SPECIFIC FRACTIONATION OF HUMAN ANTIDEXTRAN ANTIBODIES

II. Assay of Human Antidextran Sera and Specifically Fractionated Purified Antibodies by Microcomplement Fixation and Complement Fixation Inhibition Techniques*

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In a previous study a technique was reported for the purification and fractionation of human antidextran of $\alpha 1,6$ specificity produced in a single individual into *two* antibody populations with distinctly differing affinities for smaller *versus* larger oligosaccharides (1). The antidextran, specifically absorbed onto an insoluble dextran (sephadex G-75), was eluted successively with haptens (*cf.* reference 2) of increasing size. Thus upon extraction of the washed sephadex-antibody complex with isomaltose or isomaltotriose, followed by extensive dialysis of the extract to remove the oligosaccharides, an antibody fraction was obtained which was inhibited readily by small oligosaccharides, while a second fraction, eluted subsequently with isomaltohexaose and dialyzed, consisted of antibody readily inhibitable by the larger oligosaccharides (1). These two fractions, of similar purity with respect to their precipitability by dextran, contained only fast moving 7S gamma globulin,—as did the purified human antidextran obtained by digestion of dextran-antidextran–specific precipitates with dextranase,—and showed by double diffusion in agar complete fusion both with antidextran prepared with dextranase and with each other. These findings supported the earlier suggestion (3) that antibody to one antigenic determinant, the $\alpha 1,6$-linked glucose chain in dextran, produced in a single individual, consists of a heterogenous population of molecules that vary with respect to the sizes of their antibody combining sites.

Earlier data on these purified antidextran fractions (1) were obtained by quantitative precipitin and inhibition assays using the ninhydrin method with about 3 to 4 μg AbN per analysis. More recently prepared antidextran fractions

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have now been assayed by the quantitative microcomplement fixation technique of Wasserman and Levine (4). Small amounts of the earlier fractions (1) were also available. The findings indicate that purified human antidextran fractions fix complement in presence of dextran and that the antibody extracted by the smaller oligosaccharides was less effective per unit N than was the antibody extractable by the larger oligosaccharides. The relative capacities of oligosaccharides of the isomaltose series to inhibit complement fixation with the two antibody fractions strikingly parallel those found by assays using inhibition of precipitation. With the experience gained from complement fixation assays with purified antibody, conditions were determined under which several human antidextran sera could be shown to fix guinea pig complement and the complement-fixing properties of antidextran in serum and in the purified antibody fractions were studied.

EXPERIMENTAL

Antidextran Sera.—Three bleedings, (D_{11}, D_{12}, D_{13}) from subject 20 were available and used for antibody purification and fractionation. Data on earlier bleedings and purified fractions were published previously (3, 5, 6, 1). For complement fixation studies on whole antidextran sera recent bleedings from subjects 20, 176, and J. H. were first heat-inactivated at 56°C for 30 minutes, then stored in small aliquots in the frozen state. Sera stored in the presence of phenol and merthiolate became anticomplementary. Serum 176 D_5 required absorption of hemolysin for 10 minutes at 4°C with washed sheep cells.

Purification and Fractionation of Antidextran.—

(*a*) *Absorption:* The technique was essentially as published previously (1): to 550 ml of pooled 20 $D_{11\text{-}13}$ serum, clarified by prolonged centrifugation in the cold and containing a total of 9 mg of antibody N, in three 250 ml centrifuge bottles, 500 mg washed sephadex G-75 (obtained from Pharmacia, Upsala, Sweden) was added and the mixture incubated at 37°C for 1 hour with frequent stirring and then gently rotated on a Heller motor in the cold at

TABLE I

Antibody Fractionation Procedure

I	Absorption	9000 μg 20 $D_{11, 12, 13}$ antidextran N + 500 mg sephadex G-75* (washed 4 times with buffered saline)		
II	Extraction	220 mg IM3	224 mg IM6	260 mg IM6
III	Eluate	3280 μg N	2730 μg N	116 μg N
IV	Yield total N	37 per cent IM3 Ab	32 per cent IM6 Ab	
Total recovery			69 per cent	

* Ratio, $\frac{\text{mg sephadex}}{\text{mg AbN}} = 56$.

about 10 RPM for 8 days. After centrifugation of the sephadex the supernatant serum was found free of precipitating antidextran. The sephadex-antibody precipitate was then washed repeatedly with M/1000 phosphate-buffered saline pH 7.3 until the washings were free of protein measured at 2800 A.

