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# The Physico-Chemical Nature of Prepared Dextran Sulfates

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**Abstract**□The prepared dextran sulfates were characterized by measuring the reduced viscosity at five different concentrations to obtain an intrinsic viscosity in both phosphate and tris buffers, pH 7.4, ionic strength of 0.1. Dextran sulfates having 0.81, 1.06 sulfate groups per hexose unit have reduced viscosity value below 40 ml/g whereas dextran sulfates having 1.21, 1.43, 1.69 sulfate groups per hexose unit have reduced viscosity value over 40 ml/g. Dextran sulfate having 1.21 sulfate groups per hexose unit had highest value of reduced viscosity. The reduced viscosity of dextran sulfate in tris buffer was always higher than that in phosphate buffer regardless of the sulfate content of dextran sulfate. The influence of the sulfation of the dextran sulfate molecule on the dextran sulfate-LDL interaction was studied with three different dextran sulfate molecules. Dextran sulfate having 0.81 sulfate groups per hexose unit behaved quite differently from the other two dextran sulfate molecules having more than one sulfate group per hexose unit. The dextran sulfate having 0.81 sulfate groups per hexose unit showed considerably different precipitation curves in phosphate and tris buffers. This peculiar behavior of dextran sulfate having 0.81 sulfate groups per hexose unit in the two buffer systems was not noticed with dextran sulfate having more than one sulfate group per hexose unit.

**Keywords**□Dextran sulfate-sulfate groups per hexose unit-LDL-precipitation curve-equivalence ratio-phosphate buffer-tris buffer.

The formation of insoluble complexes between sulfated polysaccharides and certain plasma lipoproteins was independently described by several investigators[1~3]. Since then, the ability of sulfated polysaccharides to precipitate selectively low density lipoproteins from serum has been widely utilized for the isolation and estimation of the lipoproteins[4~8] and for the determination of lipid distribution in low and high density lipoproteins[9~10]. Furthermore, the apparent biological importance of the sulfated polysaccharide-lipoprotein interaction has been recognized[11]. The important biological activities of the heparin, a sulfated polysaccharide, has been established[12]. In spite of the biological importance of the sulfated polysaccharide and a number of sulfated polysaccharide preparation[13~14], the basic physico-chemical studies on sulfated polysaccharides have not been conducted extensively. The present study was directed toward the clarification of the physico chemical properties of the sulfated polysaccharides. Dextran sulfate was chosen as a model compound for sulfated polysaccharides.

## EXPERIMENTAL

*Buffer System*

Phosphate buffer of pH 7.4, ionic strength of 0.1 and tris buffer of pH 7.4, ionic strength of 0.1 were used for the entire study.

*Preparation of Washed Dialysis Bags*

Dialysis bags (Union Carbide Co., Chicago, Ill.) were heated in 1% sodium bicarbonate solution on a steam bath for 2 hours, after preliminary soaking in glass distilled water. They were transferred to the second sodium bicarbonate solution and soaked overnight at room temperature, and then rinsed with glass distilled water. The washed dialysis bags were stored at 4°C in glass distilled water until needed.

*Preparation of Dextran Sulfate*

Dextran, with an average molecular weight of 150,000 was purchased from Sigma Chemical Co. (St. Louis, Mo.). Dextran sulfate was prepared according to the method described by Ricketts(13). Dextran was first dried *in vacuo* over phosphorus pentoxide. Twenty-two ml cholorosulfonic acid was added drop by drop to 100 ml dry pyridine and the mixture was vigorously stirred. During the addition, the flask was cooled in a dry ice and ethanol bath. The temperature was then raised to 65°C in order to dissolve the pyridine salt. When the pyridine salt was dissolved completely, 15 g of finely powered dextran was added and dispersed in the reaction mixture by vigorous stirring. The temperature was maintained at 65°C-75°C for 4 hours. After cooling, 300ml of crushed ice and a sufficient 40% sodium hydroxide solution were added to make the mixture dark red and to cause separation of pyridine as an upper layer. The lower layer was diluted to 500 ml, brought to 37°C, and 500 ml of ethanol was added to the mixture. The precipitated syrup was allowed to settle for 10 minutes at 37°C to avoid crystallization of sodium sulfate. The syrup was then separated, redissolved in 200 ml water and similarly precipitated with 200 ml of ethanol. The precipitation was repeated again with 150ml of water and an equal volume of ethanol. The final syrup was dissolved in 200 ml of water, neutralized with hydrochloric acid, dialyzed against glass distilled water, treated with charcoal at 50°C. The pH of the filtrate was readjusted to 7.0–7.5 with sodium hydroxide and the solution was concentrated to 50 ml under vacuum. One hundred ml of aceton was added and the precipitated syrup was poured into ethanol, ground to a powder, washed with ether, and finally dried *in vacuo* over phosphorus pentoxide.

