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STUDIES ON SOME BIOLOGICALLY ACTIVE DEXTRANS*

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ABSTRACT

The relationship between the structures of six native dextrans and their effects on nonspecific resistance to infection (n.s.r.i.) in mice and also anticomplementary activity has been studied. The data obtained showed that the n.s.r.i. activity of dextrans generally increased with increase of extent of branching, but no direct correlation between these two factors was found. Data on exodextranase-catalyzed hydrolysis of dextrans suggest that the length of the outer chains may be important for the n.s.r.i. activity of the dextrans. Dextrans characterized by a significant extent of branching were anticomplementary, but no relationship between extent of branching and anticomplementary activity was observed.

INTRODUCTION

Interaction of native dextrans with various biological systems has been noted by many authors. One of the most interesting aspects of the biological activity of dextrans is their interaction with immunological systems. Hestrin *et al.*[1] found that some native dextrans possess an infection-promoting activity when injected simultaneously with the infectious challenge. Pillemer *et al.*[2] and Leon[3] found that the ability of native dextrans to interact with the complement system varied with the dextran being tested, but no correlation between activity and structure was found. These authors established two major requirements that may be prerequisites for the dextran–complement interaction; high molecular weight and significant percentage of non-(1→6)-glucosidic linkages in the dextran molecule. Kiser *et al.*[4] compared two native dextrans for their ability to enhance nonspecific resistance of animals to infection (n.s.r.i.). However, as the doses of the two dextrans were different, it was difficult to interpret the results.

We have isolated a series of dextrans containing various types of linkages in various proportions and have studied their ability to enhance n.s.r.i. No direct correlation between the activity and proportion of various types of linkages was found.

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

Therefore, an attempt was made to study in more detail the structure of dextrans with respect to their activity.

RESULTS AND DISCUSSION

Effect of dextrans on nonspecific resistance to infection (n.s.r.i.) in mice. — For our studies, we chose six different dextrans, three of which caused significant protection when injected into mice 24 h before infection; the other three dextrans were inactive (Table I). Increased doses of the inactive dextrans (up to 5.0 mg per mouse) did not enhance the protective action. It should be noted that hydrolyzed (clinical) dextrans and such dextrans as T-500 and T-2000 (M_w 5×10^5 and 2×10^6 respectively) exhibited no activity under our experimental conditions. Sephadexes of various types were also inactive.

TABLE I

EFFECT OF DEXTRANS ON AN INFECTION WITH *Escherichia coli* 094

Strain	*Survival of mice at 5 days*[a]		ED_{50}[b] (μg)
	Alive/total	*Survivors* (%)	
LU-122	54/60	90	90
LU-122 (NaOH tr)	10/10	100	—
63-1	150/190	79	116
63-1 (NaOH tr)	20/30	66	—
44b-2	51/60	85	93
61-2	31/160	19	>3200
SF-4	15/60	25	>3200
69	9/40	18	>3200
Control (saline)	39/620	6	

[a]Dextrans were injected intraperitoneally (800 μg in 0.2 ml of saline), and 24 h later the mice were infected intraperitoneally with *E. coli* 094. Survival of the animals was recorded within 5 days after the infection. [b]ED_{50} is the average effective dose, the dose that gives protection to 50% of the mice.

Some authors have claimed that biological activity of neutral polysaccharides is due to their contamination by traces of lipopolysaccharides of Gram-negative bacteria. In our control experiments, the dextrans were treated under alkaline conditions that caused degradation and inactivation of lipopolysaccharides[8]. As shown in Table I, the activity of the dextrans remained unchanged. It was concluded, therefore, that the activity was due entirely to dextrans themselves and not to the contaminants.

Anticomplementary activity of dextrans. — It is known that interaction with serum complement underlies a variety of biological reactions[9,10]. As already noted, dextrans are also able to interact with the complement system[2,3]. Our data on the anticomplementary activity of dextrans are shown in Fig. 1. For comparison, two samples of dextrans kindly provided by Dr. A. Jeanes were also tested.