(*b*) *Elution:* Elution of antibody fractions was carried out as shown in Table I using buffered saline solutions of isomaltotriose and then of isomaltohexaose. For this step the packed sephadex was transferred into screw capped bottles of "blood collecting outfits" (available from Travis & Co., London). These consist of twin bottles that are connected top to top by a double threaded, sieve type metal piece that carries a Whatman 50 filter paper disc. After addition of hapten to the sephadex-antibody precipitate in one bottle, the mixture was placed in a 37°C water-bath for 1 hour with frequent inversion followed by slow rotation in the cold over night. The twin bottles were then centrifuged lightly and the eluate passed through the filter disc into the second bottle. The eluate was removed and the sephadex washed repeatedly in this manner with small amounts of buffered saline until no material absorbing at 2800 A could be detected. Before elution of the sephadex-antibody precipitate with another oligosaccharide washings were continued until they were free of reducing sugar measured by the Park-Johnson method (*cf.* reference 7). Eluates and washings containing material absorbing at 2800 A were combined and concentrated by ultrafiltration in the cold under negative pressure through a 7 ml collodion membrane (membranfiltergesellschaft göttingen, Schleicher and Schüll, Keene, New Hampshire). The concentrate was diluted with buffered saline and reconcentrated until the ultrafiltrates were free of reducing sugar. This technique for concentrating the antibody fractions and for removal of hapten was much more rapid than the prolonged dialysis used earlier (1) and eliminated the pervaporation step. Total N in the antibody fractions was determined by a modified ninhydrin technique (8). For determination of precipitating AbN an appropriate volume of purified fraction containing about 3 μg AbN was added to known quantities of dextran, the solutions were mixed and incubated at 37°C for 1 hour and in the cold for 1 week with daily mixing. The precipitates were centrifuged off, washed once with saline and analyzed for N after digestion with H_2SO_4 by the ninhydrin method (8). Small amounts of antidextran fractions 20 D_2-IM3 Ab and 20 D_3-IM6 Ab (1) were available.

Dextrans.—Various dextrans were used as described in earlier papers (5, 9, 1).

Oligosaccharides.—The isomaltose series of oligosaccharides were those prepared earlier (1).

Quantitative Microcomplement Fixation.—The necessary components for the indicator system were purchased, stored, and prepared for the assays as described by Wasserman and Levine (4). Experiments were set up in 40 ml centrifuge tubes in a total volume of 7 ml. The diluent was added with a "miscomatic dispenser" plastic bottle (available from Microchemical Specialties Company, Berkeley, California). Dilutions of antidextran serum, purified antibody fractions, and dextrans in the standard diluent (7) were measured with 1 ml analytical blow out pipets. An amount of freshly diluted guinea pig serum containing 1.3 to 1.6 $C'H_{50}$ (4) was added to the chilled tubes in ice water with a Cornwall automatic syringe. After 16 to 20 hours' incubation in the cold, 50 million washed sheep erythrocytes, previously sensitized with rabbit anti-sheep hemolysin for 15 minutes at 37°C, were added to each tube. The titration of the hemolytic rabbit antibody in this system revealed a plateau type lysis curve, beginning at about 1:1500, which dilution was used throughout all the experiments (7). The reaction mixtures with indicator system were then incubated at 37°C until the complement containing control tubes showed approximately 90 per cent lysis or slightly less as compared to complete lysis of the tubes with excess complement. The mixtures after rapid chilling in an ice bath were transferred into smaller centrifuge tubes, spun at 1800 RPM for 10 minutes and the supernatant fluids analyzed for hemoglobin at 4120 A.

