Interaction between Wheat Proteins and Dextrans[1]

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ABSTRACT

Previous studies showed that certain dextrans alter the mixing curves of a flour-water dough, whereas others have no effect. This work has been extended to include a greater number of dextrans. In addition, the interaction of wheat proteins and dextrans in solution has been examined. In solutions of 0.01*M* acetic acid, some dextrans will interact with gluten, gliadin, and glutenin. The interaction produces either turbidity or a precipitate and is concentration-dependent. At constant carbohydrate concentration, turbidity increases to a maximum as the protein concentration is increased. As protein concentration is increased beyond the critical point, turbidity falls to zero. Not all dextrans or fractions of a given dextran react to the same degree; some do not interact at all. The effect of other polysaccharides on gluten solutions has also been studied.

Wilham *et al.* (1) showed that certain native dextrans alter mixing curves of flour-water doughs, but that others do not; their work has been extended to include a number of different dextrans. In addition, interaction in solution was studied by measuring the turbidity produced when solutions of gluten and dextran were mixed.

Dextran is an uncharged, branched polymer containing alpha-D-glucopyranose units. It is produced from sucrose by the action of certain microorganisms, principally *Leuconostoc mesenteroides*. The main chain of dextran contains alpha-1,6′-glucosidic linkages. Side chains are attached through alpha-1,2′-, 1,3′-, or 1,4′-bonds, depending on the strain of bacteria used. Dextrans from different strains also vary in molecular size, shape, degree of branching, and physical properties. Culture conditions and fractionation during isolation also may affect the polymer's properties. Native dextrans used here have molecular weights from 10 to 150 million. Dextrans from more than 100 different strains of microorganisms have been prepared and characterized at the Northern Laboratory (2).

METHODS

Dough-Mixing Studies

Procedure. To prepare a standard curve having a consistency of 500 B.U., add 30.5 ml. of distilled water to 49.5 g. of flour and 0.5 g. of wheat starch being mixed in a Brabender Farinograph. Begin recording consistency as soon as all the water is added. The farinograph tracing serves as a standard curve. For interaction studies, replace the starch with dextran. Examples of the curves are given in Fig. 1. In comparison with the standard curve, replacement of wheat starch with dextran B-742 produces only a small effect; dextran B-512F has a greater effect.

To assign a numerical value to the degree of interaction, two parameters of the mixing curve were measured. The first parameter measured was maxi-

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mum consistency recorded in Brabender units. The flour-wheat starch mixture which gave the standard curve has a consistency of 490. Consistencies for mixtures with the various dextrans ranged from 460 to 700 and were reproducible to 50 units. In Fig. 1, dextran B-742 gave a consistency of 530 and B-512F, 630.

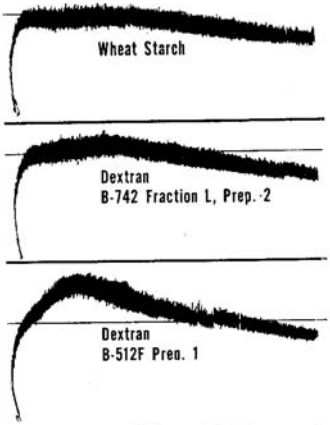

Fig. 1. Effects of mixing 49.5 g. of flour, 30.5 ml. of water, and 0.5 g. of carbohydrate.

The second parameter, based on the shape of the curve, has been designated dough stability. Stability was defined as the time (in min.) between the point where the top of the curve first intersects the maximum line and the point where it leaves the maximum line. Maximum line is the midpoint of the curve at maximum consistency. The flour-wheat starch mixture in the standard curve has a stability of 11.5 min. The effect of replacement of starch by various dextrans was evaluated by the change in stability (deviation from 11.5). Stability was reproducible to $\pm$ 1 min.

