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THE BIOLOGICAL BEHAVIOUR OF A NEW SYNTHETIC ANTICOAGULANT (DEXTRAN SULPHATE) POSSESSING HEPARIN-LIKE PROPERTIES

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The sulphuric esters of polysaccharides react readily with a variety of proteins. Heparin, which has been shown to be essentially a polysulphuric ester of mucoitin (Jorpes, 1935 ; Jorpes and Bergström, 1936), is thought by many authors (Fischer, 1931; Jaques, 1943; Jorpes, 1946) to owe its versatility of biological behaviour to the ease with which it interacts with different components of the plasma proteins, including the " clotting proteins." The sulphuric esters of other large molecular weight polysaccharides such as starch, cellulose, chitin, pectic acid, and alginic acid have been shown to possess some degree of heparin-like activity (Bergström, 1936; Chargaff, Bancroft, and Stanley-Brown, 1936; Karrer, Usteri, and Camerino, 1944; Astrup, Galsmar, and Volkert, 1944; Snyder, 1950; Kazal, Spicer, and Brahinsky, 1950; Mangieri, Engelberg, and Randall, 1951). This similarity of action has been ascribed to the strong electro-negative charges carried by the ester sulphate groups which confer great reactivity upon these molecules in the presence of molecules bearing opposite charges, such as proteins and bases (Demole and Reinert, 1930; Bergström, 1935, 1936; Jorpes, 1946).

The interaction of heparin and other naturally-occurring mucopolysaccharides with certain proteins has been shown to result in visible precipitate formation when the reaction has been carried out under suitable conditions of *p*H, temperature, salt and colloid concentrations (Fischer and Astrup, 1935; Meyer, Palmer, and Smyth, 1937; Jaques, 1943). This precipitate formation resembles that occurring between acid polysaccharides and complex bases, and has led to the assumption that, under physiological conditions, the reaction between heparin and the " clotting proteins," like that between, say, heparin and protamine, takes the form of a straight combination between the basic groups of the protein and the acidic groups of the heparin (Jaques, 1943).

The heparin-like activity of synthetic polysaccharide sulphate esters has led to their investigation as substitutes for heparin in anticoagulant therapy. Several such compounds investigated by Astrup and Piper (1946) were judged unsuitable for clinical use, since, though active anticoagulants, they caused the precipitation of plasma fibrinogen and the agglutination of blood platelets, while, when given in large doses, they caused death in experimental animals from internal bleeding.

It is the purpose of this communication to provide evidence that the compound formed between one polysaccharide sulphate ester (dextran sulphate) and fibrinogen, under physiological conditions, is a loosely combined complex which does not behave like a simple salt. The formation of this complex, it is suggested, is much more dependent upon the molecular size of the acid polysaccharide component than upon the charge borne by its acid radicals. This is of importance in determining the selection of suitable starting-material from which to synthesize non-toxic heparin analogues from dextrans, as will be more fully discussed in subsequent communications.

Dextran sulphates

Grönwall, Ingelman, and Mosimann (1945) reported an investigation of three sulphuric esters prepared from dextrans of different molecular weights. They found these possessed anticoagulant properties, but regarded them as too toxic for therapeutic use. They noted that toxicity declined as the compounds decreased in molecular weight, but when Ingelman (1947) later found that an inulin sulphate of even smaller molecular weight was more toxic than the smallest dextran sulphate, he concluded that molecular weight was not a factor in determining the toxicity of these compounds. From this author's results, it is possible to infer that the molecular weight of his least toxic preparation of dextran sulphate must have been approximately 38,000, that is, considerably larger than that of heparin, the molecular weight of which he himself estimated to be about 17,000, a value subsequently confirmed by Jensen, Snellman, and Sylvén (1948). The detailed behaviour of the dextran sulphates in relation to the plasma proteins was not reported.

A new range of dextran sulphates, extending both above and below the molecular weights of Ingelman's compounds, was prepared and described by Ricketts (1952a). These preparations were found to have anticoagulant potencies of about one-seventh that of International Standard Heparin. It was found that they also simulated other biological activities of heparin. It was noted that when a dextran sulphate approximated to or was smaller than heparin in molecular weight, its toxicity was no greater than heparin and when added to plasma it did not cause precipitation of protein. On the other hand, dextran sulphates with molecular weights considerably greater than that of heparin were more toxic, in that they produced a marked bleeding tendency in animals, and these compounds did precipitate protein from plasma.

These observations suggested (i) that molecular weight *is* an important factor in determining toxicity when a series of such compounds is examined, (ii) that " toxicity," in the sense of producing a bleeding tendency, is mediated through precipitation of " clotting proteins," and (iii) that the heparin-like activity of the dextran sulphates might be due to a similar facility for forming complexes with various plasma components.

It was found that dextran sulphates chiefly affected the stability of fibrinogen in solution, though traces of other proteins were also removed from solution when the large molecular weight compounds were added to serum. In the present communication, the interactions of these compounds with purified fibrinogen are described. Their behaviour in relation to bases, to other plasma proteins, and to the formed elements of the blood have previously been presented in summarized form (Walton, 1951) and will be reported in detail separately.

Materials and Methods

Dextran sulphates.—The structural relationship of the dextran sulphates to heparin is shown in Fig. 1; for simplicity, the basic unit of structure of heparin is taken to be glucuronic acid-glucosamine, this disaccharide unit being repeated to form a complex polymer.

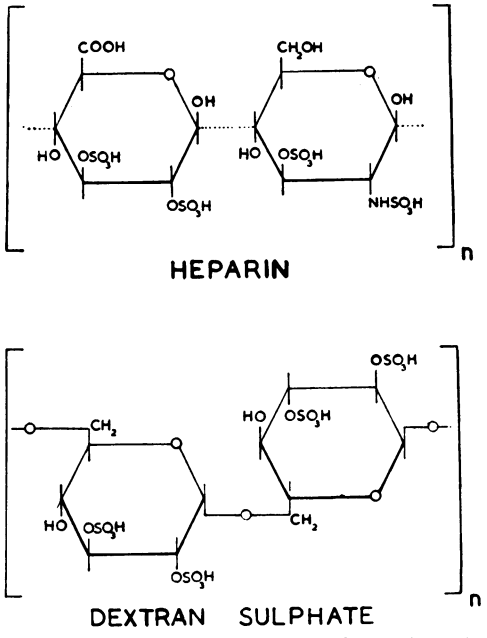

FIG. 1.—Comparison of the basic structural units of heparin and dextran sulphate.

Since the exact nature of the glucuronic acid-glucosamine link is not known, it is shown here as a dotted line. It has been shown by Jorpes and Gardell (1948) that most heparin samples are not homogeneous but consist of a mixture of mono-, di-, and tri-sulphuric esters. The spatial relationships of the sulphate radicals is not known with certainty, though Jorpes, Bostrom, and Mutt (1950) and Meyer and Schwartz (1950) have suggested that at least one sulphate group is coupled via the amino-group of the glucosamine moiety. In the formula shown, one sulphate group is coupled thus while the remainder have been inserted arbitrarily to give a di-sulphuric ester. Heparin is commonly regarded as not completely homogeneous in molecular size. The molecular weight of 17,000 for a purified sample of heparin was regarded as an average for the distribution of molecules in the preparations examined (Grönwall *et al.*, 1945; Jensen *et al.*, 1948).