Quantitative Microcomplement Fixation Inhibition.—For inhibition assays the oligosaccharides were first added to the tubes using Lang-Levy pipets and the volume made up with

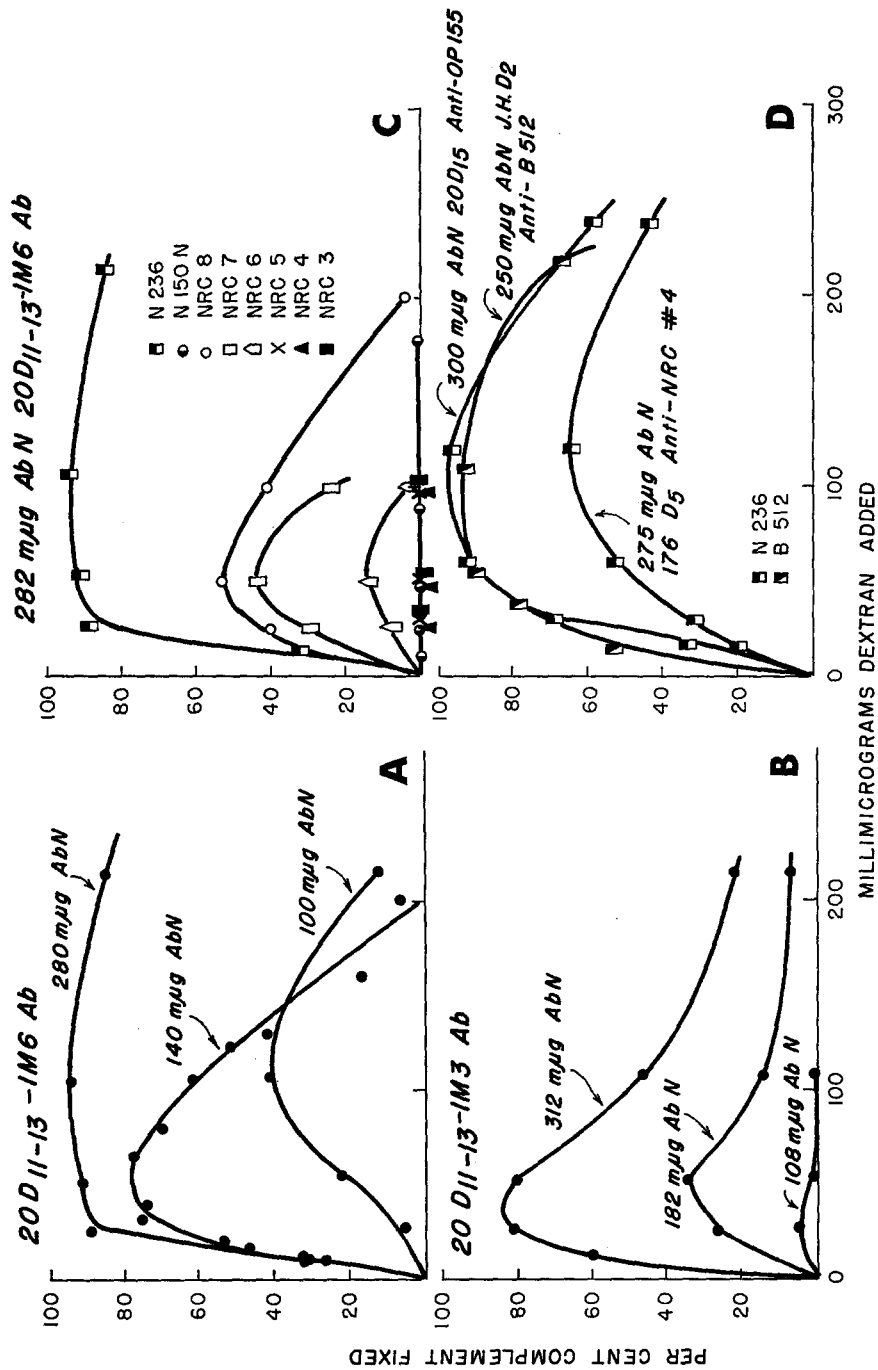
diluent. Each determination was carried out in duplicate and fixation in absence of inhibitor was assayed in triplicate. Controls for anticomplementary activity of antibody solutions, dextrans, and haptens were always included. Per cent complement fixation was calculated as the difference between the amount of released hemoglobin of antigen-antibody mixtures and the blank of antibody incubated without dextran. Inhibition was expressed as per cent fixation of reaction mixtures in presence of inhibitor compared to that in tubes without inhibitor.

RESULTS

Table I is a flowsheet of the antibody fractionation procedure: A ratio of 56 mg sephadex per mg AbN resulted in total absorption of the precipitating antidextran. The recovery in the isomaltotriose extraction was 37 per cent, and with isomaltohexaose an additional 32 per cent of the original antidextran N was recovered; total yield 69 per cent. Both fractions were 95 to 100 per cent precipitable by dextran.