*Determination of Sulafte of Dextran Sulfate*

The amount of sulfate in dextran sulfate was determined according to the procedure of Egami(15). In a 10 ml volumetric flask, 0.08 ml of 1% sodium sulfate was added and hydrolyzed with 0.25 ml concentrated hydrochloric acid in the presence of 1 ml of 8 mM barium chloride solution, and heated for 3.5 hours in a steam bath. After hydrolysis, the reaction mixture was evaporated until dryness. Two ml of 20% sodium acetate was added to the flask and the flask was kept in an ice bath for 20 minutes. One ml of 5 mM

potassium dichromate solution was then added to the flask and mixed well, the volume was brought to 10ml with glass distilled water, and the flask was kept in an ice bath for another 20 minutes. After subsequent centrifugation of the reaction mixture, 2ml of the clear supernatant solution was removed and mixed with 1 ml of 1 M sodium hydroxide solution and 7 ml of glass distilled water. The absorbance of this solution was measured at 375 nm with a spectrophotometer, and sulfate content was determined from the standard working curve obtained from a standard potassium sulfate solution.

*Density and Viscosity Measurements*

Density measurements were made using a callibrated 5 ml pycnometer at 20°C and viscosity was measured in a capillary Ostward viscometer (Cannon Instrument Co., State College, Pa.).

*Analytical Ultracentrifugation*

Ultracentrifugal analyses were performed with a Beckman-Spinco Model E ultracentrifuge utilizing schliren optics. An AN-D rotor was employed for all determination. A standard double-sector cell was used for determination of sedimentation coefficients at 52,640 rpm, 20°C and bar angle of 55 degrees. The acceleration time of the centrifuge to obtain 52,640 rpm was 5 minutes 20 seconds. A synthetic boundary double-sector cell was utilized for determination of diffusion coefficients which were done at 2500 rpm, 20°C and bar angle of 70 degrees. Synthetic boundaries were formed as described by Richards *et al.*(16): 0.44 ml of solvent was placed in the left sector and 0.14 ml of sample was placed in the right sector. For these determinations of methods outlined by Schachman(17) were used. All measurements were done on a Nikon Shadowgraph Model 6 Comparator. Areas under the peaks were measured with a planimeter on tracings of 10 x (linear) enlargements of the photographic plates.

*Preparation of LDL (low density lipoprotein)*

Human plasma LDL of the Sf 0 to 10 class was isolated and purified by ultracentrifugation according to the method of Janado and Nishida(18). The LDL was dialyzed against phosphate buffer or tris buffer of pH 7.4, ionic strength 0.1, for 24 hours under nitrogen at 1°C with four changes of the external solution. The LDL preparation used in this study contained 20.6% protein and 79.4% lipids.

*Formation of Insoluble Dextran Sulfate-LDL Complex*

The procedure of Nishida and Cogan(19) was used to study the effect of the structure of dextran sulfate on the formation of dextran sulfate-LDL complex. Mixtures containing 0.8 mg of LDL and various amounts of dextran sulfate and $CaCl_2$ solution in 1.5 ml of tris buffer were kept at room temperature for 30 minutes. The insoluble complex was centrifuged for 30 minutes at 4000 rpm (2900 × g). The supernatant solution was removed by aspiration. After removal of the supernatant solution, the tubes were kept in an ice bath for 10 minutes. The precipitates were washed twice with one ml of ice-cold water each to remove the excess $Ca^{++}$ in the supernatant which remained on the

surface of the insoluble complex. The recovery of LDL in the insoluble complex was determined by protein analysis of the complex.

## RESULTS

Dextran sulfates, whech were prepared for the study of physico-chemical properties are listed in Table I.