Interaction in Solution

Molecular weights of the dextrans were determined by light-scattering (3). Light-scattering requires centrifuging the solutions at high speed to remove foreign matter. Since extended centrifuging may fractionate the dextran, a procedure was adopted that allowed the interaction study and molecular-weight determination to be conducted on exactly the same material.

Procedure. Prepare solutions of dextran (greater than 1%) and protein (about 1%) in 0.01*M* acetic acid and dialyze overnight against 0.01*M* acetic acid. Centrifuge dextran at about 31,000 $\times$ *g* for 3 hr., glutenin, 31,000 $\times$ *g* for 40 min., and gliadin 80,000 $\times$ *g* for 1 to 2 hr., in a Spinco Model L centrifuge equipped with a No. 21 head. Remove samples of the dextran for light-scattering measurements. Determine concentrations by refractive index (4) or optical rotation. Dilute dextran to exactly 1%. To a series of test tubes, add protein and solvent to give 2 ml. of solution and the desired protein concentration. Add 2 ml. of 1% dextran to each tube and mix. Let the tubes stand 1 hr. before reading absorbance at 545 mμ with a Beckman Model B Spectrophotometer and 1 cm. cell path length. Correct for initial

turbidity of dextran with a dextran blank. (At most concentrations initial turbidity due to protein was negligible; at higher concentrations turbidity was corrected for protein with a blank.) Plot the turbidity from the interaction against the ratio of protein to carbohydrate. Typical curves are shown in Figs. 2 and 3.

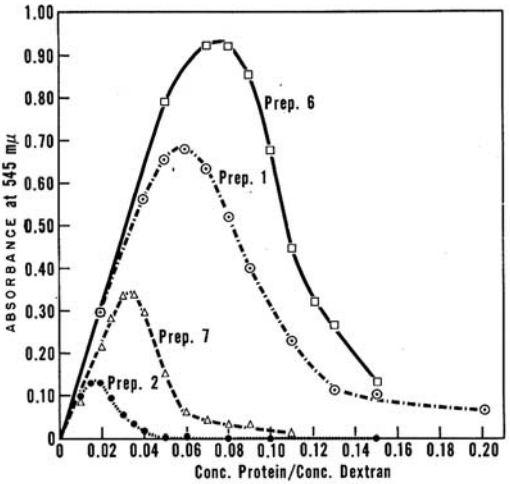

Fig. 2. Interaction between glutenin and B-512F dextrans. Dextran concentration, 0.5%. Preparations are described in Table I. Preparation 6 was purified in the laboratory.

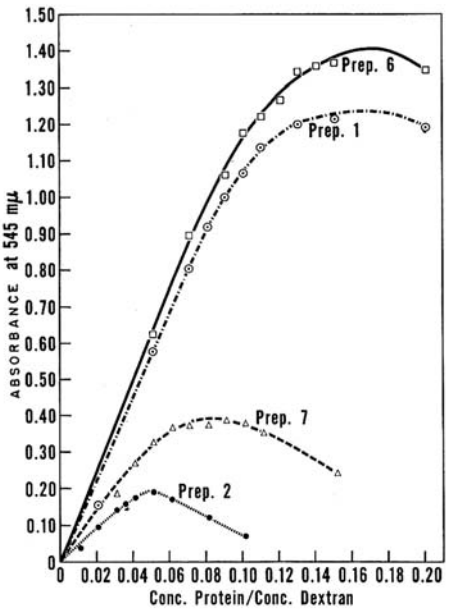

Fig. 3. Interaction between gliadin and B-512F dextrans. Dextran concentration, 0.5%. Preparations are described in Table I. Preparation 6 was purified in the laboratory.

MATERIALS

Protein

Nondefatted flour from Ponca wheat was used in the mixing studies, except where comparisons were made with flour defatted with dry n-butyl alcohol. The term *flour* refers to nondefatted flour unless specifically stated otherwise. Gluten and gluten fractions were separated from defatted Ponca flour by the method of Jones *et al.* (5).