Complement Fixation with Purified Antidextran Fractions.—The quantitative complement fixation curves obtained with the two purified 20 $D_{11\text{-}13}$ fractions are shown in Figs. 1 *A* and 1 *B*. Fig. 1 *A* shows three curves with 20 $D_{11\text{-}13}$-IM6 Ab using three levels of AbN. On incubating 100 mμg AbN with various quantities of dextran N 236 peak fixation of 40 per cent was obtained, while with 140 mμg N fixation rose to 78 per cent. At both these Ab levels inhibition of fixation occurred in antigen excess. With 280 mμg AbN per reaction mixture the percentage complement fixed rose to 94 and the resulting curve was flat over a wide range. In Fig. 1 *B* the three corresponding quantitative complement fixation curves with similar amounts of 20 $D_{11\text{-}13}$-IM3 AbN and the same quantities of dextran N 236 are given. It can be seen that the complement-fixing potency of this fraction was considerably lower since with 108 mμg AbN only 5 per cent of the complement was fixed. With 182 mμg there was 35 per cent fixation and with 321 mμg AbN 80 per cent was fixed. The 20 $D_{11\text{-}13}$-IM3 Ab gave a very narrow equivalence zone. Quantitative fixation curves of 282 mμg of the 20 $D_{11\text{-}13}$-IM6 Ab with a variety of dextrans are shown in Fig. 1 *C*. No complement fixation was found with dextran N 150 N (mol. wt. 60,000 determined by viscosity) (*cf.* reference 5). Using dextran fractions of graded molecular weight, peak fixation of 52 per cent was obtained with NRC 8 (mol. wt. 412,000) (*cf.* reference 9). Fractions of lower molecular weight gave less fixation and NRC 6 (mol. wt. 195,000) (*cf.* reference 9) was the smallest size product with which this purified antibody fixed complement.

Complement Fixation with Antidextran Sera.—The quantitative complement fixation curves of human antidextran sera are shown in Fig. 1 *D*. Reproducible fixation was found with dextrans N 236 or B 512 using 250 to 320 mμg precipitating AbN per reaction mixture. Since most human antidextrans contain only between 5 to 35 μg precipitating AbN per ml, low serum dilutions were necessary. The anti-NRC 4 serum of subject 176 required prior absorption with sheep cells for removal of hemolysin.



FIGS. 1 *A* to 1 *D*. Quantitative dextran human antidextran complement fixation curves. 1 *A*. Effect of increasing antibody N on C′ fixation with dextran N 236 and purified IM6 Ab. 1 *B*. Same as 1 *A* but with purified IM3 Ab. 1 *C*. Effect of molecular weight of dextran on C′ fixation with IM6 Ab. 1 *D*. C′ fixation with three human antidextran sera.

Quantitative Complement Fixation Inhibition Studies with Antidextran Serum 20 D_{15}.—Fig. 2 shows quantitative complement fixation inhibition curves with the isomaltose series of oligosaccharides using 43 mμg N 236 and an amount of unfractionated antidextran 20 D_{15} containing approximately 300 mμg AbN. The ratios of inhibiting power of the various oligosaccharides were very similar to those observed by inhibition of precipitation (3, 6). Thus IM3 was a poor inhibitor and only slightly better than IM2. The greatest increment in inhibiting power was found between IM3 and IM4. IM5, IM6, and IM7 were

