

ALLOTYPE EXCLUSION IN UNIFORM RABBIT ANTIBODY TO  
STREPTOCOCCAL CARBOHYDRATE\*

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The availability of homogeneous immunoglobulins of man and mice in the form of myeloma proteins has greatly facilitated the elucidation of immunoglobulin structure and has advanced our knowledge of the genetic system controlling the immune response. Because of the locations of the rabbit's allotypic markers within the IgG<sup>1</sup> molecule and the apparently multiple amino acid changes involved in their genesis, the rabbit presents advantages for the study of certain aspects of immunogenetics (1). Knowledge of the structure of rabbit immunoglobulins, particularly at the level of amino acid sequences in the variable portions of the molecule, has been hampered by the lack of homogeneous immunoglobulin.

The observation that some rabbits immunized with streptococcal vaccines responded by the production of large amounts of electrophoretically uniform antibody directed primarily against the group-specific carbohydrates of the bacterial cell wall (4) suggested that very uniform immunoglobulin for detailed structural studies might be obtained in this way. Continued investigation of these antibodies has shown further similarities to the myeloma proteins (5, 6).

An important characteristic of myeloma proteins is the selectivity they show with respect to gene expression. In man a choice is made between  $\kappa$  and  $\lambda$  light (L) chains and among the classes and subclasses of heavy (H) chain. Studies with human allotypic markers (Gm and Inv types) have shown further

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<sup>1</sup> The immunoglobulin molecules and fragments are named after the recommendations of a committee of the World Health Organization (2). For those allotype specificities which have been assigned to a definite group (3) the initial A has been omitted, e.g., a1 in lieu of Aa1.

that only one of two possible alleles for both the L chain and H chain is expressed in the myeloma proteins of individuals heterozygous with respect to these markers (7). Finally, only one of the many possible variants of each of these chain types is expressed (8).

This paper presents evidence that a similar selectivity of expression exists in some of the antibodies formed in response to immunization with streptococcal vaccine. Selection between two group a (H chain) allotypes and two group b (L chain) allotypes in a single doubly heterozygous rabbit is described. In another rabbit, homozygous with respect to both the group a and group b allotypes, the H chain is selected from the population not possessing an allotypic marker from group a (9). In other publications (6)<sup>2</sup> evidence is presented that this selectivity extends to the amino acid sequence of the L chain.

#### *Materials and Methods*

*Immunizations.*—The production of streptococcal vaccine and the immunization procedure have been described previously (4, 5). All of the antibodies described in this paper were against Group C streptococcal carbohydrate.

*Characterization of Antisera and Isolation of Specific Antibody.*—These techniques have been described previously (6).

*Isolation of IgG.*—Rabbit IgG was isolated from rabbit serum by two precipitations using 1.4 volumes of 3 M ammonium sulfate followed by chromatography on DEAE-cellulose in 0.0175 M pH 6.5 phosphate buffer following the method of Levy and Sober (10).

*Radiiodination and Counting Techniques.*—Protein was labeled with <sup>125</sup>I by the procedure of McFarlane (11). Counting was performed with a Nuclear-Chicago crystal scintillation counter.

*Antisera.*—The anti-allotype sera were prepared by the method of Oudin (12). Rabbits lacking an allotypic determinant were injected with ovalbumin specific precipitates from rabbits bearing this determinant. The goat anti-rabbit Fc $\gamma$  was obtained by absorbing goat anti-rabbit IgG with F(ab')<sub>2</sub> obtained by hydrolysis of rabbit IgG with pepsin (13).

*Precipitation Analysis with Anti-Allotype Sera.*—Antiserum (0.2 ml) was added to a series of Beckman Microfuge tubes. Radiolabeled antigen in pH 6.8 phosphate buffered saline (PBS) was added in amounts varying from 5 to 75  $\mu$ g. The volume in each tube was then adjusted to 0.35 ml with PBS. After mixing, the samples were incubated 2 hr at 37°C and then overnight at 4°C. Total radioactivity was determined by placing the Microfuge tubes with their contents in counter tubes and counting. The Microfuge tubes were centrifuged with the Microfuge for 5 min. The supernatant was discarded. The precipitates were washed 2 times in PBS and dissolved in 0.35 ml 0.2 N NaOH. The Microfuge tubes and contents were counted as before. The per cent of precipitation was calculated by dividing the counts of the precipitate by the original counts in the region of antibody excess.

