STUDIES ON THE CAPACITY OF SOME POLYSACCHARIDES TO ELICIT ANTIBODY FORMATION IN MAN*

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The demonstration that pneumococcal polysaccharides (1–6) and purified blood group substances (*cf.* reference 7) are antigenic in some species and not in others and the more recent finding that dextrans are antigenic in man (8–10) but not in the rabbit (11, 12) or guinea pig (12) prompted an investigation of the antigenicity of several other purified polysaccharides in human beings. Levan, apple amylopectin, maize glycogen, and two synthetic polyglucoses were tested for their ability to stimulate production of specific antibody. In a small series of individuals injected with these materials, antibody formation as evidenced by the appearance of precipitins and wheal and erythema type skin sensitivity was obtained only against levan.

While levan has never been reported to be antigenic in man, Horsfall (13) and Genghof (14) noted that purified levan preparations are not antigenic when injected intravenously into rabbits in amounts from 0.15 to 15 mg. Rabbit antisera to levan, however, have been obtained by immunization with heated or formol-treated suspensions of *Streptococcus salivarius* and Bacillus N-9 grown in sucrose-containing media but not with these organisms grown on glucose (15). These rabbit antisera have been shown to give complement fixation and precipitation with a number of levans isolated from supernates of bacterial cultures grown on sucrose or raffinose as well as with levans synthesized by cell-free enzyme preparations (16, 17). Living suspensions of *Aerobacter levanicum* grown on sucrose have also been employed to immunize rabbits and the antisera produced used in qualitative precipitin tests. Oligolevans were found to inhibit the levan-antilevan precipitation (18).

In this investigation antiserum from an individual who showed the greatest antibody response to levan was studied by the micro-quantitative precipitin

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method of Heidelberger and MacPherson (19) using a variety of levans. Immunochemical evidence for the existence of structural differences among levans was obtained.

Analysis of specific precipitates for distinctive constituents of the antigen has been employed to estimate degree of purity of antigens and to provide evidence that a given antibody combines with and specifically precipitates a given antigen (*cf.* reference 7 for review). Distinctive constituents of antigens such as iodine (20), copper (21), hexosamine (22–24), methylpentose (25, 26), radioactivity (9), galactose and uronic acid (27) and hydroxyproline (28) have been employed in the analysis of specific precipitates. Antibody involved in the present study can be shown to be antilevan by its specific capacity to precipitate levan as demonstrated by the presence of fructose in the specific precipitate.

A non-specific anamnestic rise in antidextran or anti-A precipitins resulting from levan immunization was not found. In addition, purified soluble laminarin, a neutral polysaccharide obtained from *Laminaria digitata*, was found to give precipitation with normal human sera.

Materials and Methods

Four levans, designated by the number of the causative strain, were supplied by the Northern Utilization Research Branch, United States Department of Agriculture, Peoria, Illinois, through the courtesy of Dr. Allene Jeanes, as follows: NRRL P-6 (3727-10), the product of an unidentified microorganism; and NRRL B-523 (3664-46 fraction M), NRRL B-512-E (3525-77) and NRRL B-512 (P.P.2, 3801-30 fraction B) which were by-products of dextran production by strains of *Leuconostoc mesenteroides* (42). Levan P-6 contains 94.5 per cent polymerized fructose with a nitrogen content of 0.05 per cent, while the other three samples each contain 96 per cent combined fructose (29).

A native levan prepared from culture filtrates of *Aerobacter levanicum* grown on sucrose (30 *a*), and purified by repeated methanol precipitation, was kindly supplied by Dr. Shlomo Hestrin of the Hebrew University, Jerusalem; it had a molecular weight of 60×10^6 (determined by light scattering), a nitrogen content of 0.1 per cent and is a branched molecule made up mainly of 2,6′ linkages with about 10 per cent 1,2′ linkages (30 *b*). Levan fractions A and B were prepared in Dr. Hestrin's laboratory by methanol precipitation of a partial acid hydrolysate (acetate, pH 3.4 at 60° for 1 hour) of the native levan from *Aerobacter levanicum*. Fraction A was precipitated at 55 per cent methanol by volume, while fraction B was obtained between 55 and 63 per cent. Fractions A and B show different degrees of polymerization in that the ratios of reducing ability after hydrolysis to those before hydrolysis were 610 and 254 respectively (30 *b*). Perennial rye grass levan (32) was obtained from Dr. E. L. Hirst.

