton and to thank Professor J. R. Squire for his encouraging interest in this work.

REFERENCES

Bailey, J. M., Whelan, W. J., and Peat, S. (1950). *J. chem. Soc.*, p. 3692.

Bayly, R. J., and Bourne, E. J. (1952). *Nature, Lond.*, **171**, 385.

Dale, H. H., and Laidlaw, P. P. (1911–12). *J. Path. Bact.*, **16**, 351.

Ricketts, C. R. (1952a). *Biochem. J.*, **51**, 129.

—— (1952b). *Chem. & Ind.*, p. 982.

—— and Walton, K. W. (1953). *Brit. J. Pharmacol.*, **8**, 476.

—— —— van Leuven, B. D., Birbeck, A., Brown, A., Kennedy, A. C., and Burt, C. C. (1953). *Lancet*, **2**, 1004.

Schaffer, P. A., and Hartman, A. F. (1921). *J. biol. Chem.*, **45**, 377.

Whistler, R. L., and Durso, D. F. (1950). *J. Amer. chem. Soc.*, **72**, 677.