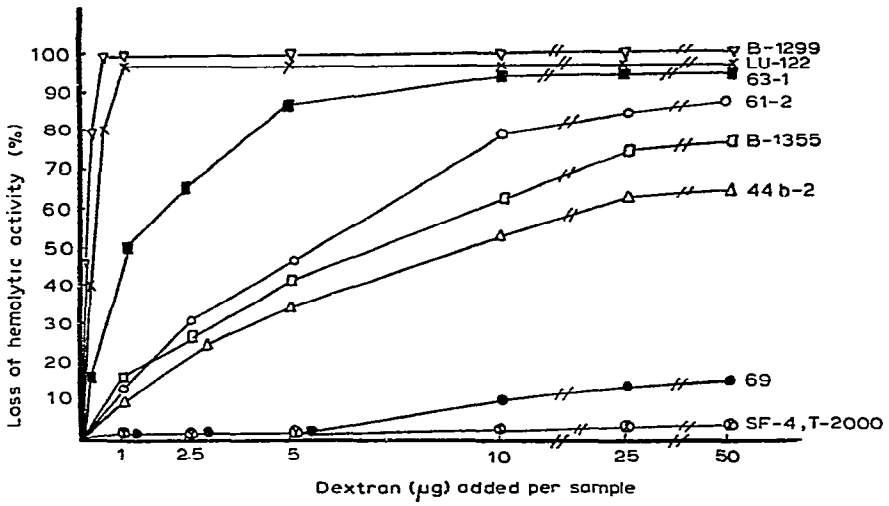

Fig. 1. Anticomplementary activity of dextrans. Increasing amounts of each preparation were incubated with 0.1 ml of guinea-pig serum, and loss of hemolytic activity was measured.

Dextrans B-1299 and LU-122 in doses of 1 μg/0.1 ml of guinea-pig serum caused complete loss of hemolysis, whereas the same doses of dextrans 61-2, B-1355, and 44b-2 decreased hemolysis by only 10–15%; much higher doses of these dextrans (>10 μg) were necessary to produce noticeable inhibition of hemolysis. Dextrans SF-4, 69, and the clinical dextrans had no effect on hemolysis in doses as high as 50 μg per 0.1 ml of serum. In alkali-treated, active dextrans, as well as in experiments on enhancing n.s.r.i., no essential changes in activity were observed. Comparative studies of the effects of dextrans on the n.s.r.i. and on complement fixation revealed no direct correlation between these two reactions. For instance, dextrans LU-122 and 44b-2 exhibited essentially the same activity in experiments *in vivo*, but differed in their anticomplementary activity *in vitro*. Dextran 61-2 was inactive *in vivo* but possessed considerable anticomplementary activity.

Structure of the dextrans. — (*a*) *Periodate oxidation.* Periodate-oxidation studies showed that all of the dextrans that enhance n.s.r.i. (LU-122, 63-1, and 44b-2) had a considerable percentage of non-(1→6)-glucosidic linkages (Table II). On the other hand, dextran 61-2, although inactive, also contained about 50% of non-(1→6)-linkages. These data demonstrate that a large proportion of glucosidic linkages other than (1→6) is not by itself important for activity.

(*b*) *Smith degradation.* Determination of periodate-unoxidized glucose after 120 h of incubation showed that two of the dextrans (44b-2 and 61-2) contained a large proportion of it, whereas unoxidized glucose only constituted 2–3% in other dextrans. The Smith-degradation products included glycerol and glucose; no erythritol was detected. These data confirmed the absence of (1→4)-glucosidic linkages in the dextrans.