Dextran is a glucose polymer consisting of long branching chains in which glucose units are linked mainly by 1 : 6-α-glucoside links (Stacey and Ricketts, 1951). For comparison with heparin, the basic unit of dextran sulphate is also shown as a disaccharide in which, again, sulphate radicals have been inserted in arbitrary positions. It has been assumed that the parent dextrans in the preparations used were of similar chemical composition but of differing mean molecular weight corresponding to observed differences in intrinsic viscosity. The sulphuric esters prepared from these dextrans were then assumed to differ in mean molecular weight correspondingly, allowance being made for the extent to which sulphate radicals were introduced into the parent polysaccharide. The method

of preparation of the compounds used and the methods of estimation of their molecular weights have been previously described by Ricketts (1952a). The limitations imposed by the available methods of estimating molecular weights when applied to these compounds were discussed by Ricketts, who stressed the difficulty of obtaining precise estimates for the compounds at the lower end of the scale. The compounds formed are in two molecular weight groups: " large," an extended range of the compounds investigated by Ingelman *et al.* (1945) ranging from molecular weight 39,800 to 515,000; and " small," designed to approximate in size to heparin. The " large " had three subgroups, D (3 samples), A (2 samples), and E (2 samples) of average molecular weights 458,000, 129,000, and 47,000 respectively. The " small " had only one subgroup, I (3 samples), designed to be the same molecular weight as heparin (17,000), but osmotic pressure measurements showed that they were, in fact, of molecular weight, 7,000 to 8,000. The subgroups had different sodium dextran sulphates derived from the same parent dextran of the subgroup but containing varying percentages of sulphur as SO_4 and differing in molecular size within the group. Precise assay of their anticoagulant activity was vitiated by their precipitation of proteins in whole blood or plasma. Assay of the small molecular weight dextran sulphates, and of the commercial samples of heparin used, was performed by the method of Kuizenga, Nelson, and Cartland (1943) against a sample of the International Standard Heparin. The I size compounds were found thus to assay at 15–20 International (Heparin) Units per mg.

In one series of experiments designed to investigate the effect of further variation of sulphur content upon biological activity, a separate range of dextran sulphates was prepared and employed. These compounds originated from the same dextran as that used for the I size compounds, but their sulphur contents varied from 9 to 17 per cent. These compounds are listed separately in Table I.

TABLE I

SMALL MOLECULAR WEIGHT DEXTRAN SULPHATES PREPARED FROM THE SAME PARENT DEXTRAN BUT VARYING IN SULPHUR CONTENT

Serial No.	% Sulphur	SO_4 per glucose unit	Anticoagulant activity, units per mg.
AO	9.2	0.64	2.6
BB	11.8	1.0	3.75
BD	14.2	1.3	15.0
BE	15.9	1.6	15.0
I/4	17.1	1.9	18.6

Heparin.—For experiments other than assay, heparin was obtained from commercial sources. Batch No. F.85020 of Evan's heparin, assaying at 90 i.u. per mg. and containing 8.61 per cent sulphur, and " Liquemin " Roche (batch dated 31.8.48), assaying at 120 i.u. per mg. and containing 9.3 per cent sulphur, were employed throughout this investigation.

Fibrinogen and thrombin.—Lyophilized human fibrinogen and thrombin were supplied by the Blood Products Research Unit of the Lister Institute, London. The method of preparation and limits of purity of these products have been described by Kekwick, Mackay, and Record (1946).

Buffer solutions.—A stock solution of the universal buffer described by Teorell and Stenhagen (1938) was prepared by the method of Ostling and Virtama (1946). This buffer varies in ionic strength with *p*H between 0.07 and 0.10 μ over the range *p*H 2.5–9. In

use, appropriate amounts of 0.1 N-hydrochloric acid were added to give solutions of the required *p*H and these were finally checked with a glass electrode *p*H-meter. In certain specified experiments, Sorensen's 0.66 M-phosphate buffers at *p*H 6.8 and *p*H 7.0 were employed.

Toluidine blue.—Batches No. 948 and No. 1234 of toluidine blue G were supplied by Messrs. G. T. Gurr Ltd., London. When a 1 per cent (w/v) solution of this dye in distilled water was added to aqueous solutions of the dextran sulphates or heparin its normal blue (orthochromatic) colour changed to a vivid reddish purple (metachromatic) colour, with aggregation of the dye in particles at high concentrations of the acid polysaccharides.

EXPERIMENTAL

On mixing the various dextran sulphate preparations with normal human plasma, it was observed that, at certain concentrations, the compounds of large molecular weight (D, A, and E size) caused the appearance of a dense flocculent white precipitate. When the same compounds were added to normal human serum only a moderate turbidity occurred. It was therefore thought that the precipitate formed in plasma must consist mainly of fibrinogen. This conclusion was supported by the finding that similar precipitates to those produced in plasma could be obtained by mixing these compounds with solutions of purified human fibrinogen.

On the other hand, admixture of the small molecular weight (I size) dextran sulphate with either plasma, serum, or a solution of fibrinogen produced no visible change.

The interaction of the dextran sulphates with fibrinogen

Experiment 1.—0.5 ml. of 1 per cent (w/v) aqueous solutions of the dextran sulphates D/3, A/1, E/1, and I/3 were added to 5 ml. quantities of 0.6 per cent (w/v) human fibrinogen in distilled water. The molecular weights of these four sulphates were 440,000, 137,000, 39,800, and 7,000–8,000 respectively. A control tube contained 5 ml. of the same fibrinogen solution and 0.5 ml. of distilled water.

Within a few seconds, the mixtures containing the D, A, and E size compounds became cloudy and opaque. The opacity rapidly increased and within a few minutes the particles aggregated to form a granular precipitate. The particles then slowly increased in size to form heavy white floccules which settled to the bottom of the tube. Maximal precipitation at room temperature (19° C.), as judged naked-eye, occurred in 20–30 minutes. Throughout this period the mixture containing the I size dextran sulphate did not differ in opacity from the control. The relative precipitations were: D/3 +++, A/1 ++, E/1 +, and I/3 nil.

After maximal precipitation had occurred in the tubes containing the D, A, and E sizes, the tubes were centrifuged at 2,500 r.p.m. for 10 minutes. The supernatant fluid was removed and the precipitate washed three times with physiological saline to free it from excess fibrinogen or dextran sulphate. The precipitates from each tube were then redissolved in 1 ml. of 10 per cent (w/v) calcium chloride and treated identically. The new solution (in calcium chloride) was divided into two parts. To one part was added one drop of concentrated human thrombin solution (50 units/ml.). Within a few seconds a fibrin clot formed. To the other part one drop of 1 per cent (w/v) toluidine blue was added. This produced the bright purplish-red " metachromatic reaction " which is given by pure dextran sulphate. Neither the original fibrinogen solution nor the calcium chloride solution gave a similar metachromatic reaction.

From this experiment it was concluded: (i) that the precipitate which appeared in some instances was composed of both dextran sulphate and fibrinogen, (ii) that

the volume of the precipitate appeared to bear some relation to the molecular weight or size of the dextran sulphate employed, and (iii) that the slow rate of maximal appearance of the precipitate and the ease with which it could be redissolved might be due to a loosely-formed complex rather than to the formation of a true salt. The last conclusion was supported by the observation that the fibrinogen in forming the precipitate did not appear to be denatured, since it could be subsequently redissolved and clotted by the action of thrombin.

Experiments were next carried out to investigate the effects on this complex-formation of variations of *p*H colloid concentration, temperature, and salt concentrations, with simultaneous variation of the mean molecular weight of the dextran sulphates by the employment of selected numbers of the available series of compounds. In each experiment, heparin was employed in parallel to provide a standard of comparison. The alterations of the environment of the reaction as outlined above are known to influence the stability of systems comprised of a mixture of colloids (Glasstone, 1947), and in particular the solubility of the plasma proteins (Oncley, 1950).