The prepared dextran sulfates were characterized by measuring the reduced viscosity at five different concentrations to obtain an intrinsic viscvosity in both phosphate and tris buffers, pH 7.4, ionic strength of 0.1 (Figures 1,2). Dextran sulfates having 0.81, 1.06 sulfate groups per hexose unit have reduced viscosity value below 40 ml/g whereas dextran sulfates having 1.21, 1.43, 1.69 sulfate groups per hexose unit have reduced viscosity value over 40 ml/g(Figure 1). It is interesting to note that the reduced viscosity of dextran sulfate in tris buffer was always

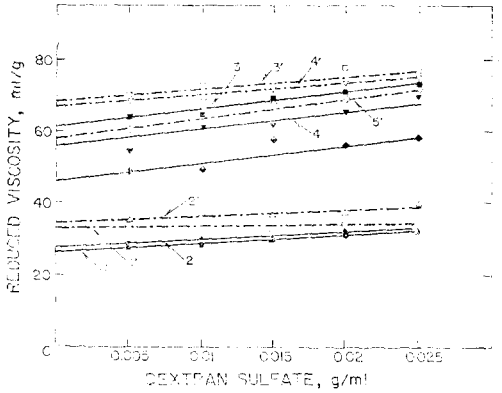


Fig. 1: Reduced viscosity of dextran sulfates in phosphate and tris buffers, pH 7.4, ionic strength of 0.1 as a function of the concentrations of dextran sulfate. The reduced viscosities of dextran sulfate having 0.81, 1.06, 1.21, 1.43, and 1.69 Sulfate groups per hexose unit in phosphate buffer are shown by curves 1, 2, 3, 4, and 5, respectively, and those in tris buffer are given by curves 1′, 2′, 3′, 4′, and 5, respectively.

**Table I: Dextran sulfate preparations[a].**

| Dextran Sulfate | S content, %[b] | $SO_3Na$ content, %[c] | S content, %[d] | No. sulfates per hexose | No. sulfates per g of dextran Sulfate, $\times 10^{3}$[f] | Molecular weight, $\times 10^{-5}$[g] |
|---|---|---|---|---|---|---|
| 1 | 10.6 | 34.1 | 65.9 | 0.81 | 3.29 | 2.28 |
| 2 | 12.6 | 40.6 | 59.4 | 1.06 | 3.93 | 2.52 |
| 3 | 13.5 | 43.5 | 56.5 | 1.21 | 4.23 | 2.65 |
| 4 | 14.8 | 47.6 | 52.4 | 1.43 | 4.63 | 2.86 |
| 5 | 16.1 | 51.8 | 48.2 | 1.69 | 5.03 | 3.11 |

[a]Dextran sulfates were prepared as described in the text.

[b]S content was obtained from the sulfate determination of the prepared dextran sulfate and was used for the calculation of the parameters.

[c]$SO_3Na$ content was calculated from S content.

$$\frac{\text{S content}}{\text{atomic weight of S}} \times \text{mol. wt. of } SO_3Na$$

[d]Hexose content was obtained from the subtraction of the $SO_3Na$ content from 100.

[e]No. sulfate per hexose was calculated from the equation,

$$\frac{\text{S content}}{\text{atomic weight of S}} \times \frac{\text{mol. wt. of (hexose-}H_2O)}{\text{hexose content}}$$

[f]No. sulfate per g of dextran sulfate was obtained from the equation. No. sulfates per hexose

Mol. wt. of (hexose-$H_2O$)+[No. sulfate per hexose$\times$ (mol. wt. of $SO_3Na$-atomic weight of H)]

[g]Mol. wt. of dextran sulfate was calculated from the equation,

$$\text{Mol. wt. of dextran} \times \frac{100}{\text{hexose content, \%}}$$

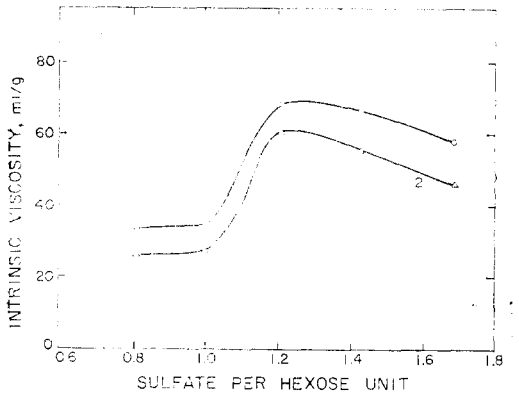


**Fig. 2**: Intrinsic viscosity of the dextran sulfates in phosphate and tris buffers, pH 7.4, ionic strength of 0.1 as a function of the sulfate content per hexose unit. Curves 1 and 2 represent the plots of intrinsic viscosity values obtained in tris and phosphate buffer, respectively.

higher than that in phosphate buffer regardless of the sulfate content of dextran sulfate. Dextran sulfate having 1.21 sulfate groups per hexose unit had highest value of reduced viscosity.