Dextran

Crude B-512F dextran is the 35% ethanol-solubles from a whole enzymatic-synthesis mixture. It contains dextran, residual nutrients from preparation of dextransucrase, fructose, residual traces of sucrose, and polymeric by-products (2,6). Native dextrans are high-molecular-weight (MW) polymers that have been well purified in the laboratory (2) or in the pilot plant (6). Dextran fractions listed in Tables I and III are those described by Wilham *et al.* (7) and are identified according to their system and that used in reference 2.

Dextran B-512F fractions of graded molecular weight listed in Table IV were obtained by fractional precipitation of purified dextran by alcohol from water solution. The 50% ethanol-soluble fraction was further purified by precipitation from absolute ethanol and exhaustive dialysis. This procedure removed most of the color, fructose, sucrose, and other small molecules. The purified 50% ethanol-solubles used for interaction study consisted of primarily low-MW dextran and some levan. This fraction has been described by Jeanes *et al.* (8).

RESULTS

Dough Mixing Studies

Table I summarizes mixing parameters established by addition of dextrans from different strains, different preparations, and different fractions, and by changes in techniques of dextran isolation. Also, the effect of adding dextran as a solid or in solution is given in Table I. In this table, the dextrans are listed in the order of increasing maximum consistency. Only with dextran B-1308 does this order differ significantly from that obtained by listing the dextrans according to decrease in stability.

On the basis of change in dough stability, native dextrans can be divided into two classes: (a) those that produce little change in stability time (1 to 3.5 min.) and (b) those that cause a great change (6 to 8 min.). In Fig. 1, dextran B-742 produces a decrease in stability of 2.5 and dextran B-512F of 7 min. If dough stability is used as a criterion, 10 dextrans were in class a and 16 in class b (Table I).

Those dextrans that produce a great change in stability tend to have a high maximum consistency. There is no sharp separation, however, among those dextrans that alter the maximum consistency and those that do not. There is a smooth transition from 460 to 700 B.U.

Correlation appears between the available data on structure and properties of most of the dextrans and the results of the dough-mixing experiments

TABLE I

EFFECT OF DEXTRAN ON FARINOGRAPH CURVES

Test	Dextran				Percent Linkages		Maximum Consistency	Decrease in Stability
	NRRL Strain	Fraction[a]	Prep.	$[\eta]$	1,6′-Like	1,4′-Like		
							B.U.	
A	B-742	L	2	.....	81	19	460	2.5
B	B-1355	S	.....	0.192	57	8[b]	470	1
C[c]	B-1145	.....	.....	1.029	96	2[b]	490	3.5
D	B-512F	M	5	0.555	95	5	500	2
E[c]	B-512F	S	5	0.992	95	5	510	2
F	B-742	L	2[d]	.....	81	19	510	2.5
G	B-742	S	2	.....	57	17[b]	510	3
H	B-742	L	1	0.152	81	19	520	1
I	B-742	S	1	0.326	57	17[b]	520	3
J	B-512F	.....	4	.....	95	5	530	6
K	B-512F	.....	7	1.07	95	5	530	6
L	B-512F	Crude	7	.....	95	5	530	6
M	B-1308	.....	.....	0.476	95	5	560	3
N	B-1399	L	.....	1.125	81	19[e]	560	6
O	B-512F	Crude	6	.....	95	5	560	6.5
P	B-512F	.....	6[f]	.....	95	5	570	6.5
Q	B-1146	.....	.....	1.078	97	3	580	6
R	B-512F	.....	2	1.498	95	5	580	7
S	B-1064	.....	.....	.....	96	4	580	7
T	B-512F	.....	6[g]	1.403	95	5	610	6.5
U	B-512F	.....	1[g]	1.456	95	5	620	7
V	B-512F	.....	3[g]	1.504	95	5	620	7
W	B-640	.....	.....	1.280	95	5	650	8
X	B-1064[h]	.....	.....	.....	96	4	670	8
Y	B-512F	.....	2[d,g]	1.498	95	5	690	8
Z	B-512F	A	5	1.069	95	5	700	8

[a] Unless otherwise stated, the dextrans were native, of high molecular weight, and well purified.
[b] Balance of linkages are 1,3′-like.
[c] Shows anomalous behavior.
[d] Added in solution.
[e] Contains 1,2′-bonds.
[f] Pilot-plant-purified.
[g] Laboratory-purified.
[h] Defatted flour used.