*Precipitation Analysis with Streptococcal Group C Carbohydrate.*—Antibody to Group C carbohydrate in preparations from R27-11 was determined in the following manner. Antiserum (25  $\mu$ l containing 1.2 mg antibody) from R27-11 was added to a series of Microfuge tubes. The <sup>125</sup>I labeled antibody fractions in 0.1 ml PBS buffer containing 25–100  $\mu$ g protein were added and mixed. Group C carbohydrate was added in amounts approximating a prede-

<sup>2</sup> Hood, L., J. Ohm, K. Eichmann, and R. M. Krause. Rabbit antibody structure; The N-terminal sequence of light chains with apparent homogeneity. To be submitted for publication.

terminated equivalence value of 27  $\mu\text{g}$  of carbohydrate per mg of total antibody. The precipitation and counting were performed as described above.

*Sephadex Gel Filtration.*—Gel filtration was done in a column (90  $\times$  2.5 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) in 0.1 M pH 6.8 potassium phosphate buffer containing 0.03 %  $\text{NaN}_3$  at room temperature.

*Gradient Centrifugation.*—Gradient centrifugations were performed at 4°C in a 5–20% sucrose gradient for 24 hr at 35,000 rpm in the SW40 head of a Spinco L2-65B centrifuge. Fractions of 0.5 ml each were collected by puncturing the bottom.

## RESULTS

*Measurement of Allotypic Specificities.*—Antibodies were isolated from the antisera of three rabbits which had been immunized with streptococcal Group C

TABLE I  
*Precipitation of Radioiodinated Antibody and IgG*

Rabbit	Allotype	Sample	Precipitation by antisera*					Fc $\gamma$
			a1	a2	a3	b4	b5	
			%	%	%	%	%	%
R24-35	a2, b4	Preimmune IgG		54		73		85
		Antibody $\ddagger$		69		88		91
R14-81	a1, a3, b4, b5	Preimmune IgG	36		12	84	20	97
		Antibody $\ddagger$	64		3	99	5	100
R27-11	a1, b4	Preimmune IgG	62			93		97
		Antibody $\ddagger$	10			99		92
		Purified antibody $\S$	0.6			99		98
		Nonantibody IgG $\parallel$	62			97		96

\* Determined by counting  $^{125}\text{I}$  in the precipitate.

$\ddagger$  Antibody against carbohydrate from Group C streptococci isolated by electrophoresis (6, 15).

$\S$  Antibody in second peak (Fig. 1) from Sephadex G-200 filtration.

$\parallel$  IgG isolated from supernatant (10) after precipitation of antibody with Group C carbohydrate.

vaccine (6). Two of the antisera were from rabbits homozygous with respect to both the group a and group b allotypic specificities. The third was from an antiserum from a rabbit heterozygous with respect to both groups.

Each of these antisera contained a prominent monodisperse antibody component when examined by electrophoresis. Each component was isolated by preparative electrophoresis as previously described (5, 14). Antiserum R27-11, which possessed antibody with a remarkable degree of uniformity, is described in detail elsewhere (6). Because antibody in antiserum R14-81 was similar to the above antibody, the details of uniformity will not be presented here. Antiserum R24-35, which possessed an antibody component somewhat less uniform than the other two has been presented in detail elsewhere (6, 15).

The antibodies from rabbit R24-35 showed an increase in the percentages of molecules carrying the specificities a2 (from 54 to 69%) and b4 (from 73 to 88%) over those found in the preimmune serum (Table I). This result is typical of those obtained in a few other cases. It is possible that subsequent purification of the antibody component in serum R24-35 by immune precipitation would enhance the allotypic shift observed here.