Samples of maize glycogen, apple amylopectin, and soluble laminarin were supplied by Dr. W. Z. Hassid. Polyglucose 2947-53 was obtained from Drs. Zambito and Denkewalter of Merck and Company, Inc., while polyglucose P-218-56 was obtained from Dr. P. T. Mora of E. I. Dupont de Nemours and Company.

Skin Testing and Immunization Procedures.—Polysaccharides used for immunization and skin testing were made up in saline, containing 0.25 per cent phenol, to a concentration of 1 mg. per ml. Volunteers were initially bled, skin-tested, and then each received subcutaneous injections of 0.5 ml. (0.5 mg.) antigen on each of 2 successive days. Intracutaneous skin tests were carried out by injecting about 0.02 ml. antigen solution along with a control con-

sisting of phenolized saline solution. Both sites were examined after 15 minutes, at which time the size of wheal and erythema reactions was recorded. Cutaneous reactions were compared in size with those elicited at the control site and were graded from − to ++++. 3 weeks after the last injection, a postimmunization bleeding was taken and subjects were retested for skin sensitivity.

Quantitative Precipitin Studies.—Procedures used in preliminary testing of pre- and postimmunization sera for antibody N were those described in detail for antidextran (8 *b*), using 3 ml. of serum and 10 and 25 μg. of antigen. Postimmunization bleedings were obtained from a subject (No. 1) who had shown the greatest antibody response and were used in quantitative precipitation studies. Subject 1 had previously been immunized with dextran and purified blood group substances (hog A, horse A and B). Anti-levan serum 1 $D_{11}L_2$[1] was absorbed with dextran and hog A substance and the absorbed serum employed in the precipitin reaction. Precipitin curves with absorbed antiserum 1 $D_{11}L_2$ were obtained by the quantitative precipitin method of Heidelberger and MacPherson (19) employing 1.0 ml. serum which was added to analytically measured amounts of polysaccharide. The total volume was 2.0 ml. Washed specific precipitates were analyzed for antibody N by the Folin-Ciocalteu tyrosine method (*cf.* reference 33), employing reagents standardized against known amounts of normal human gamma globulin (34) and antibody (35).

Ketosugar Estimations on Specific Precipitates.—Based on the quantitative precipitin curves, washed specific precipitates obtained in the region of antibody excess were analyzed for ketosugar by the cysteine-carbazole method of Dische and Borenfreund (36). Precipitates were formed and washed in 15 ml. conical centrifuge tubes. After breaking up and suspending the washed precipitates in 1.0 ml. distilled H_2O, 0.2 ml. cysteine hydrochloride (0.15 per cent aqueous solution) was added followed by addition with constant shaking of the sulfuric acid reagent (6 ml. of a mixture of 190 ml. H_2O and 450 ml. concentration H_2SO_4 C.P.). 0.2 ml. of the carbazole reagent (0.12 per cent alcoholic solution) was added immediately, the mixture shaken and allowed to stand at room temperature for 24 hours. Reagent blanks, levan standards, controls of normal gamma globulin, and gamma globulin plus levan were set up at the same time and included in each determination. Concentrations of gamma globulin N included in controls for each levan were comparable in N to those contained in the various specific precipitates as determined from the precipitin curves. Human gamma globulin was found not to interfere with the color intensity or development in the concentrations employed (up to 25 μg N), thus corrections for color due to antibody were not needed. The violet color developed after 24 hours was read in the Beckman spectrophotometer as the difference in optical densities at 5600 and 7500 A. Concentrations of antigens were chosen so that precipitates did not contain more than 10 μg. levan.

