very similar to patterns shown by BSA in appropriate media (see Fig. 2 of ref. 5 and Fig. 7 of ref. 6). This change in electrophoretic behavior is undoubtedly related to the change in macromolecular structure accompanying oxidation. When the disulfide cross-linkages of the very compact RNAase molecule are oxidatively cleaved with performic acid, the molecule unfolds and appears to assume a flexible or random-coil type of structure rather than a hydrogen-bonded structure.[10] This suggests that the flexibility conferred upon the molecule by cleavage of the -S-S-bonds is responsible for the change in its electrophoretic behavior in acetate-containing media.

Furthermore, these results indicate that there is some structural feature of oxidized RNAase which is also present in ovalbumin and BSA and which is presumably related to a certain degree of flexibility of the polypeptide chain. Although ovalbumin and BSA are compact molecules in the *p*H range from about 4 to 10, it is conceivable that portions of these molecules may be flexible. A large net positive charge acquired by binding H^+ evidently modifies this structural feature (perhaps through internal electrostatic repulsions) such that the protein molecule interacts less strongly with buffer acid at low *p*H values than near the isoelectric *p*H. Indeed, BSA undergoes profound changes in molecular configuration below about *p*H 4 as revealed by changes in viscosity,[14,15] optical rotation[14] and sedimentation constant.[16,17] These changes in properties have been interpreted in terms of reversible expansion or unfolding of the protein molecule. We find that there is a striking relationship between the change in configuration of BSA with *p*H and the change in effectiveness of un-ionized buffer acid in altering its electrophoretic patterns. Thus, the viscosity of BSA increases progressively on going from *p*H 4.0 to 2.7; and, as shown in Fig. 2, the effectiveness of buffer acid decreases progressively over the same *p*H range. It would appear that expansion of the BSA molecule decreases the strength of its interaction with the buffer medium. There would also seem to be a correlation between a small charge in the molecular configuration of ovalbumin and the *p*H-dependence of the effectiveness of buffer acid in altering its electrophoretic patterns. Small changes in optical rotation[14,18] and a barely perceptable but significant change in sedimentation constant[17] indicate that ovalbumin underoges some change in molecular configuration below *p*H 4 but to a much smaller extent than with BSA.

It is conceivable that un-ionized buffer acid may interact with the protein molecule by forming double hydrogen bonds either with two different segments of the same peptide chain or with two different chains in the molecule. If this were the case, then such interaction would be sensitive to the relative orientation of the chains and/or their various side groups. Such orientation might in turn be sensitive to electrostatic repulsions within the molecule and most certainly to gross configurational changes of the kind observed with BSA below *p*H 4.

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[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL]

Immunochemical Studies on Dextrans. II.[1] Antidextran Specificities Involving the $\alpha 1 \rightarrow 3$ and the $\alpha 1 \rightarrow 2$ Linked Glucosyl Residues

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Human antidextran sera with a specificity directed against $\alpha 1 \rightarrow 3$ linked glucosyl residues were obtained by immunization with dextran NRRL B 1355S-4. In addition, sera with a specificity involving the $\alpha 1 \rightarrow 2$ linked glucosyl unit were obtained by immunization with dextran NRRL B 1299S-3. Quantitative precipitin curves determined for various dextran preparations show the immunochemical reactivity of dextrans in $1 \rightarrow 3$ specific sera to be related to their content of $1 \rightarrow 3$ like linkages while reactivity in sera of $1 \rightarrow 2$ specificity is related to the proportion of $1 \rightarrow 2$ linkages. Quantitative oligosaccharide inhibition data show nigerose to be the best inhibitor of precipitation of $1 \rightarrow 3$ specific antidextran and kojibiose the most effective inhibitor of precipitation of $1 \rightarrow 2$ specific antidextran by their respective homologous dextrans.