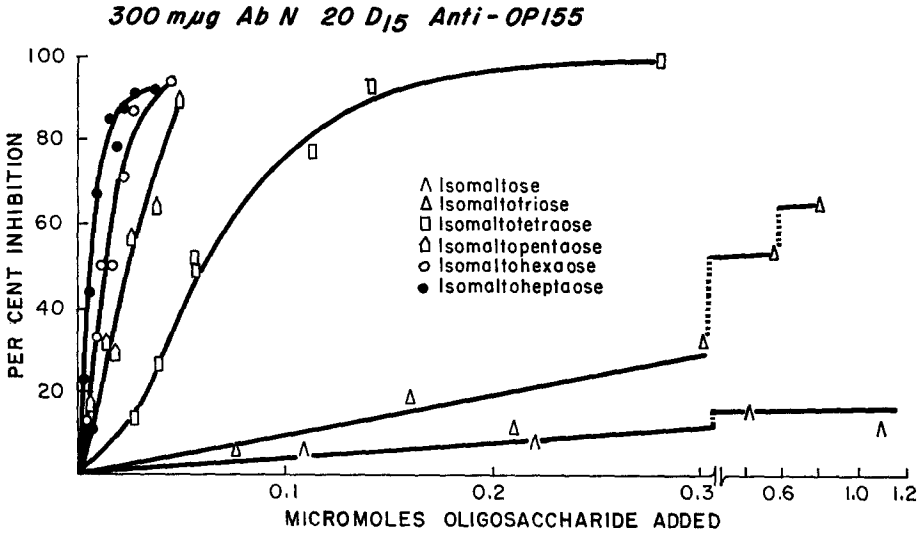
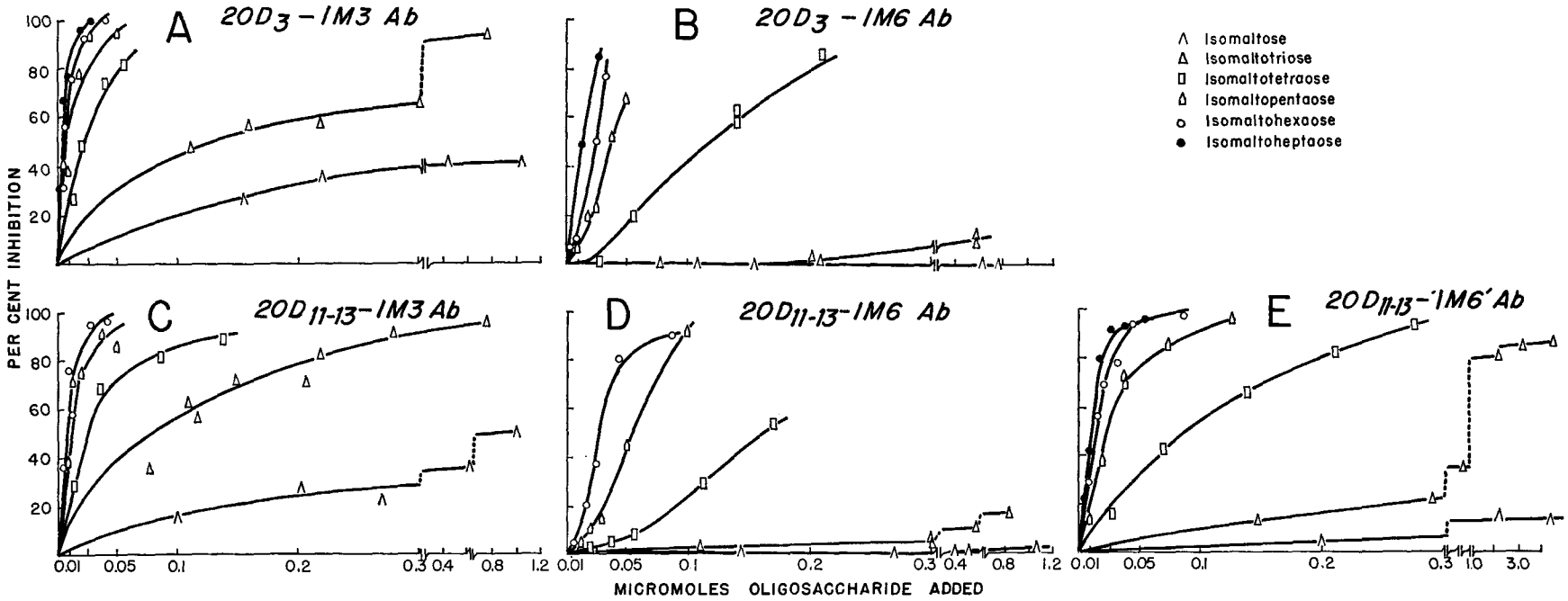


FIG. 2. Inhibition by oligosaccharides of C′ fixation of antidextran serum 20 D_{15} with dextran.

progressively better inhibitors on a molar basis than IM4, the increment decreasing with increasing chain length.