TABLE II

SUMMARY OF EFFECTS OF VARIABLES ON FARINOGRAPH CURVES

Variable	Consistency	Decrease In Stability
High $[\eta]$	High	Large
High % 1,6′-linkages	High	Large
$[\eta]$ *vs.* % 1,6′-linkages	Effect of $[\eta]$ predominates	
Lyophilized (O,L) *vs.* alcohol precipitated (J,T,V) dextran	Unimportant	Unimportant
Dextran added dry *vs.* added in solution	Added in solution higher	Unimportant
Purity of dextran	Unimportant	Unimportant
Defatted *vs.* nondefatted flour	Defatted higher	Unimportant
Clinical size ($M_w = 75{,}000 \pm 25{,}000$) *vs.* native dextran ($M_w = 50\text{–}150 \times 10^6$)	Clinical size lower	Unimportant

(summarized in Table II). Before these correlations are discussed in detail, however, it is desirable to segregate two dextrans, tests C and E in Table I, that show anomalous behavior; that is, they do not show the correlation given in Table II. Dextran B-512F fraction S (test E) gave anomalous results in all tests made, that is, dough consistency, dough stability, and interaction in solution. The only significant way fraction S is known to differ from typical preparations of native dextran from this strain is in slightly lower intrinsic viscosity and considerably lower molecular weight (10×10^6). No clue is available to account for the anomalous behavior of dextran B-1145 (test C).

Both high intrinsic viscosity of dextran and high percentage of 1,6′-glycosidic linkages in dextrans produce a large effect on mixing curves. Effect of viscosity always predominates over effect of percent 1,6′-bonds. This is illustrated in tests D, M, and N. When the greater influence of $[\eta]$ is considered and the anomalous dextrans (C and E) are disregarded, all dextrans producing a large effect on mixing curves have intrinsic viscosities greater than 1.0 and contain at least 95% 1,6′-bonds. All dextrans producing a small effect on mixing curves have intrinsic viscosities less than 0.6 and contain 81% or less 1,6′-bonds. The correlation between high percentage of 1,6′-linkage and reactivity is probably a function of low degree of branching rather than the presence of 1,6′-bonds. The less highly branched, more linear polymer would have a greater viscosity for a given molecular weight than would a highly branched molecule. Dextrans containing a high percentage of 1,6′-bonds but having low viscosities do not react.

The physical conditions of dextrans that result from the method of isolation do not produce a major change in dough stability. Addition of lyophilized samples (O and L, Table I) gave stability values similar to those for samples precipitated from alcohol (J, T, and V). When dextran was dissolved in water before it was mixed with flour, there was no change in stability (A *vs.* F and R *vs.* Y); however, the maximum consistency increased significantly.

In the absence of dextran, defatted flour has the same stability but 30 B.U. more consistency than nondefatted flour. When dextran is added to the dough, defatting of flour has little effect on stability, but defatted flour has a much higher consistency than nondefatted flour (S *vs.* X).

Purification of an added dextran sample produced no significant changes in either stability or maximum consistency of dough (laboratory P and T *vs.* crude pilot plant, O).

The effect on mixing curves of dextrans in the clinical size range was studied. Clinical dextrans (M_w about 75,000) can be produced by partial hydrolysis and fractionation of native dextran (M_w about 100×10^6), or can be prepared by direct enzymatic synthesis. Clinical dextran B-512F produced by either method did not change mixing stability, but it did decrease the maximum consistency to about 400 B.U. The difference in behavior between clinical and native dextran is probably due to difference in molecular weight or the sharp molecular weight distribution of the clinical material.