RESULTS

Table I summarizes responses obtained on injection of human volunteers with various polysaccharides. A rise of 2 μg. or over, in precipitable N per ml. serum is considered significant. This occurred in four individuals (Nos. 1, 131, 144, 148) following subcutaneous injection of levan. Subject 1 showed a rise in N specifically precipitable by levan from 0.4 to 16.7 μg./ml. while subjects 131, 144, and 148 went from 1.0 to 3.4 μg., 0.0 to 3.1 μg., and 0.9 to 3.7 μg.N/ml., respectively. Six individuals injected with maize glycogen, five

[1] D_{11} signifies the 11th bleeding following dextran immunization and L_2, the 2nd bleeding 70 days after immunization with levan.

TABLE I

Response to Injection of Various Polysaccharides in Man

Polysaccharide	Subject No.	Maximum μg. N precipitable from 1.0 ml. serum		Cutaneous reaction	
		Pre-immunization	Post-immunization	Preimmunization	Postimmunization
		μg.	μg.		
Levan P-6 (NRRL)	1	0.4	16.7	+	+±
	129	0.2	0.7	±	±
	130	0.1	0.0	+	+
	131	1.0	3.4	+	+
	132	0.2	0.0	±	±
	133	0.0	0.4	−	±
	134	1.6	1.0	±	±
	140	0.1	0.0	±	±
	141	0.0	0.1	±	+±
	142	1.1	0.8	−	−
	143	0.3	0.4	±	±
	144	0.0	3.1	−	+±
	145	0.0	0.1	+	++
Native levan *Aerobacter levanicum*	146	0.1	0.0	−*, ++++‡	±, ++++‡
	147	0.2	0.0	−, ++++‡	++++*
	148§	0.9	3.7	−*, ++++‖	
Maize glycogen	121	0.4	0.7	−	−
	122	1.0	0.2	+±	−
	123	0.4	0.4	−	−
	124	0.3	0.9	±	−
	125	0.3	0.2	−	−
	126	1.3	0.8	±	−
Apple amylopectin	135	0.7	0.8	−	−
	136	0.4	0.7	−	−
	137	0.6	1.0	−	−
	138	0.2	0.1	−	−
	139	1.1	0.1	−	−
Polyglucose 2947-53 (Merck)	59	3.3 (0.2)¶	3.9 (0.0)	−	−
	60	1.5 (0.0)	2.0 (0.0)	−	−
	61	1.5 (0.4)	1.8 (0.0)	−	−
	62	1.6 (0.0)	1.5 (0.5)	±	−
	63	2.3 (0.3)	2.7 (0.5)	+	−
	64	3.0 (0.0)	2.4 (0.0)	−	−
	77	1.2 (0.0)	1.2 (0.3)	−	−
	78	2.3 (0.2)	3.2 (0.3)	−	−
	79	2.4 (0.0)	1.6 (0.0)	−	±
	80	0.9 (0.1)	0.3 (0.1)	−	−
	81	0.0 (0.0)	0.1 (0.1)	−	−
	82	0.3 (0.2)	1.2 (0.3)	−	−
Polyglucose P218-56 (DuPont)	71	0.2	0.0	−	−
	72	0.0	0.1	−	−
	73	0.2	0.2	−	−
	74	0.1	0.2	−	−
	75	0.1	0.3	−	−
	76	0.1	0.1	−	−

* Reading after 15 minutes.

‡ After 6 hours.

§ Subject received only single subcutaneous injection (0.5 mg.) of antigen.

‖ After 2½ hours.

¶ Values in parentheses indicate μg.N/ml. precipitable by DuPont P 218-56 polyglucose.

with apple amylopectin, twelve with Merck synthetic polyglucose 2947-53, and six with Dupont synthetic polyglucose P218-56 showed no increase in nitrogen precipitable by the homologous polysaccharides.

Quantitative precipitin curves were obtained with various levans employing 1.0 ml. of absorbed antilevan serum 1 $D_{11}L_2$. Antilevan levels measured before and after absorption with dextran and blood group substance were 26.6 μg.