The sedimentation coeffecient of dextran sulfate having 1.69 sulfate groups per hexose

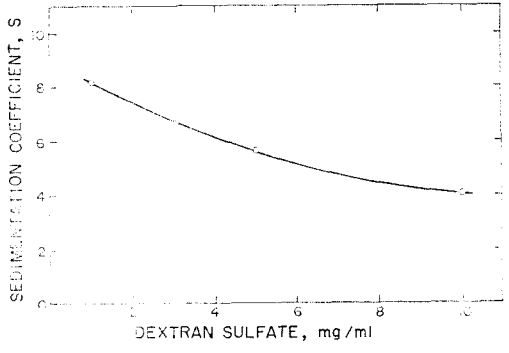


**Fig. 3**: Apparent sedimentation coefficient of the dextran sulfate as a function of the concentration of dextran sulfate.

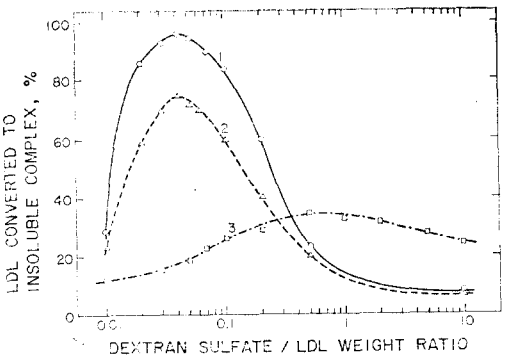


**Fig. 4**: Effect of the sulfation degrees of dextran sulfate on dextran sulfate–LDL interaction in the absence of divalent metal ions. Curves 1, 2, and 3 represent the interactions of LDL with dextran sulfates having 1.69, 1.21, and 0.81 sulfate groups, respectively, per hexose unit. Insoluble complex was formed with 0.8mg of LDL and various amounts of dextran sulfates in 1.5ml of phosphate buffer (pH 7.4, ionic strength 0.1).

unit was determined as a function of the concentration (Figure 3).

In order to study the influence of the sulfation of the dextran sulfate molecule on the dextran sulfate-LDL interaction, three different dextran sulfate molecules were employed(Figure 4). Dextran sulfate having 1.69 sulfate groups per hexose unit converted almost all of the LDL into the insoluble complex in the absence of $Ca^{++}$. Dextran sulfate having 1.21 sulfate groups per hexose unit exhibited behavior similar to that of dextran sulfate having 1.69 sulfate groups per hexose unit; the equivalence ratio was not changed but the formation of insoluble complex was slightly reduced.

Dextran sulfate having 0.81 sulfate groups per hexose unit behaved quite differently

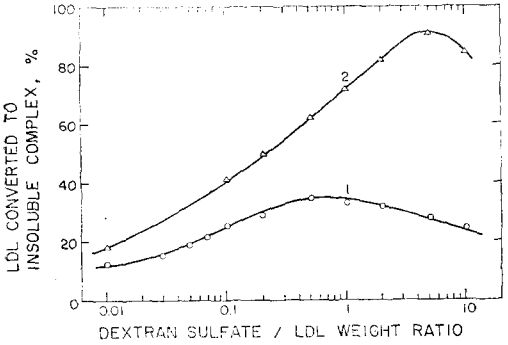


**Fig. 5**: Effect of $Mg^{++}$ on the interaction of LDL with dextran sulfate having 0.81 sulfate groups per hexose unit in phosphate buffer. Curve 1 shows the conversion of LDL to the insoluble complex in the absence of $Mg^{++}$. Curve 2 shows the conversion of LDL in the presence of 10 mM $MgCl_2$.

from the other two dextran sulfate molecules having more than one sulfate group per hexose unit. Only 35% of the LDL was converted into the insoluble complex at the equivalence ratio which corresponded to fourteen times more dextran sulfate than those having 1.21 and 1.69 sulfate groups per hexose unit. In the presence of 10 mM concentration of $MgCl_2$, the equivalence ratio was shifted further to a higher value and almost all of the LDL was converted to the insoluble complex (Figure 5).