1. *The effect of variation of* p*H*

Experiment 2.—Buffer solutions at *p*H 2.5, 4.0, 6.8, and 9.0 were prepared, as described, from the stock Teorell and Stenhagen solution. These buffers offered the advantage of constant content of cations, other than hydrogen ions, over this *p*H range. Heparin, dextran sulphates of the D, E, and I sizes, and human fibrinogen solutions were prepared in the buffers at each reaction so as to give separate 0.1 per cent (w/v) concentrations. Each anticoagulant at a given reaction was then mixed with fibrinogen at the same reaction,

TABLE II

EFFECT OF DEXTRAN SULPHATES OF VARYING MOLECULAR WEIGHTS ON FIBRINOGEN AT DIFFERENT REACTIONS

	Tube No.	1	2	3	4	5
Anticoagulant	ml. of 0.1% soln.	4	3	2	1	0
Fibrinogen	ml. of 0.1% soln.	0	1	2	3	4
(a) *p*H 2.5						
Dextran sulphate D/3		0	+++	++++	++++	0
E/1		0	+	++	+++	0
I/3		0	+	++	+++	0
Heparin		0	+	++	+++	0
(b) *p*H 4.0						
Dextran sulphate D/3		0	+	++	+++	0
E/1		0	±	++	+++	0
I/3		0	±	++	+++	0
Heparin		0	±	++	+++	0
(c) *p*H 6.8						
Dextran sulphate D/3		0	0	±	++	0
E/1		0	0	±	+	0
I/3		0	0	0	0	0
Heparin		0	0	0	0	0

in similar proportions. The tubes containing these mixtures were placed in a water-bath at 37° C. and observed, at intervals, for thirty minutes.

It was observed that maximal precipitation occurred immediately with all the acid polysaccharides at *p*H 2.5 and *p*H 4.0. On the other hand, flocculent precipitation developed slowly at *p*H 6.8 and then only in the mixtures containing the D and E size dextran sulphates. No precipitation occurred with heparin or the I size dextran sulphate at this *p*H. Further increase of alkalinity to *p*H 9.0 abolished precipitate formation in all the mixtures tested. These results are summarized in Table II.

The iso-electric point of human fibrinogen is given as *p*H 5.2 (Morrison, Edsall, and Miller, 1948). From these results it appeared that heparin and the small molecular weight dextran sulphate behaved similarly to the large molecular weight compounds in producing precipitates at reactions below this point. But at a reaction above the iso-electric point of the protein and close to physiological *p*H the previous divergence of behaviour with regard to precipitation was again observed, the large molecular weight compounds causing precipitation but heparin and the small molecular weight compound failing to produce any visible change.

2. *The effect of variation of colloid concentration*

Experiment 3.—Varying concentrations of heparin and the different dextran sulphates were titrated against a constant concentration of fibrinogen at physiological *p*H and temperature (*p*H 7.0 and 37° C.) in the following manner:

In a series of small glass tubes, doubling dilutions of the acid polysaccharides were made in 0.66 M-phosphate buffer at *p*H 7.0 from stock 1 per cent (w/v) solutions. To each tube was added an equal volume of 1.26 per cent (w/v) human fibrinogen in distilled water. A control tube contained a similar volume of fibrinogen plus an equal volume of phosphate buffer alone. The tubes were placed in a water-bath at 37° C. and observed at intervals for thirty minutes. The results are shown in Table III.

TABLE III

TITRATION OF VARYING DILUTIONS OF 1 PER CENT HEPARIN AND DEXTRAN SULPHATES AGAINST 0.63 PER CENT FIBRINOGEN AT *p*H 6.8 AND 37° C.

Tube No.	1	2	3	4	5	6	7	8	9	10	Control
Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
Heparin ..	0	0	0	0	0	0	0	0	0	0	0
Dextran sulphate I/3	0	0	0	0	0	0	0	0	0	0	0
E/1	±	±	+	++	+++	++	++	±	0	0	0
D/1	0	±	+	++	+++	++++	++++	++	++	+	0

Experiment 4.—The converse experiment of titrating various concentrations of fibrinogen against constant concentration of the acid polysaccharides was performed using the same solutions as in Experiment 3 and a similar technique. The results are shown in Table IV.

From Tables III and IV it will be seen: (i) that as before, no precipitation occurred with heparin or the I size dextran sulphate at physiological *p*H, whereas (ii) at the same *p*H, precipitates were produced by the large molecular weight dextran sulphates, and (iii) these precipitates were greater in volume and occurred at greater dilutions in step with the increase in molecular weight of members of the D and E groups.

TABLE IV

TITRATION OF VARYING DILUTIONS OF 1.26 PER CENT FIBRINOGEN AGAINST 0.5 PER CENT DEXTRAN SULPHATES AT *p*H 6.8 AND 37° C.

Tube No.	1	2	3	4	5	6	7	8	9	10	Control
Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
Heparin ..	0	0	0	0	0	0	0	0	0	0	0
Dextran sulphate											
I/3	0	0	0	0	0	0	0	0	0	0	0
E/1	±	+	++	+	±	0	0	0	0	0	0
D/1	0	±	++	+++	+++	++	+	±	0	0	0

Inhibition of precipitation by heparin and I size dextran sulphate

It was found that, at physiological *p*H, heparin and the small molecular weight dextran sulphate, if present in high concentrations, inhibited or greatly delayed the precipitation of fibrinogen by the large molecular weight dextran sulphates. Freshly-formed precipitates produced by the interaction of the large molecular weight compounds with fibrinogen could be made to redissolve by high concentrations of heparin or the I size dextran sulphate. Similar precipitates which had been allowed to stand for twenty-four hours or longer were less easily and less completely redissolved.

Experiment 5.—0.1 ml. quantities of 10 per cent (w/v) heparin and of 10 per cent (w/v) dextran sulphate I/4 respectively were added to tubes each containing 1 ml. of 0.6 per cent fibrinogen. A control tube contained 1 ml. of 0.6 per cent fibrinogen plus 0.1 ml. of distilled water. The tubes were placed in a water-bath at 37° C. and 0.1 ml. of 1 per cent dextran sulphate D/3 was added to each. A heavy flocculent precipitate appeared in the control tube, but no precipitation occurred in the tubes containing the heparin or the I size dextran sulphate. Lower concentrations (1–5 per cent) of the latter compounds were not similarly effective in inhibiting precipitate formation by the D size compounds. 0.1 ml. quantities of 10 per cent heparin or of 10 per cent dextran sulphate I/4 redissolved the precipitate formed in the control tube.

Further study of the precipitation of fibrinogen by the large molecular weight dextran sulphates was carried out by experiments in which all practicable dilutions of fibrinogen were tested with all practicable dilutions of each of the dextran sulphates in " chess-board " patterns. One such experiment is illustrated in Table V.