TABLE II

PERIODATE OXIDATION AND ACETOLYSIS OF THE DEXTRANS

Strain	D-*Glucose residues linked through*			*Unoxidized glucose* (%)	*Acetolysis product (the major disaccharide)*
	(1→6) only (%)	*(1→2)-like* (%)	*(1→3)-like* (%)		
LU-122	68	32	0	2	α-D-Glc*p*-(1→2)-D-Glc (kojibiose)
63-1 *f*	75	22	3	3	α-D-Glc*p*-(1→2)-D-Glc (kojibiose)
44b-2	70	1	29	28	α-D-Glc*p*-(1→3)-D-Glc (nigerose)
61-2	55	6	39	32	α-D-Glc*p*-(1→3)-D-Glc (nigerose)
SF-4	95	2	3	3	—
69	93	5	2	2	—

(*c*) *Acetolysis.* Acetolysis was conducted for identification of the types of linkages in the dextrans. The disaccharide α-D-Glc*p*-(1→3)-D-Glc (nigerose) was detected chromatographically in all of the adcetolyzates, but only in those from dextrans 61-2 and 44b-2 was its content high enough to indicate a high percentage of (1→3)-linkages; this result was also confirmed by periodate-oxidation and Smith-degradation studies. From the acetolyzates of dextrans LU-122 and 63-1 we isolated α-D-Glc*p*-(1→2)-D-Glc (kojibiose), thus providing evidence for the presence of (1→2)-glucosidic linkages in these dextrans. Maltose was not detected in the dextrans studied.

Among the three dextrans having pronounced ability to produce n.s.r.i., two of them thus possessed a considerable percentage of (1→2)-glucosidic linkages and one had (1→3)-glucosidic linkages. On the basis of these data, it may be concluded that a particular type of linkage in the dextran molecule is not essential for activity. Considering the results of experiments with complement fixation, it was noted, however, that the dextrans having (1→2) linkages possessed a somewhat higher activity than those containing (1→3) linkages.

(*d*) *Interaction of dextrans with concanavalin A.* As it was found that neither the types of glucosidic linkages nor the total proportion of (1→6) linkages in the dextran molecule were essential factors for the exhibition of n.s.r.i. activity, we assumed that the branched structure of the dextran molecule might have a role in the activity. Preliminary information on the relative extent of branching of various dextrans is provided by studies on their interaction with the phytohemagglutinin concanavalin A[11] (con-A). As shown by Goldstein *et al.*[12], con-A forms complexes with dextrans through the terminal, nonreducing α-D-glucopyranosyl residues. It appears, therefore, that the intensity of the dextran–con-A interaction reflects, in a way, the extent of branching of the dextran molecule. There are also other factors affecting the dextran–con-A interaction[13], but the number of non-reducing terminal residues appears to be the preponderant one. Fig. 2 presents data on the relation between the amounts of precipitated con-A and con-A added to a mixture containing 0.1 mg of dextran. As shown in Fig. 2, the biologically active dextrans precipitated con-A more readily than

the inactive dextrans, suggesting that the former had more-branched structures. To verify this suggestion, the dextrans were subjected to methylation.

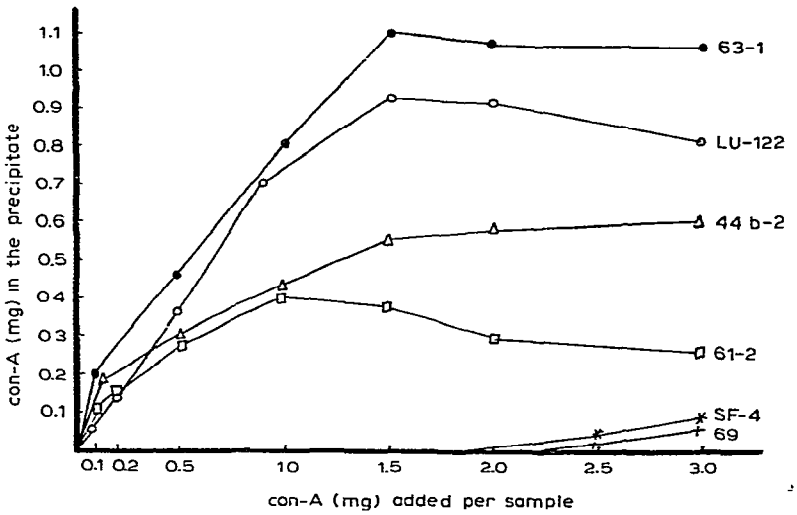

Fig. 2. Curves of quantitative precipitation of dextrans with concanavalin A (see Experimental).