Immunochemical studies with human antidextran based on quantitative precipitation and oligosaccharide inhibition[2-5] have thus far demonstrated two kinds of antidextran. One type shows a specificity directed against a terminal non-reducing sequence of up to six or seven glucose residues in

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α 1→6 linkage[3a,b,e,5] while the other type possesses a specificity involving a terminal non-reducing chain of about four α1→4 linked glucose residues.[3a]

Linkages in various dextrans have been characterized by oxidation with periodate and the proportion of anhydroglucopyranosyl units in 1→6, 1→4 like and 1→3 like linkage determined[7-11] (for other literature *cf.* 6). Classification into the latter two categories is ambiguous in that glucosyl units with substitutions on Cl and C2 cannot be distinguished by periodate oxidation from 1,4-linked units, while units linked through C2 and C4 or C2 and C3 are indistinguishable from 1,3-linked units.[12]

The presence of 1,4 and 1,3-linked glucose residues in some dextrans, however, has been established by methylation *cf.*[6,13-15] and infrared studies.[9,16,17] Resolution of the 1→4 like units and estimation of the proportions present as 1,4- and 1,2-linked units has recently been accomplished by Scott, *et al.*,[18] for several dextran preparations. This was done by optical rotation measurements of cuprammonium–dextran complexes.

An antidextran serum was described in earlier studies[4] in which part of the antibody showed a specificity which appeared related to units in 1→3 like linkage. Moreover, oligosaccharides of the maltose and isomaltose series did not inhibit precipitation of this type of antibody by a dextran (NRRL B1355S-4) with a high proportion of 1→3 like linkages.[3a] The present study extends these earlier findings. Quantitative precipitin studies employing antisera prepared by immunizing humans with dextran B1355S-4 showed that the capacity of various dextrans to remove antibody N was related to their content of units in 1→3 like linkage. That the specificity of this type of antidextran probably involves glucosyl residues in α1→3 linkage was indicated by the increased potency of 3-O-α-D-glucopyranosyl-D-glucopyranose (nigerose) over 4-O-α-D-glucopyranosyl-D-glucopyranose (maltose), 6-O-α-D-glucopyranosyl-D-glucopyranose (isomaltose), 2-O-α-D-glucopyranosyl-D-glucopyranose (kojibiose) and 3-O-β-D-glucopyranosyl-D-glucopyranose (laminaribiose) in inhibiting precipitation of antibody by the homologous dextran.

In addition, immunization of humans with a dextran (NRRL B 1299S-3) possessing a high proportion of 1→2 linkages[18] provided antisera whose specificity differed from that of other human antidextrans previously studied. The capacity of dextrans to precipitate this type of antidextran could be correlated with the proportion of 1→2 linkages as determined by Scott, *et al.*,[18] from optical rotation data. Moreover, quantitative inhibition findings showed kojibiose to be the best inhibitor of precipitation. Maltose, isomaltose and nigerose showed significantly lower inhibitory capacity, further suggesting an antibody specificity directed against glucosyl residues in α1→2 linkage.

Experimental

Dextrans.—Apart from two preparations (F90A) and S-5-A1.0) all the dextrans employed were obtained through the kindess of Dr. Allene Jeanes of the Northern Utilization Research Branch, U. S. Department of Agriculture, Peoria, Illinois. Proportions of 1 → 6, 1 → 4 like and 1 → 3 like linkages present in the dextran samples, as indicated by periodate oxidation, have been published by Jeanes, *et al.*[9] Resolution of periodate oxidation values for 1,4-like linked units to give the proportions of 1,4- and 1,2-linked units has been made by Scott, Hellman and Senti[18] for several of these preparations.

Dextran B 1299S-3, used in immunization, contained 50% 1 → 6 linkages and 50% 1 → 4 like linked units by periodate oxidation analysis.[9] The latter linkage type, however, was resolved into 12% 1 → 4 linked units and 38% 1 → 2 linked units by optical rotation data.[18] This dextran produced by strain "K" of Neill, *et al.*,[19] was prepared and characterized at the Northern Utilization Research Branch at the request of Drs. Hehre and Neill and was previously shown by Hehre[20] to have unusual immunological properties.