Interaction in Solution

Figures 2 and 3 show the results of protein-dextran interaction obtained with glutenin and gliadin and four preparations of B-512F dextran. Whole gluten gave curves intermediate between its two fractions. Dextran concentration was held constant. As protein concentration was increased, turbidity increased until a maximum was reached. Turbidity then dropped toward zero. Dextran B-512F Prep. 5, B-1355 fraction S, and B-640 did not react. They were tested at various protein-to-dextran ratios ranging from 0.005 to 1. Tables I and III give more information about these carbohydrates.

TABLE III
INTERACTIONS OF DEXTRAN AND GLUTEN PROTEINS

DESIGNATION IN TABLE I	DEXTRAN NRRL STRAIN	PREP. NO.	$M_w \times 10^{-6}$	REACTION WITH PROTEIN
T	B-512F	6	107	Yes
U	B-512F	1	150	Yes
L	B-512F	7	83	Yes
R	B-512F	2	98	Yes
E	B-512F	5	10	No
B	B-1355	...	...	No
W	B-640	...	...	No

When dextran was added to gluten proteins, the maximum turbidity was reached in less than 1 hr. Turbidity remained constant for as long as 2 weeks. After the colloid was formed, addition of more protein did not reduce turbidity. The order of addition was unimportant.

When dextran concentration was increased above the 0.5% used in preparing Figs. 2 and 3, turbidity increased, but the maximum occurred at the same ratio of protein to carbohydrate as at the lower concentration. If the concentration was high enough, a precipitate formed rather than a stable colloid.

When a stable colloid formed, the nature of the "suspended material" could not be determined. Centrifugation to bring down the suspension also threw dextran from solution. When a precipitate was formed and allowed to stand overnight, the suspension settled out and all the polysaccharide was in the supernatant. At the peak of the precipitation curve most of the protein precipitated. The turbidity, therefore, appeared to be caused by precipitation of protein rather than formation of a protein-carbohydrate bond. If the percentage of polysaccharide in the precipitate were small, the loss of dextran from the supernatant might not be detectable. Dextran concentration is determined by a phenolsulfuric acid method (9). The loss of carbohydrate from solution could be easily detected if equal moles of protein and carbohydrate were precipitated. If equal weights were precipitated, the loss could also be measured. If the weight of polysaccharide precipitated is less than one-half that of the protein, the limit of the analytical method would be reached.

The colloid was dissolved when small amounts of urea, dimethyl sulfoxide, or guanidine hydrochloride were added to the suspension. Electro-

TABLE IV
REACTION OF DEXTRAN FRACTIONS WITH GLUTENIN

Dextran Product	$M_w \times 10^{-6}$	Absorbance at Maximum
B-512F Prep. 7		
Crude	83	0.50
Purified	71	0.17
Fraction, purified		
1	109	No reaction
2	99	No reaction
3	86	No reaction
4	82	No reaction
5	72	No reaction
6	68	No reaction
Fraction, purified		
7	46	0.09
8	37	0.05
9	9	No reaction
Soluble in 50% ethanol	0.2[a]	Precipitate
B-512 Prep. 6		
Crude	....	0.45
Purified	....	0.20
Soluble in 50% ethanol	....	1.10

[a] Approximate.

phoresis (10) of the colloid shows that when whole gluten was used, glutenin precipitated first and then gliadin.

Table IV lists the results obtained with various dextran fractions. Crude dextran gave a greater reaction than the purified. The bulk of the reacting material was in the 50% ethanol-soluble fraction. Most of the reactivity of purified dextran was apparently in the soluble fraction that was discarded. Possibly fractions 7 and 8 (MW's of 46 and 37 million) that reacted slightly are contaminated with the more soluble low-MW fraction.