(*e*) *Methylation studies.* These studies employed the method of Hakomori[14]. The degree of methylation was judged by i.r. spectroscopy. After hydrolysis of the methylated dextrans, the methyl ethers of D-glucose were converted into the corresponding alditol acetates, which were analyzed by gas–liquid chromatography and mass spectrometry. The results are presented in Table III. As dextrans SF-4 and 69 had high percentages of (1→6)-glucosidic linkages, as shown by periodate-oxidation studies, and were inactive in the con-A precipitation, it was evident that both were more or less linear, and so these dextrans were not subjected to methylation.

TABLE III

MOLE PERCENTAGE OF METHYLATED D-GLUCOSE COMPONENTS IN HYDROLYZATES OF METHYLATED DEXTRANS

Strain	*Methyl ethers of* D-*glucose*				
	2,3,4,6	*2,3,4*	*2,4,6*	*2,4*	*3,4*
LU-122	25.4	47.5			27.1
63-1	15.0	69.8			15.2
44b-2	20.7	59.9		19.4	
61-2	9.8	46.9	32.8	10.5	

As shown in Table III, the dextrans having a high capacity to enhance n.s.r.i. had non-(1→6)-glucosidic linkages only at the branching points, and were characterized by a considerable extent of branching. Dextrans LU-122 and 44b-2 contained one branching point per 4 and 5 glucose residues, respectively. Dextran 63-1 was somewhat less branched (1 branching point per 7 glucose residues). The average extent of branching of dextran 61-2 was lower than those of the other three dextrans. This dextran contained one branching point per ten glucose residues and, as with dextran B-1355, had two types of glucosidic linkages, (1→3) and (1→6), in the linear chain of the molecule[15].

The data obtained provide evidence for a dominant role of the extent of branching in the ability of dextrans to enhance n.s.r.i.

As already noted, dextran 61-2 was inactive in experiments on production of n.s.r.i., but exhibited anticomplementary activity. It is likely that certain structural features, other than the extent of branching, are essential for complement fixation, as evidenced, in particular, by the somewhat enhanced activities of dextrans 61-2 and B-1355 in comparison to that of dextran 44b-2. The role of certain physicochemical properties of dextrans is not to be excluded[16,17].

(*f*) *Enzymic degradation of dextrans.* Some data on dextran structure are obtainable by use of the dextran-splitting enzymes, endo- and exo-dextranases[13,18]. In our studies, two such enzymes were used: the endodextranase produced by the mould *Penicillium purpurogenum* (Ftoli) and an acid α-D-glucosidase from pig spleen possessing an exodextranase activity.

As was demonstrated earlier[19,20], the endodextranase of *Penicillium purpurogenum* specifically splits (1→6)-glucosidic linkages in dextrans without affecting the branching points. The main low-molecular-weight products formed upon enzymic hydrolysis of dextrans containing (1→2) linkages are glucose, isomaltose, and tetrasaccharides of two types: branched (2^2-α-D-glucosylisomaltotrioses) and linear (2^3-α-D-glucosylisomaltotriose). Table IV shows the extent of splitting of various dextrans by the dextranase of *Penicillium purpurogenum* and the products formed upon exhaustive hydrolysis of these dextrans. Oligosaccharides designated as B_4, B_5, and so on, are a series of branched oligosaccharides resistant to dextranase action. The presence of residual amounts of isomaltotriose may be attributed to its very low rate of splitting.