From such experiments, it became clear that the reaction between the large molecular weight dextran sulphates and fibrinogen at physiological *p*H, like that between certain other colloids bearing similar charges, could serve as a model of an antigen-antibody precipitation reaction. For instance: (*a*) The pattern of maximal and minimal precipitation, as shown in Table V, closely resembled that seen with precipitin reactions. (*b*) Maximal precipitation occurred with constant optimal proportions of fibrinogen, and of any one given large molecular dextran sulphate. (*c*) The dilution giving maximal precipitation when varying concentrations of dextran sulphate were titrated against a constant concentration of fibrinogen was not identical with that obtained in the converse titration with the same components. (*d*) Precipitation was inhibited in the region of dextran sulphate or fibrinogen excess (prozone phenomenon). The reaction differed, however, from a true antigen-antibody

TABLE V

EFFECT OF VARYING DEXTRAN SULPHATE–FIBRINOGEN RATIOS ON RATE AND AMOUNT OF PRECIPITATE

Fibrinogen dilution	Dextran sulphate dilution										
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1/1	0	±[2]	+	++	+++[1]	+++[1]	++	++	+	±[2]	0
1/2	0	0	±[2]	±	+	++[1]	++[1]	+	±	±[2]	0
1/4	0	0	0	0	±[2]	±	+[1]	+[1]	±	±[2]	0
1/8	0	0	0	0	0	±[2]	±	+[1]	+	±[2]	0
1/16	0	0	0	0	0	0	0	±[2]	±[1]	±[2]	0
1/32	0	0	0	0	0	0	0	0	0	0	0

Dilutions of dextran sulphate D/3 in 0.9% saline from a stock solution of 1% dextran sulphate. Dilutions of fibrinogen in 0.9% saline from a stock solution of 0.9% fibrinogen. *p*H of solutions 6.8. Temperature 19° C. [1] Maximal precipitation. [2] Minimal precipitation.

reaction in being non-specific. Subsequent attempts to sensitize animals to the various dextran sulphates were unsuccessful and no evidence was obtained that these compounds could behave as true antigens *in vivo* (Walton, 1951).

Titration of the various dextran sulphates against fibrinogen at *p*H 2.5 and at *p*H 4.0 showed two important points of difference from the reaction at physiological *p*H. These were: (i) Simple decrease of precipitation with decrease of dextran sulphate concentration (i.e. absence of maximal precipitation with constant optimal proportions and of " prozoning "); and (ii) the volume of the precipitate bore no obvious relation to the molecular weight of the compound concerned. This latter point was subsequently confirmed quantitatively (see below).

It was possible to infer from the minimal concentrations of fibrinogen and of the large molecular dextran sulphate producing precipitation at physiological *p*H *in vitro* that similar precipitation might occur *in vivo* with intravenous administration of these compounds in doses sufficient to produce an anticoagulant effect. It was subsequently confirmed that, under such circumstances, these compounds caused a temporary drop in the level of circulating fibrinogen. On the other hand, the I size compounds, which did not precipitate fibrinogen from solution in the test-tube, produced no alteration of the plasma fibrinogen level *in vivo* (Walton, 1951).

3. *The effect of variation of temperature*

Because of the observed differences in the reaction with fibrinogen at *p*H levels, above and below the iso-electric point of the protein, the effect of variation of temperature was observed at both *p*H 6.8 and *p*H 4.0.

(*a*) *At* p*H* 6.8.—It was found that variation of the temperature between 0° C. and 37° C. produced no visible alteration in the reaction between fibrinogen and either heparin or the I size dextran sulphates, in that no precipitation occurred over this temperature range. On the other hand, the precipitate produced by the large molecular weight dextran sulphates was reversibly altered in character and amount by variation of the temperature within this range, as illustrated in the following experiment.

Experiment 6.—Doubling dilutions of an E size dextran sulphate were made in triplicate in 0.4 ml. volumes of 0.66 M-Sorensen's phosphate buffer at *p*H 6.8 from a stock solution of 1 per cent (w/v) dextran sulphate E/1. To each tube was added 0.4 ml. of 1.6 per cent (w/v) human fibrinogen in the same phosphate buffer. Control tubes in each rack contained a similar volume of the fibrinogen solution plus an equal volume of phosphate buffer alone. Rack A was allowed to stand on the bench (room temperature 19° C.). Rack B was placed in a water-bath at 37° C. Rack C was placed in a refrigerator at 1° C. The racks were inspected after thirty minutes and the nature and volume of the precipitate noted. The positions of Racks B and C were now reversed—i.e., Rack B was placed in the refrigerator and Rack C in the water-bath. At the end of a further half-hour they were again inspected and the nature and amount of the precipitate once more recorded. Finally, all three racks were left upon the bench at room temperature overnight, and their contents inspected and the results recorded the following morning. The results of this experiment are shown in Table VI.

TABLE VI

THE EFFECT OF VARIATION OF TEMPERATURE ON THE REACTION BETWEEN 1.6 PER CENT FIBRINOGEN AND 1 PER CENT DEXTRAN SULPHATE E/1 AT *p*H 6.8

(a) At the end of 30 minutes

	Tube No.	1	2	3	4	5	Control	Nature of precipitate
	Dilution	1/2	1/4	1/8	1/16	1/32		
Rack A ..	19° C.	+	++	++	+++	++	0	Coarse granules
Rack B ..	37° C.	±	+	++	++	+	0	Fine granules
Rack C ..	1° C.	++	+++	+++	++++	+++	0	Heavy floccules

(b) After second 30 minutes (Racks B and C reversed in refrigerator and water-bath respectively)

	Tube No.	1	2	3	4	5	Control	Nature of precipitate
	Dilution	1/2	1/4	1/8	1/16	1/32		
Rack A ..	19° C.	+	++	++	+++	++	0	Coarse granules
Rack B ..	1° C.	++	+++	+++	++++	+++	0	Heavy floccules
Rack C ..	37° C.	±	+	++	++	+	0	Fine granules

From Table VI (a) it will be seen that at the end of the first half-hour the precipitate varied in character and amount in the three racks, being greatest in bulk and composed of large floccules at the lowest temperature, intermediate in amount and character at room temperature, and least in bulk and made up of fine granules at body temperature. From Table VI (b) it will be seen that at the end of the second half-hour period, Rack B, which had been first at 37° C. and then at 1° C., showed an apparent increase in the amount of precipitate on cooling with a change from fine granules to heavy floccules. Rack C, on the other hand, which had been first at 1° C. and then at 37° C., showed apparent lessening of the bulk of the precipitate on warming, with the opposite change from heavy floccules to fine granules. Rack A, which was kept at 19° C. throughout both half-hour periods, showed a precipitate intermediate in amount and character. When all three racks were left upon the

bench overnight, the precipitate in corresponding tubes of all three racks became identical in character and amount. The changes described could be repeated many times at will by the appropriate alteration of temperature.

The increase in precipitation on cooling was shown to be an actual one in the following further experiment:

Experiment 7.—The titration of dextran sulphate E/1 against fibrinogen was repeated as in the preceding experiment and the rack was placed in the water-bath at 37° C. for thirty minutes. The tubes were then spun in a warm centrifuge at 2,500 r.p.m. for fifteen minutes, and the water-clear supernatant fluid decanted into fresh tubes which were placed in the refrigerator at 1° C. After half an hour, the tubes were inspected and a fresh flocculent precipitate was seen to have formed. When the rack was now replaced in the water-bath at 37° C., this precipitate dissolved, leaving the fluid water clear once more.

It was found that the precipitates formed by the A and D size compounds with fibrinogen behaved similarly under the conditions of Experiments 7 and 8, over the temperature range 0°–37° C. Further increase in temperature (to 45°–50° C.) caused complete solution of the precipitate, but it reappeared on cooling. Increase to 56° C. caused denaturation of the fibrinogen. The influences of the dextran sulphates and heparin on the denatured protein are described below.

(*b*) *At* p*H* 4.0.—When similar experiments were performed at *p*H 4.0, the precipitates formed by heparin and all the sizes of dextran sulphate showed little or no difference in amount or character over the range 0°–37° C. Increase of temperature caused, if anything, further aggregation of the precipitate with the formation of larger, coarser floccules, but once maximal aggregation had occurred no further change was noted on heating or cooling.