Crude B-512F Prep. 6 and the 50% ethanol-solubles from this dextran contain a considerable amount of levan, as indicated by the qualitative test of Jeanes *et al.* (8). Other dextrans that react strongly with gluten contain little or none. Therefore, the substance responsible for turbidity when mixed with gluten protein is apparently not levan.

Five other carbohydrates were tested for interaction with glutenin. Sweet-corn glycogen reacted to a greater extent than dextran. A precipitate which settled out was formed with glutenin at a glycogen concentration of 0.5%. However, when animal glycogen was added to glutenin, only a slight turbidity of about 0.01 was produced. Waxy maize amylopectin and levan B-512F did not react with glutenin. Levan B-523 reacted slightly with glutenin.

DISCUSSION

Although it is difficult to explain an interaction between a protein and a neutral polysaccharide, such a reaction has been reported before. A mixture of concanavalin-A and glycogen produces a precipitate (11). The degree of interaction varies markedly with the source of the glycogen. Dextrans react with *Pneumococcus* antiserums (12), properdin (13), and conglutenin (14). Differences among dextrans were found that could not be detected by chemical means. Dextrans and other neutral polymers reduce the solubility of protein (15–18). Some authors have proposed that protein is precipitated because the other polymer removes water from the protein-solvent system. This property of insolubility of protein in the presence of carbohydrate has been suggested as one way to fractionate proteins. These workers (15–18)

have in general used only one concentration. Others have worked over a wider range and noted that the interaction of dextran with properdin (19), human plasma (20), and concanavalin-A (21) resembles an antigen-antibody reaction; that is, it is inhibited by an excess of either component. The precipitation curves are similar to those in our work.

Dextran B-1355 fraction S, which did not react with gluten either in our mixing or solution studies, reacts both with concanavalin-A (21,22) and with the conglutinating system of bovine serum (14).

The effect of dextran on mixing curves and that on solution turbidity may not be due to the same phenomenon. Dextrans that react in the mixer do not always react in solution. Dextrans that affect dough-mixing curves are, in general, high-MW, high-viscosity polymers having relatively few branches. The dextran fraction that produces the greatest turbidity with gluten proteins is the lowest-MW component. Purity of the dextran used has a marked effect on turbidity formation but no effect on dough-mixing curves. The mixing results alone could be explained on the basis of competition for water with no actual bonds between dextran and gluten. However, as reported by Wilham *et al.* (1), a gluten ball falls apart when kneaded in a 5% dextran solution. This disintegration suggests an actual interaction between gluten and dextran.

The precipitation curves obtained with gluten and dextran certainly resemble the typical antigen-antibody reaction. However, in immunochemical reaction both components are usually precipitated. In our experiments it appears that both dextran and glycogen remained in solution, although the data are not conclusive. With the information that is now available, a definite explanation of the nature of the interaction cannot be given.

Acknowledgment

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Literature Cited

1. WILHAM, C. A., DIMLER, R. J., and SENTI, F. R. A note on the effects of neutral polysaccharides on dough-mixing properties. Cereal Chem. 36: 558–563 (1959).
2. JEANES, ALLENE, HAYNES, W. C., WILHAM, C. A., RANKIN, J. C., MELVIN, E. H., AUSTIN, MARJORIE J., CLUSKEY, J. E., FISHER, B. E., TSUCHIYA, H. M., and RIST, C. E. Characterization and classification of dextrans from ninety-six strains of bacteria. J. Am. Chem. Soc. 76: 5041–5052 (1954).
3. SENTI, F. R., HELLMAN, N. N., LUDWIG, N. H., BABCOCK, G. E., TOBIN, R., GLASS, C. A., and LAMBERTS, B. L. Viscosity, sedimentation, and light-scattering properties of fractions of an acid-hydrolyzed dextran. J. Polymer Sci. 17: 527–546 (1955).
4. TAYLOR, N. W., and CLUSKEY, J. E. Wheat gluten and its glutenin component: Viscosity, diffusion, and sedimentation studies. Arch. Biochem. Biophys. 97: 399–405 (1962).
5. JONES, R. W., TAYLOR, N. W., and SENTI, F. R. Electrophoresis and fractionation of wheat gluten. Arch. Biochem. Biophys. 84: 363–367 (1959).
6. SOHNS, V. E., ROGOVIN, S. P., CONWAY, H. F., and LANGFORD, C. T. Pilot-plant production of clinical-sized dextran by acid hydrolysis of enzymatically synthesized high polymer. U. S. Dept. Agr. Bull. AIC–372, 30 pp. (June 1954).
7. WILHAM, C. A., ALEXANDER, B. H., and JEANES, ALLENE. Heterogeneity in dextran preparations. Arch. Biochem. Biophys. 59: 61–75 (1955).