As may be seen in Table IV, dextrans 63-1, LU-122, and B-1299 differed in both the extent of splitting and the composition of the products formed. The extent of splitting decreased with increase in the extent of branching. A somewhat proportion quantity of relatively low-molecular-weight products (B_4, B_5) formed upon hydrolysis of dextran 63-1 suggests that branching points in this dextran are more widely spaced than those in dextrans LU-122 and B-1299. Comparison of the products formed shows that branching points are located very irregularly in the dextrans. In the regions constituting 30,55, and 73% of molecules of dextrans 63-1, LU-122, and B-1299, respectively, the branching points were very closely spaced, thus rendering these regions more resistant to dextranase action. The remaining regions of the dextran

TABLE IV

ACTION OF *Penicillium purpurogenum* DEXTRANASE ON DEXTRANS CONTAINING HIGH PROPORTIONS OF (1→2)-α-D-GLUCOSIDIC LINKAGES

Strain	*Degree of hydrolysis*[a] (%)	*Products of dextranase action (percent by wt)*							
		D-*Glucose*	*Isomaltose*	*Isomaltotriose*	*Branched oligosaccharides*[b]				
					B_4	B_5	B_6	B_7	B_8 *and unresolvable material*
63-1	31	17.1	22.7	1.0	10.8	3.4	7.9	6.4	30.7
LU-122	24	5.7	24.6	0.3	4.4	1.3	4.4	4.0	55.1
B-1299	6	3.3	6.6	1.7	6.4	1.1	4.4	6.6	73.5

[a]Liberation of reducing sugars calculated as glucose. [b]B_4, B_5, B_6, B_7: tetra-, penta, hexa-, and hepta-saccharide fractions.

molecules contained either linear segments or segments having widely spaced branches, and were readily split by dextranase with the formation of D-glucose and isomaltose.

As shown earlier[21,22], acid α-D-glucosidase is capable of splitting off D-glucose units from the non-reducing ends of the dextran molecule. It readily splits (1→3)-glucosidic linkages, both in linear chains and at branching points, but does not split (1→2)-linkages at branching points[22]. The tetrasaccharide 2-α-D-glucosylisomaltotriose is resistant to the action of acid α-D-glucosidase, which allowed the use of this enzyme in structural studies of dextrans containing (1→2)-glucosidic linkages. Table V presents data on the splitting of the three dextrans by acid α-D-glucosidase. As the latter splits off D-glucose residues only from the non-reducing ends of the molecule, leaving attached one D-glucose residue at each branching point (plus one residue from the non-reducing end of the main chain), the average length of the outer branches in the molecule may be calculated, as has been done for glycogen and amylopectin[23]. The number of branching points was established on the basis of the methylation data. Table V summarizes the results of these calculations. The outer branches of dextrans 63-1 are longer than those of dextran LU-122, and these latter are longer than the branches of dextran B-1299. On the basis of the data obtained[24] for dextran B-512, and assuming that most dextrans contain short branches, it may be concluded that the outer chains of dextran B-1299 contain, in the majority of instances, only one D-glucose residue. The outer chains of dextran LU-122 contain one and two D-glucose residues (in approximately equal quantities), and the outer chains of dextran 63-1 contain 2–3 glucose residues. The data obtained provide structural information on the dextran; however, these data are as yet insufficient to prepare a detailed structural model of these dextrans.