Antibody R14-81 (Table I) showed an increase in a1 (from 36 to 64%) accompanied by a decrease in its allele, a3 (from 12 to 3%) when compared to the rabbit's own preimmune IgG. Both of these specificities are present on the H chains. Similarly for the allotypes present on the L chains, an increase

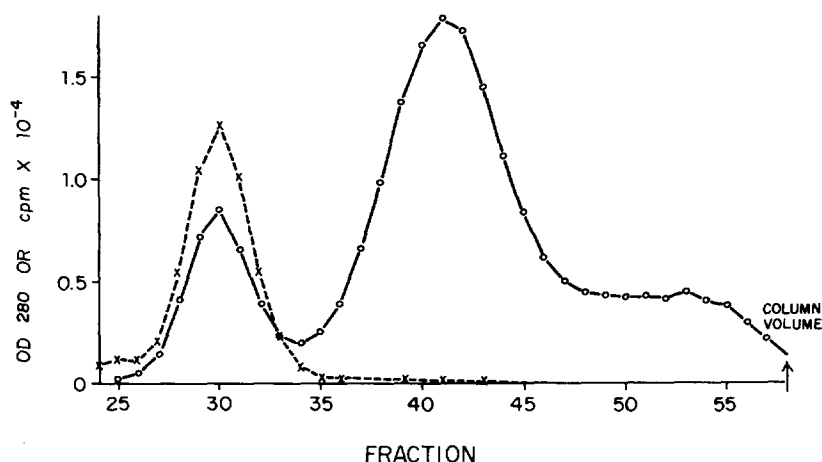


FIG. 1. Gel filtration of antibody from rabbit R27-11. A 5.0 ml sample containing 0.10 mg of radioiodinated (<sup>125</sup>I) antibody isolated by electrophoresis (6) was mixed with 25 mg of normal IgG and was applied to a column (90 × 2.5 cm) of Sephadex G-200 in 0.1 M pH 6.8 potassium phosphate buffer. X---X, OD 280; O—O, radioactivity. Fraction size was 8.0 ml.

in b4 (from 84 to 99%) was accompanied by a decrease in b5 (from 20 to 5%) when compared to the rabbit's own preimmune IgG. The small amount of residual a3 and b5 specificity may represent: nonspecific precipitation; cross reaction; the presence of immunoglobulin not part of the specific antibody population; or possibly the presence of a minor population of specific antibody not reflecting the restrictions imposed on the major antibody population.

*Antibody R27-11 and its Purification by Reaction with Sephadex G-200.*—The initial antibody preparation from rabbit R27-11 showed a decrease of the H chain allotype a1 (from 62 to 10%) and a slight increase in the L chain allotype b4 (from 93 to 99%) (Table I). Chance provided a way to eliminate this minor population of a1 molecules from the streptococcal antibodies of rabbit R27-11.

To determine if these antibody molecules were the same size as those of normal IgG, a small portion of it, labeled with  $^{125}\text{I}$ , was mixed with a much larger quantity of IgG from a rabbit with the same allotype and passed through a column of Sephadex G-200. The result was unexpected. The iodinated material emerged as two major peaks and some slower material (Fig. 1). The first and smaller of the two peaks coincided with the peak of unlabeled IgG as determined by OD 280. The major portion of the iodinated material emerged later as a distinct second peak, which was followed by an even slower tail.