8. JEANES, ALLENE, WILHAM, C. A., and MIERS, J. C. Preparation and characterization of dextran from *Leuconostoc mesenteroides*. J. Biol. Chem. 176: 603–615 (1948).
9. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., and SMITH, F. Colorimetric method for determining sugars and related substances. Anal. Chem. 28: 350–356 (1956).
10. WOYCHIK, J. H., BOUNDY, JOYCE A., and DIMLER, R. J. Starch gel electrophoresis of wheat gluten proteins with concentrated urea. Arch. Biochem. Biophys. 94: 477–482 (1961).
11. CIFONELLI, J. A., MONTGOMERY, R., and SMITH, F. The reaction between concanavalin-A and glycogen. J. Am. Chem. Soc. 78: 2485–2489 (1956).
12. HEHRE, E. J. Contribution of classical immunology to the development of knowledge of dextran structure. Bull. Soc. Chim. Biol. 42: 1581–1585 (1960).
13. PILLEMER, L., SCHOENBERG, M. D., BLUM, LIVIA, and WURY, LEONA. Properdin system and immunity. II. Interaction of properdin system with polysaccharide. Science 122: 545–549 (1955).
14. SAGE, H. J., RANSBY, A., and LEON, M. A. Quantitive studies of a conglutinating system in normal bovine serum. J. Immunol. 90: 347–357 (1963).
15. LAURENT, T. C. The interaction between polysaccharides and other macromolecules. 5. The solubility of proteins in the presence of dextran. Biochem. J. 89: 253–257 (1963).
16. LAURENT, T. C. The interaction between polysaccharides and other molecules. VI. Further studies on the solubility of proteins in dextran solutions. Acta Chem. Scand. 17: 2664–2668 (1963).
17. POLSON, A., POTGIETER, G. M., LARGIER, J. F., MEARS, G. E. F., and JOUBERT, F. J. The fractionation of protein mixtures by linear polymers of high molecular weight. Biochim. Biophys. Acta 82: 463–475 (1964).
18. YEPPEYAUER, M., and BRISHAMMER, S. Protein precipitation by uncharged water-soluble polymers. Biochim. Biophys. Acta 94: 581–583 (1965).
19. LEON, M. A. The reaction between dextrans and the properdin complement system. I. Inhibition by excess dextran. J. Immunol. 85: 190–196 (1960).
20. FESSEL, W. J. Dextran turbidity: Acute distress-phase reaction. Nature 205: 771–773 (1965).
21. GOLDSTEIN, I. J., HOLLERMAN, C. E., and SMITH, E. E. Protein-carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin-A with polysaccharides. Biochemistry 4: 876–883 (1965).
22. GOLDSTEIN, I. J., and SO, LUCY L. Protein-carbohydrate interaction. III. Agar gel-diffusion studies on the interaction of concanavalin-A, a lectin isolated from jack bean, with polysaccharides. Arch. Biochem. Biophys. 111: 407–414 (1965).

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