As already noted, highly branched dextrans are also characterized by pronounced ability to enhance n.s.r.i. However, the extent of branching is not the only

TABLE V

ACTION OF ACID α-D-GLUCOSIDASE ON DEXTRANS CONTAINING HIGH PROPORTIONS OF (1→2)-α-D-GLUCOSIDIC LINKAGES

Strain	*Conversion into* D-*glucose* (%)	*Average exterior chain-lengths, expressed as* D-*glucose residues*
63-1	21	2.5
LU-122	14	1.6
B-1299	6	1.2

factor involved in determining the latter. It is likely that a certain length of outer chains is also essential for the n.s.r.i. activity. For instance, dextran 63-1 possessed the same activity as dextran LU-122, but the branches in dextran 63-1 were longer and less numerous than those in dextran LU-122. It may be assumed that the density and length of outer chains in the dextran molecule determine the adoption of a certain conformation that is responsible for interaction with biological systems controlling resistance to infection.

EXPERIMENTAL

Dextrans. — Dextrans T-500 and T-2000 und Sephadexes were products of Pharmacia Fine Chemicals Co. (Sweden). Most of the native dextrans were synthesized by strains of *Leuconostoc mesenteroides.* Dextran LU-122 was synthesized by *Leuconostoc dextranicum* strain. Isolation of the dextrans has been described previously[5,25].

The strains of *Leuconostoc mesenteroides* NRRL B-1299 and NRRL B-1355 were obtained from Dr. A. Jeanes. The organisms were cultured in media A and D as described[26]. For the isolation of these two dextrans, the culture media were dissolved in water and centrifuged (4500*g* for 30 min). The precipitate was discarded, and ethanol (up to 45%) was added to the solution. The precipitate formed was separated by centrifugation (1800*g* for 15 min), reprecipitated, and deproteinized by repeated treatment with chloroform by the Sevag method[27]. After reprecipitation, the dextran solution was centrifuged (30,000*g* for 40 min) in a Spinco Model L-2 ultracentrifuge. The water-soluble supernatant solution was subjected to fractional precipitation with 5% incremental increase in the concentration of ethanol. The major proportion of the product was precipitated at 35% ethanol (minor precipitates formed at lower concentrations of ethanol were discarded). The fraction thus obtained was separated by centrifugation, dissolved in water, lyphilized and used in subsequent studies. The detailed structure of these two dextrans has been described[15,28].

Alkali-treated dextrans was prepared by heating dextrans in 0.25M sodium hydroxide for 1 h at 56°. The solution was neutralized with acetic acid, and dextran was precipitated by ethanol, dissolved in water, and freeze-dried.

Periodate oxidation. — Periodate-oxidation studies, assay of unoxidized glucose, and determination of Smith-degradation products were performed as described[21,29].

Acetolysis. — This procedure was conducted by the method of Suzuki and Hehre[30] as described[6].

Quantitative determination of con-A binding. — Concanavalin A was isolated by the method of Agrawal and Goldstein[31]. Dextran and con-A were dissolved in 0.02M phosphate buffer (pH 7.0) prepared in M sodium chloride (solution B). To 100 μg of the dextran in 0.1 ml of solution B were added the corresponding amounts of con-A in 0.4 ml of the same solution. The mixture was incubated for 24 h at 25° and then centrifuged at 3000*g* for 30 min. The solution was then carefully decanted, the precipitate was washed twice by suspending it in 0.5 ml of solution B, and it was allowed to drain with the tubes in an inverted position. The drained precipitate was dissolved in 0.5 ml of 0.1M D-mannose solution prepared in solution B, and con-A was assayed by the method of Lowry[32], with dried con-A as standard.