Dextran fraction B 1355S-4 used in immunization to obtain antidextran of 1 → 3 specificity possessed 57% 1 → 6, 8% 1 → 4 like and 35% 1 → 3 like linkages by periodate oxidation analysis. Preparation F90A was supplied by Dr. E. J. Hehre who described its preparation and partial characterization.[21] Clinical fraction S-5-A-1.0 was prepared at the National Bureau of Standards[22] and obtained from Drs. S. G. Weissberg and H. S. Isbell.

Antisera.—Four human antidextrans (sera 326, 327, 332 and 333) were employed in the present study. Antisera 326 and 327 were produced in response to injection of dextran B 1355S-4, while antidextran sera 332 and 333 were obtained by immunization with dextran B 1299S-3. Immunization of human subjects and preliminary testing of sera were carried out as previously described.[2,23] Post-immunization bleedings taken at various times after injection were labeled with the subscripts 2, 3 etc. In order to remove small amounts of antidextran of 1 → 6 specificity from antisera 326 D_4, 332 D_3 and 333 D_2, used in inhibition studies, sera were absorbed with dextran B 512 containing 96% 1 → 6 linkages. Amounts of dextran used to absorb each serum were determined from quantitative precipitin curves.

Quantitative Precipitin Studies and Quantitative Inhibition Assays.—Precipitin curves obtained with various dextrans were determined in the same way as those reported in earlier studies for antidextran sera of 1 → 6 and 1 → 4 specificity.[2-5] Antibody N in specific precipitates was estimated by the Folin–Ciocateu tyrosine method[24-26] employing reagents standardized against known amounts of human γ-globulin and antibody.[27]

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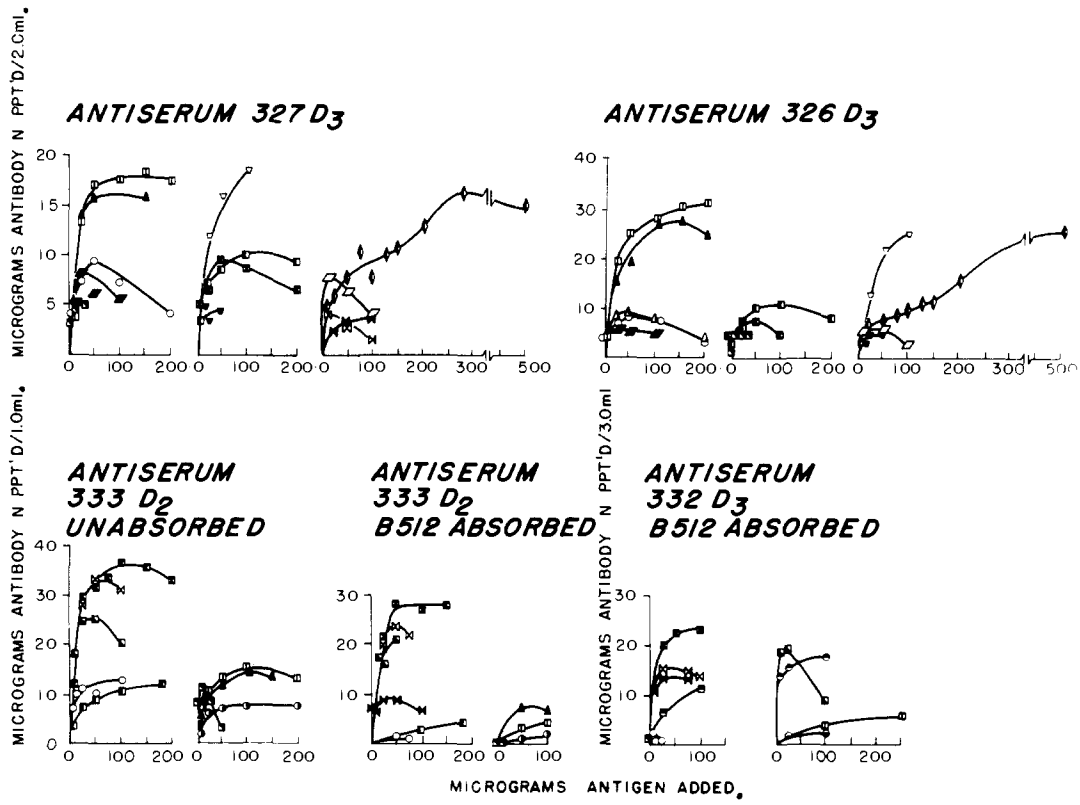