It was found that dextran sulfate having 0.81 sulfate groups per hexose unit showed considerably different precipitation curves in phosphate and tris buffers, both having the pH of 7.4 and ionic strength of 0.1 (Figure 6). The dextran sulfate converted 35% of the LDL into the insoluble complex at the equivalence ratio in phosphate buffer,

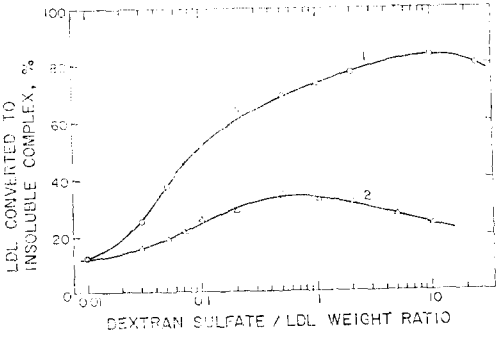


**Fig. 6**: Effect of buffer on the interaction of LDL with dextran sulfate having low sulfate content. Curve 1 shows the formation of the insoluble complex of LDL with dextran sulfate having 0.81 sulfate groups per hexose unit as a function of the dextran sulfate to LDL weight ratio in tris buffer. Curve 2 shows the formation of the insoluble complex in phosphate buffer. Experimental conditions were the same as those described for Curve 3 in figure 4.

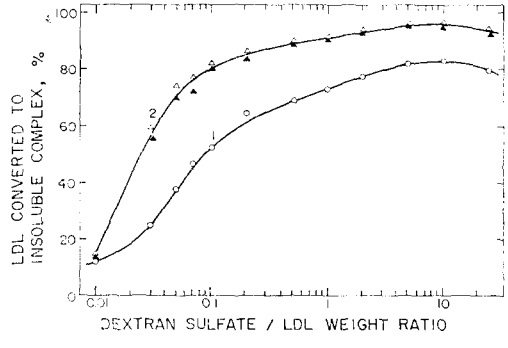


**Fig. 7**: Effect of $Mg^{++}$ and $Ca^{++}$ on the interaction of LDL with dextran sulfate having 0.81 sulfate groups per hexose unit in tris buffer. Curve 1 shows the conversion of LDL to insoluble complex in the absence of divalent metal ions in tris buffer. Curve 2 shows the conversion of LDL to insoluble complex in the presence of 10 mM $MgCl_2$(-▲-▲-) and $CaCl_2$(-△-△-) in tris buffer.$Ca^{++}$ and $Mg^{++}$ exerted the same degree of enhancement of the insoluble complex formation.

while it converted 84% of the LDL into the insoluble complex in tris buffer at the equivalence ratio which was much higher than in phosphate buffer. This peculiar behavior of dextran sulfate having 0.81 sulfate groups per hexose unit in the two buffer systems was not noticed with dextran sulfate having more than one sulfate group per hexose unit.

The effect of divalent metal ions on the interaction of LDL with dextran sulfate having 0.81 sulfate groups per hexose unit in tris buffer was also studied (Figure 7). Both $Ca^{++}$ and $Mg^{++}$ enhanced the complex formation in a similar manner. However, the precipitation curves were markedly different from those obtained in the presence of $Ca^{++}$ with dextran sulfate having 1.69 sulfate groups per hexose unit.