Methylation of dextrans. — Methylation was performed in one step by the Hakomori method[14] as modified by Lindberg[33]. The extent of methylation was monitored by i.r. spectroscopy with use of KBr pellets and a Perkin–Elmer Model 700 i.r. spectrometer. The i.r. spectra of the dextrans showed no absorption at 3400 cm^{-1}, confirming permethylation. The methylated dextrans were subjected to formolysis (2 h, 100°, 90% formic acid) and to subsequent hydrolysis (0.25M sulfuric acid, 100°, overnight), and the methylated glucoses were studied (*1*) by thin-layer chromatography on Silica Gel G plates in two systems: (*a*) butanone–water azeotrope and (*b*) 9:1 chloroform–methanol, and (*2*) by gas–liquid chromatography (g.l.c.), as alditol acetates. The alditol acetates were prepared as described[33]. G.l.c. was performed with a Hewlett–Packard Model 5830 instrument equipped with a flame-ionization detector and two types of columns: (*1*) Teflon (1800 × 1.58 mm) containing 3% of ECNSS-M on Gas Chrom Q, and (*2*) stainless steel (480 × 2.16 mm) packed with 10% of UCW-982 on Chromosorb WAW. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. The initial temperature was 150°, and was programmed at 5° per min. Molar ratios of the methylated glucoses were calculated from the peak-area response-factors proposed by Sweet *et al.*[34]. Mass spectrometry of the alditol acetates of the methylated glucoses was performed as described[20].

Enzymes. — The isolation of a dextranase from *Penicillium purpurogenum* (Ftoll) and its properties have been described[19,20]. The isolation and properties of acid α-D-glucosidase from pig spleen have also been described[21,22].

Quantitative determination of fragments formed from dextrans by the action of dextranase of Penicillium purpurogenum (*Ftoll*). — Dextran (20 mg) was dissolved in 18 ml of water with subsequent addition of 2 ml of dextranase (0.4 units) in 0.05M acetate buffer, pH 5.7. The mixture was incubated for 3–4 days at 37°, and 0.1 ml of dextranase solution was added daily until the increase in the content of reducing products[35] terminated. After incubation, the content of D-glucose was determined by using D-glucose oxidase[36]; three volumes of ethanol were added, and the resulting mixture was centrifuged at 4500*g* for 30 min, after which time the solution was.

evaporated to low volume and subjected to chromatographic separation on the previously washed Whatman 3MM paper in the system 14:2:7 propanol–ethyl acetate–H_2O. D-Glucose and isomaltosyl oligosaccharides of various degrees of polymerization were used as standards. The areas of chromatograms corresponding to oligosaccharides of various degrees of polymerization were eluted from the paper with water into volumetric flasks, and the solutions were filtered through glass-wool filters. The content of each fraction was determined by the phenol–sulfuric method[37]. Paper strips of the same size were eluted as controls, and the low values obtained were subtracted.

Determination of the biological activity of dextrans. — (*a*) *Test for production of resistance to infection.* The experiments were performed on white male mice of 18–22 g body weight. Dextran was injected intraperitoneally (800 μg in 0.2 ml of 0.9% sodium chloride solution); The control group was injected with 0.2 ml of 0.9% sodium chloride. The mice were challenged intraperitoneally 24 h after the injection with a virulent strain of *E. coli* 094. The doses were so chosen as to bring the control death-rate to about 90% (1.3–1.5 LD_{50}). Deaths occurred within 5 days after the infection. The average infectious dose of *E. coli* (LD_{50}) and the average effective dose of dextran (ED_{50}) were determined by the method of Kärber[38].

(*b*) *Complement fixation.* This was determined by the method of Galanos *et al.*[8] Fresh guinea-pig serum (100 μl) was mixed with graded amounts of dextran dissolved in 20 μl of distilled water, and incubated for 1 h at 37°. The mixtures were diluted to 300 μl with veronal buffer (pH 7.5), and 7-μl aliquots of the solutions were added to 1 ml of the buffer, and then 0.5 ml of a suspension of sheep erythrocytes sensitized with amboreptor were added. After incubating for 1 h at 37°, the samples were centrifuged and the absorbance of the supernatant solution was measured at 546 nm. Under these conditions, the complement control (no dextran added) gave an absorbance of 0.500, whereas the erythrocyte control (0.5 ml of the erythrocyte suspension completely lysed with 1 ml of distilled water) gave an absorbance of 1.2. Anticomplementary activity is expressed as percent inhibition of hemolysis obtained with the respective amounts (μg) of dextran added to 100 μl of undiluted serum.

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