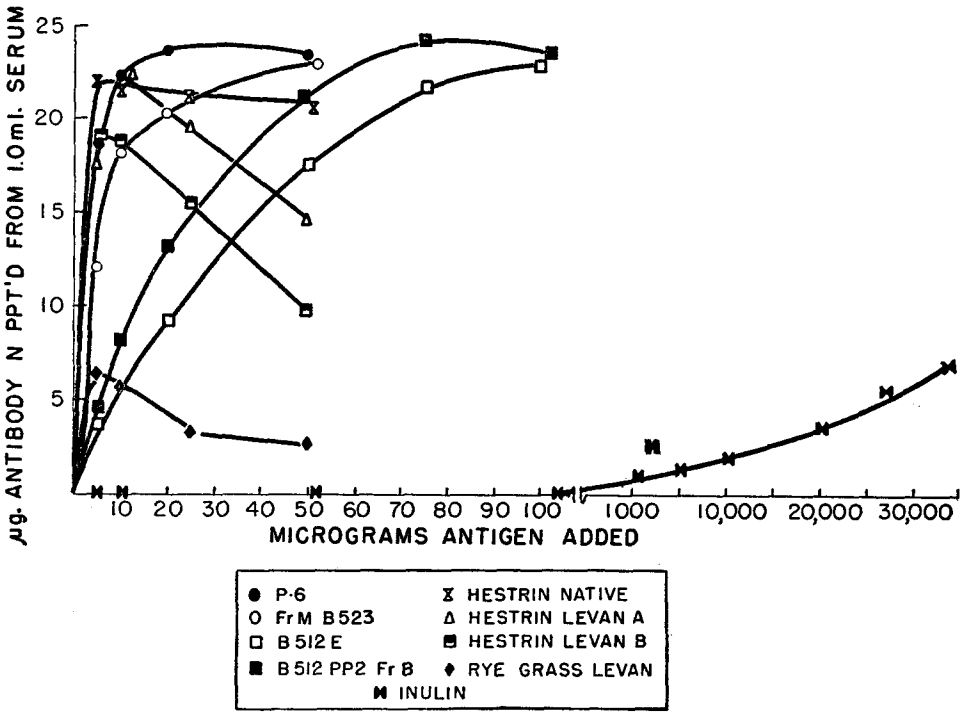

FIG. 1. Precipitin reaction of levans with 1.0 ml. antiserum 1 D_{11} L_2 (dextran and A absorbed).

and 23.6 μg.N/ml., respectively, as shown in Table II. In view of the multiple absorptions this is within experimental error.

As can be seen from Fig. 1, six of the levan preparations tested were able to precipitate from 21.9 to 24.2 μg. antibody N/ml., amounts comparable within experimental error to the 23.6 μg.N precipitable by the homologous levan P-6. Hestrin levan fraction B, precipitated somewhat less antibody (18.8 μg.N/ml.) while rye grass precipitated only 6.3 μg. antibody N. Amounts of inulin from 5 to 104 μg. gave no precipitation; in high concentrations, however, 33.6 mg. of inulin precipitated 6.8 μg. N. Although preparations B523 fraction M, B512 E, B512 P.P.2. fraction B, Hestrin native levan, and

Hestrin fraction A remove all antibody to levan P-6, as evidenced by failure of supernates at the point of maximum precipitation with these levans to give further precipitation on addition of levan P-6, they differ in their capacity to precipitate antibody per unit weight just as has been found with dextran (8, 37). Thus levan P-6, Hestrin native and fraction A levans require only 3 μg. fructosan to precipitate 50 per cent of the maximum antibody N, while preparations B523 fraction M, B512 P.P.2 fraction B, and B512 E require 5, 20 and 34 μg. levan, respectively.

As shown in Table II, levels of antibody N precipitable by levan showed a rise from 0.4 to 16.7 μg. antibody N/ml. within 3 weeks following levan injections, while antidextran and anti-A precipitin levels remained essentially un-

TABLE II

Circulating Precipitin Levels in Subject 1 before and after Levan Injection

	Maximum μg. antibody N/ml. precipitated			
	Before levan injection	After levan injection		
	D_8L_x bleeding (Jan. 28, 1954)	$D_{10}L_1$ bleeding (Feb. 17, 1954)	$D_{11}L_2$ bleeding (Apr. 8, 1954)	
			Unabsorbed	Absorbed*
Levan P-6	0.4	16.7	26.6	23.6
Dextran native NRRL B1255‡	17.1	17.1	16.4	—
Hog 30§	21.6	21.3	20.9	—

* Serum absorbed with dextran B1255 and purified blood group substance (hog A preparation).