4. *The effect of variation of electrolyte concentration*

(*a*) *At physiological* p*H*.—It was found that the precipitates produced by the large molecular weight dextran sulphates at this reaction could be redissolved by increase of the electrolyte concentration. The effect of various cations in this respect was investigated as follows:

Experiment 8.—All solutions were prepared in carbon-dioxide-free redistilled water at *p*H 7.0. Precipitates were produced by adding 0.2 ml. of 1 per cent (w/v) dextran sulphate D/2 to 2 ml. of 0.9 per cent (w/v) human fibrinogen in the presence of 1 ml. of 1 per cent (w/v) solutions of the chlorides of magnesium, calcium, strontium, barium, potassium, and sodium. In each case the mixture was centrifuged and the supernatant removed and replaced by 3 ml. of 10 per cent (w/v) solutions of the same salts. The effects were observed at room temperature (19° C.) and at 37° C.

It was found that the higher concentrations of cations caused redissolution of the precipitate to varying degrees. In the presence of high concentrations of the divalent cations Mg^{++} and Ca^{++}, the precipitate was completely redissolved at 19° C., giving water-clear solutions. The divalent cations Sr^{++} and Ba^{++} also dispersed the particulate precipitate but yielded opalescent solutions at room temperature. These lessened in opalescence at 37° C. The monovalent cations K^{+} and Na^{+} caused partial solution of the precipitate at 19° C. and further solution at 37° C. In this respect these cations behaved in correspondence with their positions in the lyotropic series of Hofmeister.

The effect of various anions was also observed in a similar manner, the citrate, tartrate, chloride, nitrate, iodide, and thiocyanate of sodium being investigated. These anions again behaved in accordance with their positions in the Hofmeister series, the iodide and

thiocyanate (monovalent anions) being more effective in redissolving the precipitate than the tri- or di-valent members of the series.

Still higher concentrations (over 20 per cent) of these electrolytes merely caused " salting out " and denaturation of the protein which was then completely insoluble except in strong alkali.

(*b*) *At* p*H* 2.5 *and* p*H* 4.0.—The precipitates formed by heparin and all sizes of the dextran sulphates at these reactions were not dissolved by similar increase of the electrolyte concentration.

The experiments so far described revealed a number of differences in the characteristics of the interactions between fibrinogen and heparin or the dextran sulphates at reactions above and below the iso-electric point of the protein. These differences are summarized in Table VII.

TABLE VII

DIFFERENCES BETWEEN THE INSOLUBLE COMPLEXES FORMED ABOVE AND BELOW THE ISO-ELECTRIC POINT OF FIBRINOGEN, BY THE LARGE MOLECULAR WEIGHT DEXTRAN SULPHATES

Above the iso-electric point	Below the iso-electric point
1. Molecules of fibrinogen and dextran sulphate bear similar nett charges	1. The molecules of fibrinogen and dextran sulphate bear opposite nett charges
2. Precipitation of fibrinogen occurs only with compounds of molecular weight 40,000 and above	2. Precipitation of fibrinogen occurs with all sizes of dextran sulphate and with heparin
3. Precipitation becomes maximal at 37° C. in 20–30 minutes	3. Maximal precipitation occurs immediately
4. The process resembles antigen-antibody precipitin reactions in that:	4. The process bears no resemblance to antigen-antibody precipitin reactions
(i) " Pro-zoning " occurs at high concentrations	(i) to (iii)
(ii) Maximal precipitation occurs with optimal proportions which differ in a constant dextran sulphate concentration titration from those of a constant fibrinogen concentration titration	These conditions do not apply
(iii) Precipitation is inhibited by high concentrations of heparin or the small molecular weight homologues	
(iv) Precipitates are reversibly dissociated and re-formed by alteration of temperature	(iv) Precipitates cannot be dissociated and re-formed by alteration of temperature
(v) Precipitates are inhibited or redissolved by increase of electrolyte concentration	(v) Precipitates are not inhibited or redissolved by increase of electrolyte concentration
5. The amount of precipitation is not quantitatively related to the content of acidic groups (i.e. to the charge) of the acid polysaccharide component	5. The amount of precipitation gives a linear correlation with the content of acidic groups of the polysaccharide component
6. The amount of precipitation is related to the molecular size of the dextran sulphate	6. There is no obvious relationship between volume of precipitate and molecular size of dextran sulphate

Consideration of these differences suggested that, *at reactions below the iso-electric point of the protein*, the forces engaged in conjugating the components resulted in the formation of strong bonds, capable of uniting the polysaccharide and protein moieties regardless of their molecular size and capable of withstanding the disrupting

effect of thermal agitation of the molecules and the effects of variation of the ionic atmosphere of the reaction. At these reactions, the components bear opposite nett charges and union could be effected by coupling of the negatively-charged acidic radicals of the polysaccharide with the positively charged basic groups of the protein. In such a situation the amount of salt-formation occurring with a given amount of fibrinogen should be dependent on the number of acid radicals borne by the polysaccharide (i.e. in the dextran sulphates, on the content of sulphate groups alone; in heparin, on the content of both sulphate and carboxyl groups). The relationship between fibrinogen precipitation and sulphur content was therefore investigated quantitatively in the following experiment:

Experiment 9.—A 0.1 per cent (w/v) solution of human fibrinogen was prepared in citric acid phosphate buffer at *p*H 2.5; 0.1 per cent (w/v) solutions of heparin (Evans), of the small molecular weight dextran sulphates listed in Table I, and of the parent dextran from which the latter were derived, were prepared in the same buffer; 0.2 ml. quantities of heparin, the dextran sulphates, and the dextran were added to 1.0 ml. of the fibrinogen solution. A control tube contained 1 ml. of fibrinogen plus 0.2 ml. of buffer solution alone. Precipitates formed with the heparin and dextran sulphates. No precipitate was given by the dextran. The precipitates which appeared were centrifuged off and 1.0 ml. aliquots were taken from the supernatants, and from the dextran and control tubes. The fibrinogen content of these samples were estimated by the bromsulphalein method of Grief (1950). Previous experiments had shown that concentrations below 100 mg./ml. of heparin and the I size dextran sulphates did not interfere with this reaction, and that the values obtained for small quantities (0–2,000 μg./ml.) of fibrinogen by this method were in good agreement with those given by a colorimetric method employing the Folin–Ciocalteau reagent (Quick, 1942) or by estimation of protein nitrogen by the micro-Kjeldahl technique. After estimation of the amount of fibrinogen remaining in solution after treatment with heparin or the dextran sulphates, by simple subtraction from the figure obtained for the control, it was possible to derive the amount precipitated in each case by a constant weight of the polysaccharide (200 μg.). The results are shown in Table VIII. When the amount of fibrinogen precipitated was plotted against the sulphur content of the polysaccharide, a linear correlation was obtained, as seen in Fig. 2.

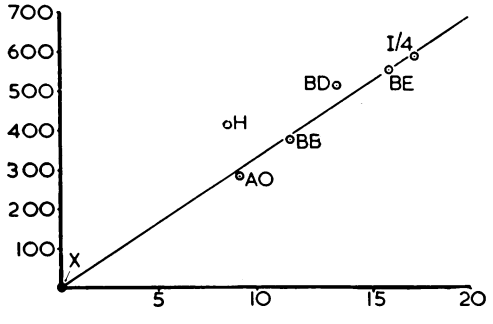

FIG. 2.—Precipitation of fibrinogen by heparin, dextran, and dextran sulphates at *p*H 2.5. Ordinate: Micrograms fibrinogen precipitated by 200 micrograms of the polysaccharides. Abscissa: Percentage sulphur content of the compounds. H = Heparin. X = Dextran (unsulphated). AO, BB, BD, BE, I/4 = Dextran sulphates prepared from dextran X but containing differing amounts of sulphur as sulphate.