Fig. 1.—Quantitative precipitin curves of antidextran sera with various dextrans. Antiserum 327 D_3 and 326 D_3 to dextran B 1355S-4; antiserum 333 D_3 and 332 D_3 to dextran B 1299S-3. ⊞, B 1355 S-4; ▲, B 1498 S; O, B 1255; ◆, B 1141; ◥, B 512; ▽, B 1501 S; ⊠, B 1299S-3; ◧, B 742 C 3 R; ▼, B 1142; □, B 1425; ◀>, F 90 A; ▶◀, B 1399 Enz. Dig; ▷◁, B 1399; ◭, B 1375; ◣, B 1424; ◑, B 742LR.

Sugars of known structure were assayed for their capacity to inhibit precipitation of 22.4, 23.1 and 28.0 μg. of antibody N (AbN), respectively, from 3.0 ml. of absorbed sera 326 D_4, 332 D_3 and 1.0 ml. 333 D_2. Dextran B 1355S-4 was used in 100 μg. amounts to precipitate antidextran from serum 326 D_4 and 50 μg. dextran B 1299S-3 used for sera 332 D_3 and 333 D_2 (absorbed). Inhibition assays with nigerose and kojibiose employing serum 326 were determined on a pool of the D_3 plus D_4 bleedings, containing 25.1 μg. of AbN per 3.0 ml. These points are indicated by an asterisk in Fig. 2.

Mono- and Oligosaccharides.—Sugars employed in inhibition assays included: isomaltose, methyl α-isomaltoside and isomaltotriose supplied by Dr. Allene Jeanes; panose and maltoheptaose obtained from Dr. Dexter French of Iowa State College; maltotriose, maltotetraose and maltopentaose from Dr. Roy L. Whistler of Purdue University; laminaribiose from Dr. Paul A. Rebers of the Institute of Microbiology, Rutgers State University. A sample of nigerose was obtained from Dr. John H. Pazur and additional amounts of this sugar were prepared in this Laboratory by Mrs. Rose Mage and Mr. Joel W. Goodman. Kojibiose and kojibiose octaacetate[28-30] were supplied by Dr. Kyoshi Aso of the Faculty of Agriculture, Tohoku University. The octaacetate of kojibiose was deacetylated by treatment with methanol–ammonia and isolated and purified by chromatographing on paper. Maltose was obtained from Eastman Kodak Company and galactinol from Dr. C. E. Ballou of the University of California. 3-O-Methyl-D-glucose, 6-O-methyl-D-glucose, methyl-β-glucoside, methyl 3-O-methyl-α- and β-D-glucoses were from Dr. N. K. Richtmyer, 3-O-methyl-D-glucose from Dr. S. A. Barker, trehalose and turanose from Mann Chemical Company and methyl-α-glucoside from Bios Laboratories.

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Results

Quantitative precipitin curves (Fig. 1) were obtained with antidextran sera 326 D_3 and 327 D_3 employing twelve dextran preparations with varying proportions of 1→2, 1→3, 1→4 and 1→6 linkages. As can be seen from Fig. 1, the maximum amount of antibody N precipitable from 2.0 ml. of serum varied for the individual dextran samples tested. While the bulk of antibody N could be removed by dextrans B 1355S-4, B 1498S, B1501S and F 90A, which removed 25.6–31.6 μ g. of AbN from 2.0 ml. of antiserum 326 D_3 and 16.3–18.7 μ g. of AbN from 2.0 ml. of antiserum 327 D_3, the remaining eight preparations removed only a portion (16–55%) of the total antibody N.