‡ See reference 8 *b*.

§ Purified blood group A substance, see Leskowitz, S., and Kabat, E. A., *J. Am. Chem. Soc.*, 1954, 76, 4887; Kabat, E. A., and Leskowitz, S., *J. Am. Chem. Soc.*, 1955, 77, 5159.

changed. Bleeding $D_{11}L_2$ taken 70 days following initial immunization showed an increase in antilevan over the 20 day sample attaining a peak of 26.6 μg. antibody N/ml. Despite this further increase, antidextran and anti-A remained unchanged from prelevan immunization values. Subsequent determinations after immunization showed gradual declines in all three antibodies comparable to those observed with pneumococcal anticarbohydrate (38).

Antiserum 1 $D_{11}L_2$ can be shown to be antilevan by its specific capacity to precipitate levan as evidenced by the presence of ketosugar (fructose) in specific precipitates. Table III shows ketosugar determinations on washed specific precipitates determined by the cysteine-carbazole method. Precipitates formed in the region of antibody excess employing 5 μg. levan P-6, B523 fraction M, and Hestrin native and fraction A levans show 86–100 per cent of the levan added as antigen to be recoverable in the washed precipitates. In the equivalence zone or at the point of maximum precipitation

74–94 per cent recovery was obtained. Analysis of precipitates formed at the point of maximum precipitation with 5 μg. Hestrin levan fraction B and rye grass levan show low levan recoveries.

Subject 144, injected with levan P-6, showed increased skin reactivity accompanying a small increase in precipitins, while subjects 1 and 131 who were initially reactive, showed little or no increase in cutaneous sensitivity despite a rise in precipitins. Two subjects (141 and 145) showed a slight increase in skin reactivity without production of circulating precipitin, nor was non-

TABLE III

Ketosugar Estimations on Specific Precipitates

Levan preparations	Amount levan added	Antibody N precipitated	Levan recovered in specific precipitate	Recovery
	μg.	μg.	μg.	*per cent*
Levan P-6	5.0	18.6	4.4	88
	10.0	22.3	8.6	86
B523 fr. M	5.2	12.2	4.6	88
	10.4	18.2	9.8	94
Hestrin native	5.0	21.9	5.0	100
	10.0*	21.8	8.5	88
Hestrin fraction A	5.0	17.5	4.3	86
	10.0	21.9	7.4	74
Hestrin fraction B	5.0	18.8	3.2	64
	10.0	18.3	5.7	57
Rye grass	5.0	6.3	0.3	6
	10.0	5.6	1.2	12

* Average of analyses run in triplicate, all other analyses in duplicate.

precipitating (coprecipitating) antibody found when these sera were tested for their ability to add N to a specific levan-antilevan precipitate. Preliminary sensitivity to levan P-6 was shown by four subjects (1, 130, 131, 145) prior to injection; two of these (130, 145) failed to produce antibody. The best response to levan was obtained in an individual (No. 1) who had shown an excellent capacity to produce antibodies to dextran and blood group substances.

Initial skin testing of three individuals with the *Aerobacter levanicum* sample of native levan showed this preparation to have some degree of toxicity and it elicited intense local reactions which appeared after 2½ to 6 hours. Toxicity is associated with individual batches containing trace contaminants and

Hestrin's data indicate that it is not associated with the levan itself (31) but is probably due to some contaminant. One subject (No. 148) showed such severe local reaction including marked edema about the site following a single subcutaneous injection that immunization with this preparation was immediately discontinued. This single injection resulted in the appearance of signifi-