It should be noted that the amount of fibrinogen precipitated by heparin is plotted against its sulphur content alone. Its apparent extra capacity for precipitating fibrinogen under these conditions, as shown by its deviation from the curve given by the dextran sulphates, was thought probably to be due to the extra base binding capacity conferred upon it by the possession of carboxyl radicals which were not present in the dextran sulphates.

TABLE VIII

PRECIPITATION OF FIBRINOGEN BY HEPARIN, DEXTRAN, AND SMALL MOLECULAR WEIGHT DEXTRAN SULPHATE AT *p*H 2.5 AND 37° C.

Compound*	Sulphur content (%)	S_2/ glucose-unit	Fibrinogen precipitated (μg.)
Dextran X	—	—	Nil
Dextran sulphate AO	9.2	0.64	281
BB	11.8	1.0	375
BD	14.2	1.3	513
BE	15.9	1.6	552
I/4	17.2	1.9	587
Heparin	8.6	?	412
Control	—	—	Nil

*In each case 200 μg. of the acid polysaccharide in 0.2 ml. citric acid phosphate buffer at *p*H 2.5 was added to 1,000 μg. of fibrinogen in 1 ml. of the same buffer.

On the other hand, consideration of the characteristics of the interactions between these compounds *at reactions above the iso-electric point of the protein* suggested that much weaker intermolecular forces were engaged in conjugating the components. These forces apparently only became fully effective when the compounds approximated to one another in size, appeared to be disrupted easily by thermal agitation of the molecules and to be markedly affected by alteration of the ionic atmosphere of the reaction. The most noticeable feature of this reaction was the variation in the degree of precipitation with variation of the molecular size of the dextran sulphate component. This was also investigated quantitatively in the following experiment.

Experiment 10.—The bromsulphalein method of Grief (1950) was again employed to estimate the amount of fibrinogen remaining in solution after treatment with heparin and the dextran sulphates, but this time the reaction was carried out at *p*H 6.8 and the dextran sulphates were chosen so as to contain approximately equal contents of sulphur but to show wide variation in molecular weight.

Preliminary experiments showed that the presence of concentrations of the D size dextran sulphate greater than 0.5 mg./ml., and concentrations of the A and E sizes greater than 5 mg./ml. interfered with the subsequent precipitation of fibrinogen by bromsulphalein. Heparin and the I size compounds, as previously mentioned, only interfered with the precipitation of fibrinogen by bromsulphalein at concentrations above 100 mg./ml.

TABLE IX

PRECIPITATION OF FIBRINOGEN BY HEPARIN AND DEXTRAN SULPHATES OF VARYING MOLECULAR WEIGHT AT *p*H 6.8 AND 37° C.

Compound*	Sulphur content (%)	S_2/ glucose-unit	Approximate molecular weight	Fibrinogen precipitated (μg.)
Dextran sulphate D/1	14.6	1.4	418,000	1,260
A/1	13.7	1.3	137,000	840
E/1	13.7	1.3	39,800	262
I/2	15.2	1.5	<8,000	Nil
Heparin	8.6	?	17,000 (?)	Nil

* In each case 200 μg. of the acid polysaccharide in 0.1 ml. Sorensen's phosphate buffer at *p*H 6.8 was added to 4,000 μg. fibrinogen in 1 ml. of the same buffer.

1 ml. quantities of fibrinogen solution containing 4 mg./ml. were added to a series of tubes each containing 1 ml. quantities of 0.02 per cent heparin or the dextran sulphates. The tubes were allowed to stand at 37° C. for thirty minutes. Any precipitate was then centrifuged off and 1 ml. aliquots of the supernatant were transferred to fresh tubes. The fibrinogen contained in these samples was then estimated with bromsulphalein. From this value, the amount precipitated by the acid polysaccharides was derived as described previously. The results are shown in Table IX.

It will be observed from reference to Table IX that, weight for weight, progressively more fibrinogen was removed from solution as the dextran sulphates increased in molecular weight. It was noteworthy that though the smallest dextran sulphate contained an amount of sulphur slightly greater than that present in the larger homologues, it failed to produce fibrinogen precipitation.

A subsequent experiment employing a similar technique showed that large molecular weight compounds of differing sulphur content but derived from the same parent dextran (i.e. compounds within the subgroups D, A, or E) produced approximately the same amount of fibrinogen precipitation within each subgroup. It was therefore evident that, at this reaction, the conjugation of dextran sulphate with fibrinogen was not dependent on the content of sulphate groups of the polysaccharide but rather upon the molecular size of the compound.

Further confirmation of the relative unimportance of charge in the formation of precipitates at this reaction was provided by an observation of Ricketts (1952b) that the large molecular weight dextrans (at higher concentrations than the sulphate esters derived from them) were also capable of precipitating fibrinogen, and that these precipitates could be dissociated under the same conditions influencing the precipitates formed by the dextran sulphates.

The effect of heparin and the dextran sulphates on fibrin and the denaturation of fibrinogen

The influences of heparin and the small molecular weight dextran sulphate on fibrin clot were studied in the following experiment:

Experiment 11.—Fibrin clots were made in the presence of the I size dextran sulphate or heparin by adding 0.1 ml. of thrombin solution (containing 25 units/ml.) to a mixture of 0.1 ml. of 1 per cent heparin or dextran sulphate plus 0.9 ml. of 0.6 per cent fibrinogen in phosphate buffer at *p*H 6.8. A control clot was made without acid polysaccharide present. The clots were washed repeatedly with distilled water till the washings no longer gave a metachromatic reaction with toluidine blue. The clots were then regarded as free from excess anticoagulant. It was then found that if the clots themselves were stained with toluidine blue, those previously exposed to the heparin or dextran sulphate gave strong metachromatic reactions while the control clot stained orthochromatically. It was observed that if 10 per cent sodium or calcium chloride was substituted for distilled water in washing the clots, subsequent metachromatic staining was abolished.

A further experiment showed that heparin and dextran sulphate acted on denatured fibrinogen (coagulated by heat and alcohol-treated) in a similar fashion.

Experiment 12.—Several thin films were made from a 1 per cent solution of fibrinogen on glass slides with a platinum loop. The films were dried rapidly by passing them once or twice through the flame of a bunsen burner, and they were then " fixed " by immersion for thirty seconds in 95 per cent alcohol. They were then rinsed and allowed to dry. One such film, when immersed for two minutes in 1 per cent aqueous toluidine blue, took

a feeble orthochromatic (blue) stain. A second film was dipped into a 1 per cent solution of heparin for a few seconds, washed thoroughly to remove excess heparin, and then immersed in the dye. The film took a deep metachromatic (purplish-red) stain. A third film, similarly treated with 1 per cent dextran sulphate I/3, washed, and immersed in the dye, gave a similar strong metachromatic reaction. Once again, if 10 per cent sodium or calcium chloride was used to wash the films before they were exposed to the dye, the metachromatic staining reaction was abolished.

Effect on clotting and denaturation

Heparin and the I size dextran sulphate were found to produce very little effect on the coagulation of fibrinogen, either by heat or by the action of pure thrombin. Thus, when heparin or the I size dextran sulphate was added, in concentrations between 1 and 20 per cent to solutions of fibrinogen at *p*H 7.0, the coagulum formed on heating the mixture to 56° C., or on adding a drop of concentrated thrombin, showed only slightly greater opacity than the controls. On the other hand, when a concentration of one of the large molecular weight dextran sulphates sufficient to produce precipitation was added to a fibrinogen solution, the gel formed on heating, or the clot formed with thrombin, was markedly more opaque, white, and flocculent than the control. But if the concentration were sufficient to produce pro-zoning (to inhibit precipitation) the coagulum was more translucent than the control. It was observed that the translucent coagulum showed slow and incomplete retraction, while the opaque clot formed at lower concentrations showed more rapid retraction than the control.