The presence of at least two kinds of antidextran in these sera is indicated by precipitin curves obtained with dextran F 90A, which shows two peaks. Dextran B 512 containing predominately 1→6 linked units (95%) precipitated only 5 μg. of N from both sera, indicating that only a small portion of antibody of 1→6 specificity is present. The bulk of antidextran formed appears to be directed against units other than 1→6 linked units. That the major type of antibody present in both sera has a specificity involving 1→3 like linkages is suggested by the finding (Fig. 1) that dextrans B 1355S-4, F 90A, B 1498S and B 1501S, which show the greatest capacity to remove AbN, also show the highest proportion of 1→3 like linkages (34, 31, 27 and 20% respectively). A single preparation dex-

tran B 1142, although it contains 28% 1→3 like linkages, showed an anomalous behavior in that it removed only 5 μg. of AbN from both samples of antidextran.

Evidence that the specificity of this type of antidextran is directed at least in part against α1→3 linked glucosyl residues was obtained from quantitative oligosaccharide inhibition assays carried out with absorbed serum 326; these findings are summarized in Fig. 2. On a molar basis, nigerose was found to be the most potent inhibitor of precipitation, requiring only 10 μ*M* to give 56% inhibition of precipitation. Kojibiose and maltose were less effective than nigerose and required 10 and 55 μ*M*, respectively, to give 20 and 50% inhibition. Isomaltose showed no significant ability to inhibit when tested in amounts up to 46 μ*M*. Specificity for the 1→3 linkage in α-configuration is evident from the finding that 33 μ*M* laminaribiose gave only 10% inhibition. Thus involvement of the 1→3 glucosidic linkage in β-configuration results in a marked decrease in the capacity of the 1→3 linked disaccharide to inhibit. Trehalose, turanose, methyl α- and β-glucosides, galactinol, 2-O-methyl-D-glucose and 6-O-methyl-D-glucose showed little or no capacity to inhibit when tested in amounts greater than 55 μ*M*. Panose was tested at a single point and was somewhat poorer than nigerose.