Quantitative Complement Fixation Inhibition Studies with Purified Antibody Fractions.—Figs. 3 *A* and 3 *B* show the quantitative complement fixation inhibition data with purified antibody fractions derived from bleeding 20 D_3 on which curves by inhibition of precipitation had already been obtained (1). 398 mμg 20 D_3-IM3 AbN and 382 mμg 20 D_3-IM6 AbN with 43 mμg N 236 were used per assay giving reproducible complement fixation of 76 to 88 per cent. The degree of inhibition in presence of a constant amount of hapten was constant in the zones of equivalence and slight antigen excess. The behavior of the 20 D_3-IM3 Ab was strikingly different from that of the 20 D_3-IM6 Ab: with the former IM2 inhibited substantially and inhibition was readily obtained with IM3. On the other hand with the IM6 Ab identical amounts of IM2 showed no



FIGS. 3 *A* to 3 *E*. Inhibition by oligosaccharides of C′ fixation of purified antibody fractions from human antidextran serum. Upper curves: fractions obtained from bleeding 20 D_3 (1952). Lower curves: fractions obtained from bleedings 20 D_{11-13} (1962).

inhibition and IM3 inhibited almost negligibly over the range tested. With the 20 D_3-IM3 Ab, IM4 was a better inhibitor than IM3 and IM5 was better than IM4. IM6 and IM7 were better than IM5 and almost equally potent on a molar basis. With the 20 D_3-IM6 Ab on the other hand IM7 was significantly better than IM6.

In Figs. 3 *C*, 3 *D*, and 3 *E* the curves with the purified fractions from more recent bleedings of subject 20 are represented. 305, 280, and 300 mμg AbN respectively with 43 mμg N 236 were found to fix comparable amounts of complement, approximately 80 per cent of the quantity of complement added. At the 50 per cent inhibition point IM3 and IM4 were shown more potent relative to IM6 in inhibiting complement fixation of 20 $D_{11\text{-}13}$-IM3 Ab than they were in inhibition of complement fixation of the 20 $D_{11\text{-}13}$-IM6 and -IM6′ Ab. Thus with the IM3 Ab the relative inhibiting power of IM3, compared to IM6, was 11 per cent, whereas with the IM6′ Ab fraction IM3 was only 1.1 per cent as effective as IM6. The curves obtained with IM4, IM5, and IM6 from the 20 $D_{11\text{-}13}$-IM3 Ab were superimposable with the corresponding ones from the IM3 Ab prepared from the earlier bleeding D_3 (Fig. 3 *A*). With the 20 $D_{11\text{-}13}$-IM6 Ab, IM6 was better than IM5 which in turn was better than IM4. Almost no inhibition of complement fixation was obtained with IM2 and IM3 in the range tested. The same relationship of the various oligosaccharides to each other was found with the 20 $D_{11\text{-}13}$-IM6′ Ab. IM7 was the best inhibitor and slightly better than IM6. The curves obtained with IM7, IM6, IM5, and IM4 were also superimposable within the limits of experimental error with those from the IM6-Ab of the earlier bleeding (Fig. 3 *B*).

DISCUSSION

The findings clearly show that purified human antidextran and some human antidextran sera fix guinea pig complement in presence of dextran. In earlier studies no difference in N (*cf.* reference 7) was found between specific precipitates formed in undecomplemented and decomplemented antidextran sera (5). To obtain reproducible fixation curves 250 to 400 mμg of precipitating antidextran N were required per analysis with serum and purified antibody (Figs. 1 *A* to 1 *D*). Variations in fixing potency of antidextran serum were due to sheep hemolysins present at the low dilutions of the antidextran sera used, enhancing the lytic susceptibility of the sheep cells. Prior absorption of such sera abolished this variation (Fig. 1 *D*). Purified antidextran fractions were suitable for microcomplement fixation even when obtained from sera preserved with phenol and merthiolate for long periods and which had become anticomplementary. With the two purified 20 $D_{11\text{-}13}$-Ab fractions definite differences in complement fixing potency were established. Approximately half the amount of 20 $D_{11\text{-}13}$-IM6 AbN as of 20 $D_{11\text{-}13}$-IM3 AbN of equal purity was necessary to fix 40 per cent of the complement added in presence of dextran N 236 (Figs. 1 *A* and 1 *B*). Moreover the 20 $D_{11\text{-}13}$-IM3 Ab showed rapid inhibition with