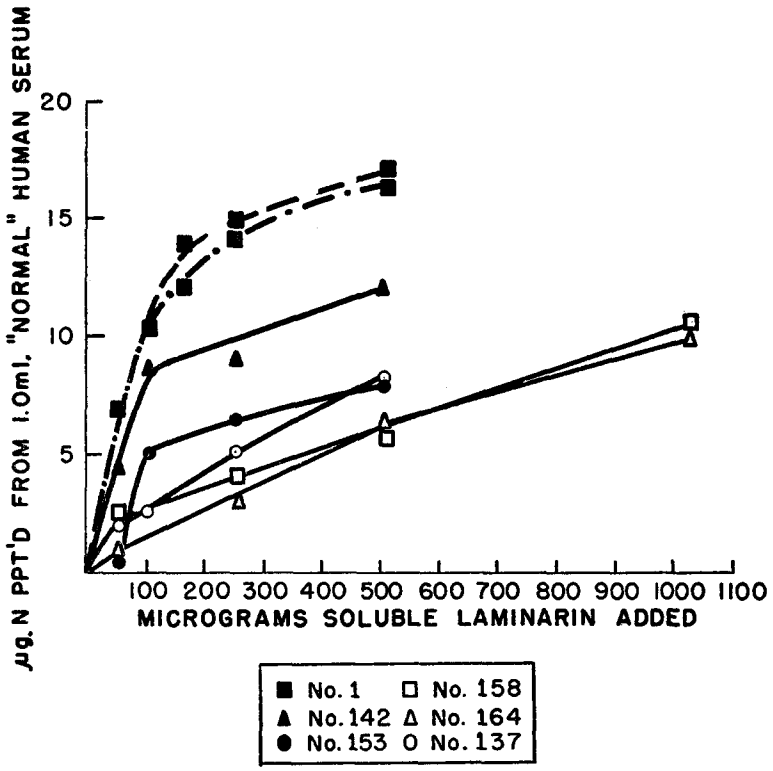

FIG. 2. Plot of N precipitated from 1.0 ml. various human sera by soluble laminarin.—— normal human sera, -·- antiserum $1D_8$ (abs.); - - antiserum 1 $D_{11}L_2$ (abs.). Antisera absorbed with dextran and blood group A substance.

cant amounts of antibody N. Intradermal injection of this preparation elicited a local reaction in the rabbit, induration and erythema appeared within 1 hour and reached maximum intensity at about 6 hours, while comparable amounts of fractions A and B showed little or no reaction over saline control sites. The other levans caused no significant reactions.

Purified soluble laminarin was found to give precipitation with absorbed anti-levan serum 1 $D_{11}L_2$. Study of preimmunization sera taken from five normal subjects and from individual 1 before and after levan injection (1 D_8 and 1 $D_{11}L_2$) also showed ability to form precipitates with laminarin. These pre-

cipitates are unlike carbohydrate-anticarbohydrate specific precipitates in appearance in that they are very finely divided and do not form a pellet on centrifugation, but pack as a fine sediment. While the nature of this precipitate has not been studied, it produces a blue color with the Folin-Ciocalteu phenol reagent. Fig. 2 shows a plot of N precipitated from 1.0 ml. serum by increasing amounts of laminarin, determined arbitrarily as gamma globulin N.

The extent of precipitation by any given quantity of laminarin, as shown by Fig. 2, varies considerably for each individual serum tested. Addition of 104 μg. laminarin precipitated only 1.6 μg.N/ml. from the least reactive serum (No. 164), while 10.4 μg.N/ml. was precipitated from the most highly reactive serum (No. 1). While immunization with levan caused a rise in antibody N so that absorbed antiserum 1 $D_{11}L_2$ contained 23.6 μg. antibody N/ml., comparison with an earlier preimmunization serum sample (1 D_8 absorbed) showed no increase in N precipitable by laminarin.

Sera tested with Merck polyglucose 2947-53 show small amounts of precipitable N both before and after immunization (Table I), while DuPont polyglucose P218-56 fails to precipitate. While values in Table I are reported per milliliter serum, the precipitin tests were actually performed with 3 ml. of serum and showed definite disc-like precipitates with the Merck sample, while no evidence of precipitation was seen with DuPont polyglucose. This difference in ability among polyglucose samples to precipitate N from both pre- and postimmunization sera is especially evident when both polyglucoses are tested with the same serum. Sera (59–64 and 77–82) which show small amounts of N precipitable by Merck polyglucose show little or no precipitate N (Table I, values in parentheses) when tested with the DuPont sample.