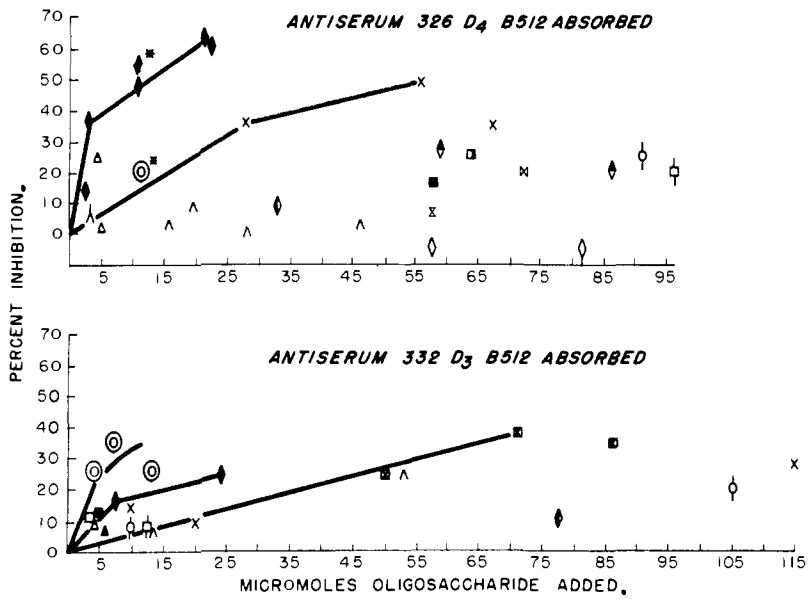

Fig. 2.—Oligosaccharide inhibition studies on the dextran antidextran reactions. Antiserum 326 D_4 to dextran B 1355S-4; antiserum 332 D_3 to dextran B 1299S-3. Each antiserum set up with homologous dextrans. The two points with asterisks were determined with a mixture of 326 D_3 and D_4 absorbed with B 512. ◆, nigerose; ◎, kojibiose; X, maltose; ▲, maltotriose; ■, maltotetraose; Λ, isomaltose; △, isomaltotriose; □, isomaltotetraose; ⋏, α-methyl isomaltoside; ◊, α-methyl glucoside; ⌷, β-methyl glucoside; ♀, 2-O-methyl-D-glucose; ◊, 6-O-methyl-D-glucose; ⋈, α-methyl-3-O-methyl-D-glucose; ⧖, β-methyl-3-O-methyl-D-glucose; ⊠, trehalose; ◈, turanose; ◧, galactinol; ◀▶, laminaribiose; △, panose.

Quantitative precipitin data obtained with antisera 332 D_3 and 333 D_2 also showed variation in the maximum amount of antibody N precipitable by individual dextrans before and after absorbing with dextran B 512 (Fig. 1). Preparations B 1299S-3, B 1399 and B 1424 maximally removed 36, 33 and 25 μ g. of AbN, respectively, in 1.0 ml. of unabsorbed antiserum 333 D_2 and 28.8, 23.6 and 21.2 μg. of AbN from 1.0 ml. of absorbed serum. The remaining dextrans, however, maximally removed only 8–14 μg. of AbN before and 2.2–7.7 μg. of AbN per ml. after absorption.

With antiserum 332 D_3 (B 512 absorbed), while 23.3, 17.8, 14.9 and 19.1 μg. of AbN could be precipitated from 3.0 ml. of serum by dextrans B 1299S-3, B 1299L, B 1399 and B 1424, preparations B 1255, B 1245L and B 1355S-4 removed only 2.1, 2.1 and 5.8 μg. of AbN.

Dextrans B 1299S-3, B 1299L, B 1399 and B 1424 are thus able to remove greater amounts of AbN from both samples of antidextran prepared against B 1299S-3, than do other preparations tested. While the proportion of 1→2 linkages in dextran B 1424 has not been determined, the relatively high percentage of 1→2 linkages in dextrans B 1299S-3, B 1299L and B 1399 (38, 34 and 29% respectively, ref.[18]) suggests that the specificity of the major portion of antidextran in these sera may be directed against α1→2 linked glucosyl units.

Quantitative oligosaccharide inhibition assays obtained with antisera 332 D_3 (B 512 absorbed) and 333 D_2 are in agreement with these findings. As shown in Fig. 2, kojibiose was found to be the most potent inhibitor of precipitation, 7.0 μ*M* giving 35% inhibition. Isomaltose, nigerose, maltose and trehalose were less effective than kojibiose and required 59.2, 24.0, 114 and 70 μ*M*, respectively, to give 24, 16, 30 and 35% inhibition. Laminaribiose, maltotriose maltotetraose, panose, methyl α- and β-glucosides showed only 10% inhibition or less in the amounts tested (4–105 μ*M*).

A limited number of assays were carried out with serum 332 D_2 employing maltotriose, maltopentaose isomaltose and panose in amounts up to 5 μ*M*. These sugars failed to show any significant degree of inhibition.

Discussion

Previous studies on human antidextran sera[2–5] employing the quantitative precipitin method and the quantitative oligosaccharide inhibition technique have established the existence of two distinct types of antidextran. One type was shown to possess a specificity directed against glucosyl residues in α1→6 linkage and to be specifically inhibited by α1→6 linked oligosaccharides. The other type was shown to be directed against glucosyl residues in α1→4 linkage and specifically inhibited by oligosaccharides of the α1→4 series.

While the existence of antibody specific for the periodate determined 1→3 like linkages of dextran was indicated by earlier findings,[3a] evidence based on quantitative inhibition indicating a specificity for the α1→3 glucosyl unit was not available.