moderate antigen excess which did not occur either with the corresponding unfractionated antidextran 20 D_{15} (Fig. 1 *D*) and the 20 D_{11-13}-IM6 Ab (Fig. 1 *A*). Similar findings were obtained with the two antibody fractions from the 20 D_3 bleeding (1). These differences between the two fractions reflect heterogeneity of the antidextran of a single individual in ability to fix complement. Approximately twofold differences in complement fixing activity per μg AbN were reported earlier between first- and second-course rabbit anti-BSA sera using 100 $C'H_{50}$ units (10). With first-course antisera the necessary range for 50 per cent fixation was 2 to 4 μg AbN per analysis whereas with second-course antisera 1 μg AbN produced 50 per cent and 2 μg about 70 to 90 per cent fixation. The narrow equivalence zone obtained with the 20 D_{11-13}-IM3 Ab indicates that extraction with IM3 yielded a fraction with reduced complement fixing capacity. In quantitative precipitin assays with low molecular weight NRC dextrans (1) the 20 D_3-IM3 Ab was found to go into the inhibition zone more readily than did the 20 D_3-IM6 Ab. The basis for the difference in complement-fixing potency is unknown.

Studies on fractions of antibody obtained by papain digestion (11, 12) ascribe to fragment III the binding site for complement on rabbit antibody while the antibody combining sites are on fragments I and II (13–15; *cf.* reference 16). With human antibody the complement-fixing site is thought to be on the F(B) (*cf.* reference 16) and the antibody combining sites were shown to be on the S(A and C) fragments (17). Thus the site for complement binding might be remote from the antibody combining sites. However the antigen-antibody bonds which are unaffected by papain digestion appear to affect the complement fixing properties. Schur and Becker (18) found that washed specific precipitates of pepsin digested 5 S rabbit antibody (*cf.* reference 19) and egg albumin fixed complement well although peptic digestion is accepted as destroying fragment III. Subsequently Reiss and Plescia (20) showed that human complement which had been fixed on to Ea–rabbit anti-Ea specific precipitates remained fixed after removal of fragment III by papain digestion. The present finding that the IM3-Ab is only half as effective per unit weight in complement fixation as the IM6-Ab indicates that the antibody-combining site is involved in strength of the complement fixation. The two purified antibody fractions are identical in mobility range in immunoelectrophoretic analysis (1), both give essentially the same pattern in starch gel electrophoresis after reduction and alkylation (21); moreover the whole antidextran of individual 20 D was Gm(a−b+) which corresponds to his whole serum Gm grouping (22). The two fractions were also found to sensitize the guinea pig for passive cutaneous anaphylaxis, the IM3-Ab being slightly poorer than the IM6-Ab (23), the property of attaching to guinea pig skin also being associated with fragment III (24, 25). How the interaction of antigen with antibody influences complement binding to fragment III in intact gamma globulin requires further study.

The quantitative complement fixation studies with 20 D_{11-13}-IM6 Ab and

dextrans of various molecular weights (Fig. 1 *C*) established that a decrease in molecular weight of the antigen causes a significant decrease in the amount of complement fixed. The smallest fraction, NRC 6, giving appreciable complement fixation, has a molecular weight of 195,000. In quantitative precipitin studies with antidextran sera, however, NRC 1 with a molecular weight as low as 10,600 precipitated about ⅓ of the antibody N and fractions with molecular weights of 50,000 or above precipitated as much antibody as did native dextran (9). Small differences in quantitative precipitin curves between purified 20 D_3-IM3 Ab and 20 D_3-IM6 Ab with graded molecular weight dextrans were reported (1). NRC 1 (mol. wt. 10,600) and NRC 2B (mol. wt. 35,000) precipitated somewhat less antibody than did NRC 7 (mol. wt. 255,000). In the complement fixation assay, on the other hand, differences between NRC 8, 7, 6 were measurable. In studies on human sera from patients with lupus erythematosus decreased complement fixation accompanied large decreases in molecular weight of the DNA used (26, 27). Extensive boiling and sonic disruption of DNA resulted in lowered complement fixation, if the decrease in molecular weight was more than four-fold (27).