## DISCUSSION

The insoluble complex formation with dextran sulfate having 0.81 sulfate groups per hexose unit was considerably less effective than with the preparation having 1.69 sulfate groups per hexose unit. This ineffectiveness was indicated by the requirement of a large amount of the dextran sulfate in obtaining the equivalence ratio which gave maximal complex formation and reduction in the insoluble complex formation, suggesting the existence of a drastic conformational difference between the two dextran sulfate molecules. The determination of the instrinsic viscosity of several dextran sulfate molecules indeed revealed the possible conformational difference depending upon their sulfate contents (Figures 1,2). Dextran sulfate having more than 1.21 sulfate groups per hexose unit gave high intrinsic viscosity values indicating their highly flexible and expanded conformation due to the repulsion between sulfate groups. On the other hand, the dextran sulfates having less than 1.06 sulfate groups per hexose unit showed considerably lower intrinsic viscosity values indicative of more rigid and compact conformation. Thus, a considerable number of the sulfate groups would not assume the optimal spatial arrangement needed for the effective interaction. This would necessitate the presence of a large amount of dextran sulfate for the interaction and hence the occurrence of a considerable number of unreacted sulfate groups which could exert a solubilizing effect. The enhancement of insoluble complex formation by divalent metal ions both below and above the equivalence ratio appeared to be due largely to the crosslinking of the excess sulfate groups.

It was reported that sulfated corn amylopectin containing from 0.85 to 2.18 sulfate groups per hexose unit could completely precipitate low density lipoproteins(11). However, the present study showed that more than one sulfate group per hexose unit of dextran sulfate was required for near complete precipitation of LDL. This discrepancy regarding the effect of the sulfate content on the interaction can be explained by the difference in the spatial arrangements of the hexose units in dextran sulfate and of

space filling molecular models, dextran sulfate which possesses (1→6) linkage between glucose units produces a compact conformation containing highly twisted helical coils. On the other hand, corn amylopectin sulfate which has (1→4) linkage between glucose units yields a helical structure with more glucose units per turn and a space inside the helices which can accomodate a large number of water molecules. Apparently, the less compact structure of corn amylopectin sulfate provides a conformational flexibility of the sulfate groups for the efficient interaction of even those having less than one sulfate group per glucose unit.

The formation of insoluble complex of LDL with dextran sulfate having 0.81 sulfate groups per hexose unit was found to be enhanced by tris buffer both in the presence and absence of divalent metal ions. Tris buffer also shifted the equivalence ratio to a higher value than that obtained in phosphate buffer. Furthermore, the intrinsic viscosities of dextran sulfate molecules in tris buffer were higher than on phosphate buffer. These observations suggest a possible ionic interaction between the sulfate groups of dextran sulfate and the trishydroxymethylaminomethane ions of tris buffer. Indeed, the space filling molecular models of these compounds can be arranged in such a way to hydroxyl groups of trishydroxymethylaminomethane ions and hydroxyl groups of the glucose units of the dextran sulfate molecules. This hydrogen bonding assisted ionic interaction would result in the expansion of dextran sulfate molecules, thus giving higher instrinsic viscosity values in tris buffer than in phosphate buffer. It is noteworthy that the interaction of LDL with dextran sulfate having a higher degree of sulfation was not influenced by the tris buffer. Presumably, the spatial rearrangement of dextran sulfate molecules occurring upon interaction with LDL would not allow the hydrogen bonding, resulting in the disruption of the ionic interaction with trishydroxymethylaminomethane ions. This disruption apparently would not take place with dextran sulfate having less than one sulfate group per hexose unit. It seems that the rigidity of the dextran sulfate of low sulfation does not permit a sufficient expansion of the dextran sulfate upon interaction with LDL. Thus, the hydrogen bonding between dextran sulfate molecules and trishydroxymethylaminomethane ions would still be maintained, reducing the number of sulfate groups available for the interaction with LDL. This, in turn, require the additional amount of sulfate groups for the maximal complex formation and hence, causes the shift of the equivalence ratio to a higher value. The enhancement of the complex formation by tris buffer seems to occur also by the ionic interaction of trishydroxymethylaminomethane ions with the sulfate groups which are not involved in the complex formation. In the absence of divalent metal ions, the shielding of the free sulfate groups by the ionic interaction is likely to nullify the repulsion between negatively charged sulfate groups. In the presence of divalent metal ions, the shielding seems to reduce the amount of

$Ca^{++}$ or $Mg^{++}$ required for the charge neutralization or crosslinking of the free sulfate groups.

It may be concluded that the sulfate content per hexose unit and the type of the linkages between the hexose units of sulfated polysaccharides were found to govern the extent of their interaction with LDL by influencing primarily the conformational flexibility is rather limited as observed with dextran sulfate preparations having less than one sulfate group per hexose unit, the trishydroxymethylaminomethane ions seems to retain their ionic interaction with the sulfate groups even after the interaction of the dextran sulfate with LDL, thus improving the complex forming ability of the preparations.

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