Although heparin and the I size dextran sulphates were ineffective in preventing the coagulation of pure fibrinogen by thrombin, it was found that the I size dextran sulphates like heparin showed anti-thrombin activity in the presence of a small quantity of fresh serum or plasma. This suggested that the dextran sulphates, like heparin, required the presence of a " co-factor " in serum or plasma for their anti-coagulant action. An investigation of this aspect of the behaviour of the I size dextran sulphates will be reported subsequently. Here it is only necessary to record the result of an experiment designed to investigate whether the large molecular weight dextran sulphates, which caused fibrinogen precipitation, owed their anti-coagulant action to the latter effect alone or whether they, too, exerted a true anti-thrombin action.

It had been noted in the previous experiments reported that for maximal precipitation of fibrinogen to occur, a mixture of the large molecular weight dextran sulphate and fibrinogen must be allowed to stand for thirty minutes at 37° C. On the other hand, a suitable concentration of thrombin clots fibrinogen within a few seconds. This difference in the time-relations of the two reactions was utilized in the following experiment:

Experiment 13.—(i) 0.4 ml. of 0.85 per cent (w/v) fibrinogen solution in distilled water at *p*H 7.0 was added to a mixture of 0.1 ml. of 1 per cent (w/v) dextran sulphate D/3 and 0.1 ml. of normal human serum, and was followed as quickly as possible by 0.1 ml. thrombin (containing 25 units per ml.). Slow precipitation of fibrinogen occurred, but the mixture did not clot.

(ii) 0.4 ml. of the same fibrinogen solution was added to 0.1 ml. dextran sulphate D/3 plus 0.1 ml. of physiological saline (control). Once again 0.1 ml. of the thrombin solution was added as quickly as possible to the mixture. A little fibrinogen was precipitated from solution, but an opaque white clot occurred within three seconds.

From this experiment it was concluded that the large molecular weight dextran sulphate owed its anticoagulant action not to fibrinogen precipitation alone, but exerted, in addition, a true anti-thrombin action in the presence of a serum co-factor.

Discussion

From the evidence presented, it is clear that both heparin and the dextran sulphates can form salts with one of the plasma proteins. The conditions under which true salt formation occurs, however, are unphysiological and therefore irrelevant to the behaviour of these compounds in the blood and tissue fluids. Under physiological conditions of *p*H, temperature, electrolyte and colloid concentrations, the behaviour of the dextran sulphate series suggests that a much looser and less stable association occurs with fibrinogen. It will be the aim of subsequent communications to show that similar association may occur with other components of the plasma-proteins and that, by analogy, heparin may be assumed to behave in a similar fashion, thus accounting for the similar versatility of behaviour of these two substances in relation to a variety of biological systems. The use of a series of dextran sulphates of varying molecular weights has shown that complex formation with fibrinogen is followed by precipitation of the complex when the dextran sulphate is above a certain critical level of molecular size. But there is indirect evidence, which will be subsequently referred to, that complex formation between dextran sulphates of smaller molecular size and fibrinogen or other plasma proteins may occur unaccompanied by visible precipitation but detectable by altered behaviour and properties of the plasma component. In the present study, the precipitation of the complex formed with fibrinogen in certain circumstances has been utilized to isolate the complex and study its properties. The nature of this complex and the biological significance of its occurrence merit further discussion.

The nature of the complex.—The interaction of the dextran sulphates with fibrinogen is evidently closely similar to that occurring between other acid polysaccharides and various proteins.

The interactions of various hydrophilic colloids were studied by Bungenberg de Jong and his associates (for bibliography see Bank and Bungenberg de Jong, 1939), who considered that, regardless of the reaction of the surrounding medium, complexes were not formed through salt linkages but that physical apposition of the micelles was brought about by their opposing charges with displacement of their water of hydration and their consequent precipitation. The visible precipitate which occurred, regardless of the *p*H of the reaction, was considered to be always a " complex coacervate." The interaction of chondroitin sulphate with proteins was explained on this basis (Bungenberg de Jong and Dekker, 1935). The formation of complexes between amylophosphoric acid and various plant proteins was similarly accounted for by Koets (1936).

This view was opposed by Meyer *et al.* (1937), who studied the formation of complexes between chondroitin sulphate and various proteins *only at reactions more acid than the iso-electric point of the proteins.* They showed that, under these conditions, true salt formation occurred in stoichiometric proportions, both ester sulphate and carboxyl groups being concerned in forming linkages with the basic groups of the protein.

It was shown by Cohen (1942) that, *at neutral reaction*, various highly asymmetrical plant-virus proteins, with molecular weights greater than 1,000,000, could be precipitated from solution by heparin, chondroitin sulphate, and hyaluronic acid. These reactions occurred on the alkaline side of the iso-electric point of the proteins and no evidence of salt-linkage was obtained.

A soluble mucoid, extracted from bovine nasal cartilage, was studied by Partridge (1948). This was found to consist of chondroitin sulphate, and a protein derived from the degradation of collagen. The association of the chondroitin sulphate with the protein was found to occur over the range *p*H 5–9, within which both the protein and the acid polysaccharide were negatively charged. The complex formation occurring within this *p*H range was considered to be different in type from the salt formation described by Meyer *et al.* (1937) as occurring below *p*H 4.85.

From these examples it is evident that much confusion exists as to the nature of the insoluble complexes formed on the admixture of strongly charged polysaccharides with proteins. It appears well established that salt formation occurs when the environmental reaction is below the iso-electric point of the protein, and this is confirmed in the present investigation, where it was shown that the amount of fibrinogen precipitated by the dextran sulphates could be correlated directly with the content of acid groups (and therefore the charge) upon the polysaccharide molecûle. With regard to the formation of complexes above the iso-electric point of the protein, the position is less clear. Although it is generally acknowledged that complexes may form between similarly charged colloid components, there is a natural reluctance to exclude polar association, since, except at very strongly alkaline reaction, the more strongly basic residues of the protein might still possess positive charges, which would bring about coupling with the strongly electro-negatively charged acidic groups of the polysaccharide. It is significant, however, that similar complex formation may occur between similarly weakly-charged protein molecules or between relatively uncharged polysaccharides and weakly electro-negatively charged protein molecules as in the antigen-antibody precipitin reaction. In reviewing the evidence concerning the nature of the forces engaged in such antigen-antibody precipitin reactions (in which, also, both colloid components are similarly electro-negatively charged, as a rule) Pauling, Campbell, and Pressman (1943) concluded that forces variously described as electronic van der Waal's attraction, secondary valencies (hydrogen bonds), coulomb attraction, attraction of electric dipoles and multipoles, etc., were those primarily engaged in bringing about the union of the antigen and antibody. These authors suggest that such forces operate most strongly in large molecules which are able to bring large areas of their surfaces into close contact and which are mutually complementary in surface configuration and distribution of active electrical charges and hydrogen-bond-forming groups. Molecules with approximately, but not completely, complementary surfaces would show weaker mutual attraction, while only very weak attraction would occur between other molecules.

Conceivably such intermolecular forces would operate equally in the reaction under discussion. It was observed that, although this reaction lacked the specificity of the antigen-antibody reaction, it showed properties analogous to the pro-zone phenomenon, maximal precipitation with optimal proportions, discrepancy between constant antigen and constant antibody ratio, and inhibition of haptene excess which

are shown by true precipitin reactions. The behaviour of the complex with alteration of electrolyte concentration and temperature was also closely similar to that of antigen-antibody complexes.