Previous studies have shown that antidextran from a single individual could be separated into two antibody portions of similar purity by absorption of the antidextran to sephadex followed by elution with haptens of increasing size (1). The experiments reported above have confirmed that despite minor differences in the antibody purification technique, an essentially identical fractionation of antidextran from later bleedings of the same individual resulted. Moreover quantitative microcomplement fixation assays gave findings comparable to those by the quantitative precipitin inhibition technique (*cf.* reference 1, Figs. 2 *C* and 2 *D*).

Thus the results provide further evidence that the 20 D_3-IM3 Ab consisted largely of molecules with a higher affinity for smaller oligosaccharides and presumably contained relatively few antibody molecules with combining sites larger than a pentasaccharide. On the other hand the 20 D_3-IM6 Ab consisted chiefly of molecules with affinity for larger oligosaccharides and hence these are inferred to possess larger size combining sites. Thus with this fraction IM7 was definitely superior to IM6 which was better than IM5 which in turn was considerably better than IM4. IM3 and IM2 were extremely poor inhibitors.

The separation of antidextran from single individuals into two populations of antibody molecules provides further indications that the increased inhibiting power of the higher oligosaccharides is specifically related to the combining site. Metzger, Wofsy, and Singer (28) have recently questioned this interpretation by attributing increased inhibition by larger sized haptens to non-specific interactions making cooperative contributions to the overall free energy. The present findings indicate that the complement-fixing properties of the two antibody fractions as well as their capacity to be inhibited by oligosaccharides of various

sizes differ significantly. To maintain the interpretation of Metzger *et al.* (28), one would be forced to assume that the hypothesized non-specific contribution to binding energy by the oligosaccharides is not the same for all of the antibody molecules. Moreover earlier studies have shown that a change in linkage of the third to the fourth glucose ring as in 4-O-α isomaltotriosyl-D-glucose produced a definite decrease in inhibiting power as compared with isomaltotriose (29, 30). Thus the supposed non-specific interaction shows at least a remarkable specificity for the α1, 6 linkage at the fourth glucose. While it is possible that minor non-specific effects occur attributable to the fifth, sixth, and seventh glucose units, it is apparent that the increased inhibitory power up to at least the tetrasaccharide is predominantly related to the size of the antibody combining site. This is further supported by the recent findings (31) that the portion of type XXII antipneumococcal antibody cross-reacting with dextran could be further fractionated into two populations of cross-reacting antibody molecules. With one fraction, the isomaltose oligosaccharides showed increasing inhibiting capacity on a molar basis up to isomaltohexaose and isomaltoheptaose which were equal and the best inhibitors. The second fraction gave maximum inhibition with isomaltose and kojibiose and all isomaltose oligosaccharides from isomaltotriose to isomaltohexaose were equal in inhibiting power to isomaltose. Once again one would be forced to attribute to individual antibody molecules differences in capacity to react non-specifically to chains of α1, 6 linked glucoses to maintain the interpretation of Metzger *et al.* (28).

Although the relative inhibiting capacities of the various oligosaccharides to one another in each fraction was the same for inhibition of precipitation and of complement fixation, the amount of inhibitor necessary for 50 per cent inhibition relative to the amount of AbN used was much greater for complement fixation inhibition. Similar findings were reported in the rabbit anti-SXIV immune system (4).

SUMMARY

Human antidextran of one individual, absorbed specifically on sephadex, was fractionated into two populations of antibody molecules by successive elution with oligosaccharides of the isomaltose series of increasing size. The purified antibody fractions and some whole antidextran sera were found to fix complement with dextrans of molecular weight of 195,000 and above.

It could be demonstrated by quantitative microcomplement fixation inhibition assays that the antibody eluted with isomaltotriose had a higher affinity for smaller oligosaccharides relative to isomaltohexaose, indicating a high content of antibody molecules with smaller combining sites, while with the second fraction, eluted with isomaltohexaose, the small haptens were very poor inhibitors and the larger oligosaccharides inhibited readily, presumably due to a higher proportion of molecules with larger combining site size. Assays of simi-

larly prepared fractions, obtained from earlier bleedings of the same individual (1), with inhibition of complement fixation were in good agreement with those obtained by inhibition of precipitation.

The two purified antidextran fractions were shown to differ with respect to their complement-fixing capacity. The fraction with molecules with smaller size–combining sites fixed only about half as much complement per unit antibody N as did the fraction containing largely molecules with larger combining sites suggesting that the strength of complement fixation is affected by the strength of the antigen-antibody interaction.

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