DISCUSSION

The data show that purified levans are antigenic in man, in that subcutaneous injection of 1 mg. of levan can give rise to the production of antibody specifically precipitable by levan. In a small number of individuals no evidence of antibody production was obtained after injection of comparable amounts of apple amylopectin, maize glycogen, and synthetic polyglucose.

Immunochemical findings that levan preparations B523, fraction M, B512 P.P.2 fraction B, and B512E show differences per unit weight in their ability in precipitating antibody are best interpreted as indicating structural differences among these preparations analogous to similar findings with dextrans (37). While structural information on these materials is fragmentary, inhibition studies undertaken in collaboration with Dr. Shlomo Hestrin employing oligosaccharides of known structure show the antilevan to have a specificity involving fructo-furanose units in 2,6′ linkage. This can also be inferred from Fig. 1, from which it can be seen that while rye grass levan and inulin have comparable molecular weights (about 5000), inulin, in which fructofuranose

residues occur in 1,2′ linkage, fails to precipitate anti-levan while rye grass levan, made up of 2,6′ linkages (32), is able to precipitate about ¼ of the anti-levan.

Inhibition of precipitation of anti-levan by levan P-6 is not observed with inulin. Addition of 25 μg. homologous levan to supernates of points set up with 10.0, 20.4, and 33.6 mg. inulin shows that of the 23.6 μg. antibody N originally present, 22.6, 22.8, and 22.2 μg. N respectively could be precipitated. The small amount of precipitate formed at high inulin concentrations is not due to antilevan since addition of 33.6 mg. inulin to serum completely absorbed of its anti-levan precipitated 6.8 μg. N from the absorbed serum. That antibody formed is levan-specific and not antibody to trace contaminants is shown by the capacity of absorbed antiserum 1 $D_{11}L_2$ specifically to precipitate levan as evidenced by the high recoveries (86–100 per cent) in specific precipitates of levan added as antigen.

Although no evidence was obtained of antibody formation resulting from injection of synthetic polyglucoses, differences among polyglucose samples were found. Absence of precipitation by DuPont polyglucose P-218-56 is in agreement with the finding (8 *b*) that Merck polyglucose 2947-53 shows cross reaction with antidextran sera, while the former does not. Moreover, Heidelberger and Aisenberg (39), have shown that polyglucose P-218-56 differs from other synthetic polyglucoses in that it shows considerably lower cross-reactivity in horse antipneumococcus sera. Structural differences among these polyglucoses which could account for differences in cross-reactivity with antidextran may also allow the Merck sample to cross-react with small amounts of some antibody present in normal sera. Immunization with levan failed to induce a non-specific anamnestic rise in antibody N precipitable by dextran or purified blood group A substance.

Precipitation of N from normal sera has been observed with several carbohydrates. Glycogen in large amounts is able to form precipitates with normal sera of several species (40) while normal human serum forms precipitates with higher concentrations of cycloheptaamylose but not cyclooctaamylose (41).

The observation that soluble laminarin, a neutral polysaccharide, is able to precipitate N from normal human sera remains to be explained.

SUMMARY

Purified levan is antigenic in man and subcutaneous injection of 1 mg. leads to the production of precipitins and skin sensitivity. Apple amylopectin, maize glycogen, and synthetic polyglucose did not stimulate antibody production in small numbers of individuals injected.

Quantitative precipitin studies carried out with several levan preparations employing human antilevan show variations in immunochemical behavior indicating structural differences among levans.

Evidence that precipitins formed in response to levan injection are antilevan antibodies was obtained by analysis of specific precipitates formed in the region of antibody excess for ketosugar.

Injection of levan giving rise to production of antilevan failed in one individual to give a non-specific anamnestic rise in either antidextran or antiblood group A precipitins.

Laminarin, a neutral polysaccharide, was found to give precipitation with normal human sera.

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