Both fibrinogen and dextran are known to be markedly asymmetrical molecules. The available evidence indicates that fibrinogen consists of very elongated ellipsoidal molecules with a molecular weight of about 400,000 and approximate dimensions of 700 A by 37 A (Oncley, Scatchard, and Brown, 1947; Edsall, Foster, and Scheinberg, 1947; Hall, 1949). Calculations by Ingelman and Halling (1949) showed that the dimensions of dextran fractions varying in molecular weight between 240,000 and 14,000 vary between 1170 A by 20 A and 200 A by 12 A. Sulphate esters prepared from progressively degraded dextrans would show a similar parallel decrease in molecular asymmetry and particularly a decrease in molecular length. If complex formation with the asymmetric fibrinogen molecule depends less on charge than on similarity of molecular shape and configuration (allowing greater opportunity for the interplay of non-ionic forces), then it would be expected that interaction would occur more markedly with the dextran sulphates with molecular dimensions approximating to those of fibrinogen and less markedly with progressively smaller dextran sulphate molecules. It might also be expected that even the parent dextran molecule itself, free from acidic groups and relatively uncharged might show some degree of similar interaction if of large molecular dimensions. Further, it would be reasonable to predict that maximal interaction of the molecules, leading to their mutual dehydration, would be accompanied by precipitation of the complex, whereas this tendency would become progressively less marked as interaction between the molecules decreased.

The present findings are in consonance with those of Cohen (1942), who found that, although a given sample of hyaluronic acid precipitated the tobacco mosaic virus protein at *p*H 7, when the hyaluronic acid was degraded with hyaluronidase, the reaction products, though still charged, now failed to cause precipitation. Similarly Partridge (1948) found that his mucoid preparation, when heated, suffered a rapid reduction in viscosity (suggesting depolymerization of its components) with parallel loss of the capacity to form insoluble complexes at *p*H 5–9.

Although the charge borne by the polysaccharide ester sulphate groups is comparatively unimportant in forming complexes with the plasma proteins, this charge is essential for anticoagulant action. Although some of the parent dextrans were also capable of forming complexes they did not affect the clotting system.

The effects of the dextran sulphates upon the appearance and texture of the coagulum formed from fibrinogen, in their presence, by heat or by thrombin, appear to be in keeping with the observations of Foster (1948) on the influence of heparin on clotted plasma. They also appear to correspond with the change from " fine " to " coarse " clots, described by Ferry and Morrison (1947) as accompanying alteration of the environment in which the conversion of fibrinogen to fibrin takes place. These authors suggested that " fine " clots were made up of a network of chains, consisting of fibrinogen molecules joined end to end, cross linked at least partially by primary chemical bonds. " Coarse " clots, on the other hand, were regarded as made up of a network of bundles of such chains, cross linked largely by secondary bonds and by lateral association. Translucent or " fine " clots could only be

produced by concentrations of the large molecular weight dextran sulphates within the " pro-zone " region. At lower concentrations where fibrinogen precipitation occurred (perhaps due, as suggested, to aggregation of the polysaccharide and protein molecules in layers by lateral association and secondary bond formation), the " coarse " nature of the clot was markedly accentuated.

Although heparin and the I size dextran sulphates produced no visible interaction with native fibrinogen, it could be shown that they were adsorbed upon the surface of the clotted or denatured protein. This may be significant in explaining their apparent regionally selective capacity in preventing the extension of intravascular thrombi.

Biological significance of complex formation.—Although the insoluble complex formed by the large molecular weight dextran sulphates under physiological conditions appeared to be composed of weakly conjoined components, it was sufficiently stable in plasma or whole blood to produce a number of pathological effects. These will be described in detail in a separate communication.

The terms " molecular weight " and " molecular size " have been used in this communication as though they were interchangeable. But, if as here contended, it is the actual shape, dimensions, and surface configuration of an acid polysaccharide which determine the degree of complex formation and the stability in solution of the complexes formed with plasma proteins, while the latter phenomenon in turn promotes the " toxicity " of the compound, then it follows that molecular weights (whether derived from intrinsic viscosities or osmotic pressures) are only acceptable as standards of reference for a given series of compounds derived from the same parent polysaccharide. Earlier reference has been made to Ingelman's (1947) comparison of the " toxicity " of an inulin sulphate with that of his smallest dextran sulphate. He found the former more " toxic " though smaller in molecular weight than the dextran sulphate, and concluded that molecular weight was not a factor in determining " toxicity." Inulin is composed of different (fructose) units which are linked differently and disposed differently (Haworth, Hirst, and Percival, 1932) from dextran, and its molecular dimensions for a given molecular weight probably differ from those of a dextran of similar molecular weight, making comparison of their respective sulphate esters on a basis of molecular weight unjustifiable. The physical data here quoted as distinguishing a " toxic " dextran sulphate from a " non-toxic " one should not be directly transferred to other polysaccharide sulphate esters. Nevertheless it appears advisable to urge (in view of the comparative ease with which anticoagulant preparations can be obtained by the sulphation of different polysaccharides) that any such preparations should be investigated for similarity of behaviour to the large molecular weight dextran sulphates before administration to man.

The small molecular weight dextran sulphates, when screened to ensure sufficient homogeneity of molecular size (absence of " contamination " by large molecules), appear not to affect the stability in solution of the macromolecular plasma proteins and also to be virtually non-toxic. They appear to exert a true antithrombin action and to behave qualitatively identically with heparin. They offer, therefore, therapeutic potentialities as inexpensive heparin substitutes. The biological testing and therapeutic trial of one such compound will be reported subsequently.

Summary and Conclusions

Observation of the interactions, with fibrinogen, of heparin and of a series of dextran sulphates of varying molecular weights showed that:

1. Visible interaction occurred as precipitate formation on adding heparin and dextran sulphates of all the available molecular sizes to fibrinogen at reactions *below* the iso-electric point of the protein.

2. Visible interaction as precipitate formation at reactions *above* the iso-electric point of fibrinogen occurred only with dextran sulphates with molecular weights above a certain critical level.

3. When the behaviour of these precipitates was examined in relation to variations of the environment in which they were formed (i.e. variations of *p*H, temperature, electrolyte, and colloid concentrations) it was found that the precipitates formed on either side of the iso-electric point of the protein differed in many respects from each other.

4. From consideration of these differences it was concluded that, at reactions below the iso-electric point of fibrinogen, precipitation is due to ordinary salt formation, and this was shown to be dependent directly upon the content of acidic groups, and therefore the electro-negative charge, borne by the acid polysaccharide. The conditions governing this variety of interaction with fibrinogen were such that the reaction was not regarded as being directly relevant to the behaviour of these compounds in normal tissue fluids.

5. On the other hand, the precipitates formed by the large molecular weight dextran sulphates, under physiological conditions, with fibrinogen, were found to be loose, easily dissociated complexes, resembling antigen-antibody complexes in some respects. Attention has been drawn to the similar behaviour of other acid polysaccharides in relation to a variety of proteins.

The formation of insoluble complexes in this second variety of interaction with fibrinogen was shown to be dependent primarily upon the molecular size of the dextran sulphate and not upon its content of acid groups. For this reason the reaction was regarded as being due mainly to the interplay of non-ionic forces between the molecules.

6. The formation of such insoluble complexes with fibrinogen (and possibly other macromolecular components of plasma) by the large molecular weight dextran sulphates is indirectly responsible for the toxicity of these compounds *in vivo*.

7. Low molecular weight dextran sulphates, like heparin, do not form insoluble complexes with the plasma proteins, under physiological conditions. For this reason, apparently, they are no more toxic than heparin, yet show qualitatively similar behaviour as anticoagulants, making it possible to employ them as heparin-substitutes.

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