In the present study quantitative precipitin data (Fig. 1) were obtained with human antisera prepared against a dextran (NRRL B 1355S-4) possessing 35% 1→3 like linkages. These data show a correlation between the immunochemical reactivity of various dextrans and their content of 1→3 like linkages. While individual preparations showed variation in their extent of reactivity, dextrans which showed the ability to remove greater amounts of antibody N from these sera also showed a relatively higher proportion of 1→3 like linkages (20–34%).

In agreement with this finding, suggesting an antibody specificity directed against glucosyl residues in α1→3 linkage, are the results of quantitative oligosaccharide inhibition assays. Inhibition data (Fig. 2) show that the antidextran present in anti-B 1355S-4 sera differ markedly in specificity from the 1→6 and 1→4 specific types of antidextran previously studied and shown to be inhibited by oligosaccharides of the isomaltodextrin and maltodextrin series, respectively.[2,3,5] The finding that nigerose is the most potent inhibitor of precipitation of this type of antidextran and shows an increased inhibitory capacity over isomaltose, maltose laminaribiose and kojibiose, further indicates a specificity directed at least in part against the α1→3 linked glucosyl residue.

The striking difference in behavior of dextran B 1142, in both samples of 1→3 specific antisera, from other preparations with comparable proportions of 1→3 like linkages suggests structural dissimilarities between dextran 1142 and dextrans B 1355S-4, B 1498S, B 1501S and F 90A with respect to the 1→3 like linkages.

In contrast to the findings with anti-B 1355S-4 sera are the results obtained with antisera prepared against dextran B 1299S-3 containing 38% 1→2 linkages. Quantitative precipitin data (Fig. 1) determined with antisera 332 D_3 and 333 D_2 show that the amount of antibody N precipitable from these sera could be correlated with the proportion of α1→2 linkages present. Preparations which removed greater amounts of AbN also showed relatively higher proportions of α1→2 linked glucosyl residues.

Further evidence that the specificity of this type of antidextran is directed at least in part against α1→2 linked units was obtained from inhibition studies. Data summarized in Fig. 2 show that on a molar basis, kojibiose was the best inhibitor of precipitation while nigerose, isomaltose and the maltodextrins—maltose, maltotriose and maltotetraose—were significantly poorer inhibitors.

These results, moreover, are in agreement with findings on the cross reaction of dextrans with Type XII antipneumococcal horse sera. The capacity of dextrans to remove antibody N from horse anti-XII sera was found by Goodman and Kabat[31] to be related to the proportion of 1→2 linkages present in the cross reacting dextran. In addition, dextran-anti-XII precipitation was somewhat more effectively inhibited by kojibiose than by other oligosaccharides. It is noteworthy that dextrans B 1299S-3, B 1299L, B 1399 and B 1424 which showed the greatest reactivity with human antidextran sera of 1→2 specificity were also found by these workers to show the greatest reactivity with horse anti-XII sera. These observations extend those of Hehre[20] that dextran from strain K (NRRL B-1299) and several other dextrans reacted strongly in high dilution with Type XII antipneumococcal rabbit serum and reacted with periodate in a manner compatible with the presence of 1→2 linked glucose units.[32]

The antisera employed in the present study could be shown to have specificity directed against disaccharides of glucose linked α1,3 or α1,2. From other studies it is generally found that the sugars at the non-reducing end of chains contribute most to the reactivity with antibody with each successive sugar residue contributing a smaller increment.[3] Thus it is probable that the α 1,3- and α 1,2-linked glucoses on the dextrans B 1355S-4 and B 1299S-3 which react with the homologous antibodies represent the two units at the non-reducing ends of the respective dextrans. Since the antidextran of α 1→6 specificity can react with chains of up to six or seven α 1→6 linked glucoses from the non-reducing end and the antidextran of α 1→4 specificity involved at least four glucoses linked α 1→4, the nature of an appreciable portion of the antigenic group on these two dextrans remains to be determined. Whether the specificity of the antigenic group involves linkages other than the α 1→3 and α 1→2 linkages is not known.

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