An independent measure of molecular size was accordingly made by gradient

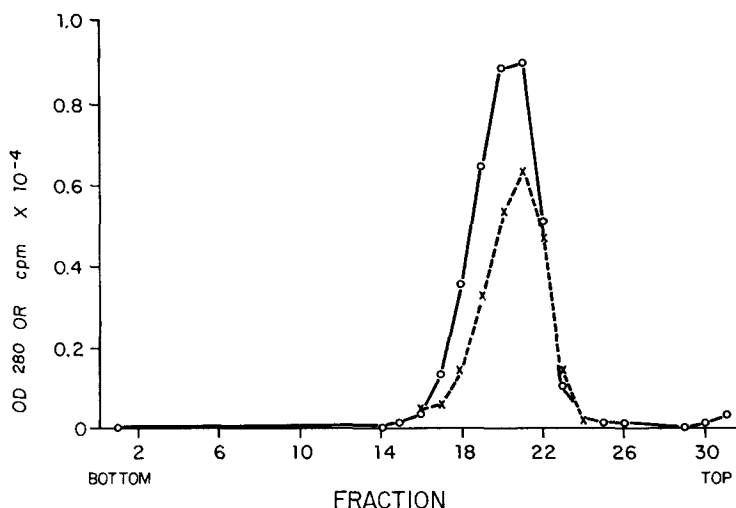


FIG. 2. Gradient centrifugation of antibody from rabbit R27-11. A 0.025 ml sample containing 0.05 mg of radioiodinated ( $^{125}\text{I}$ ) antibody isolated by electrophoresis (6) mixed with 2.0 mg of IgG in 0.3 ml of 0.1 M potassium phosphate buffer pH 6.7 was applied to a gradient of 5–20% sucrose. Centrifugation was performed for 24 hr at 35,000 rpm at 4° C in the SW40 head of a Spinco Model L2-65B centrifuge. X---X, OD 280; O—O, radioactivity.

ultracentrifugation. A small amount of antibody labeled with  $^{125}\text{I}$  was mixed with a much larger quantity of unlabeled IgG and centrifuged in a 5–20% sucrose gradient. A single radioactive peak was found, and this coincided with the peak of unlabeled IgG as revealed by OD 280 (Fig. 2). It thus seemed unlikely that the second peak found by Sephadex gel filtration could be attributed to a difference in molecular size.

Because the antibody was directed against a polysaccharide, a branched polymer of rhamnose terminating in *N*-acetylgalactosaminide residues (16), it seemed possible that it might be capable of a low degree of immunological crossreactivity with Sephadex, which is obtained by chemically crosslinking another bacterial polysaccharide, dextran. Even a weak affinity could retard

considerably the movement of the antibody through the column. If the ability to crossreact with Sephadex were independent of the degree of crosslinking of the Sephadex, one might expect that a more highly crosslinked Sephadex would be even more effective than a less highly crosslinked Sephadex in separating the antibody. This proved not to be the case. When a column of Sephadex G-25 was employed, the peak of antibody radioactivity and optical density due to the cold IgG coincided in the column effluent. This indicated that the crossreactivity of the antibody with Sephadex was a function of the degree of crosslinking of the Sephadex.

To test the ability of the antibody to crossreact with preparations of Sephadex differing in their degree of crosslinking, a simple test was made. A mixture

TABLE II  
*Reactivity of R27-11 with Sephadex\**

Sephadex	Antibody bound‡
	%
G-25	4
G-50	10
G-100	22
G-150	22
G-200	37

\* 1.0 ml bed volume of each Sephadex in 0.1 M pH 6.8 phosphate buffer and 1.0 ml of a solution containing 1 mg of unlabeled IgG and 10  $\mu$ g of antibody labeled with  $^{125}$ I ( $5 \times 10^4$  cpm) were incubated 2 hr.

‡ The supernatant was counted. The value given is calculated as follows,  $[1 - (\text{actual cpm/ml}/\text{theoretical cpm/ml})]100$ , where theoretical cpm/ml is cpm/ml in the original solution  $\times (\text{OD 280 supernatant})/(\text{OD 280 original solution})$ .

of a small quantity of highly labeled antibody and a much larger amount of unlabeled IgG to serve as an internal control on dilution and nonspecific adsorption was mixed with 1.0 ml of a variety of swollen Sephadex types. After incubation for 2 hr at room temperature the mixture was centrifuged. Radioactivity and optical density were measured on each supernatant. The results presented in Table II show that the degree of antibody binding decreases as the degree of crosslinking of the Sephadex increases. Since the binding continued to decrease as the crosslinking for a molecule the size of IgG has been passed, and since the actual amount of Sephadex used in this test increases as the crosslinking increases, it seems likely that there are fewer sites, rather than simply a reduction in the accessibility of the sites, in the more highly crosslinked Sephadex samples.

These findings presented a method to achieve further purification of antibody R27-11. As shown in Table I, the protein present in the second major

peak eluted from the Sephadex column (Fig. 1) was now 0.6% a1, whereas the starting material was 10% a1. The b4 remained at 99%. A second gel filtration was performed using the same column, but in this case 2.7 mg of labeled material without unlabeled normal IgG was used. The profile of radioactivity in

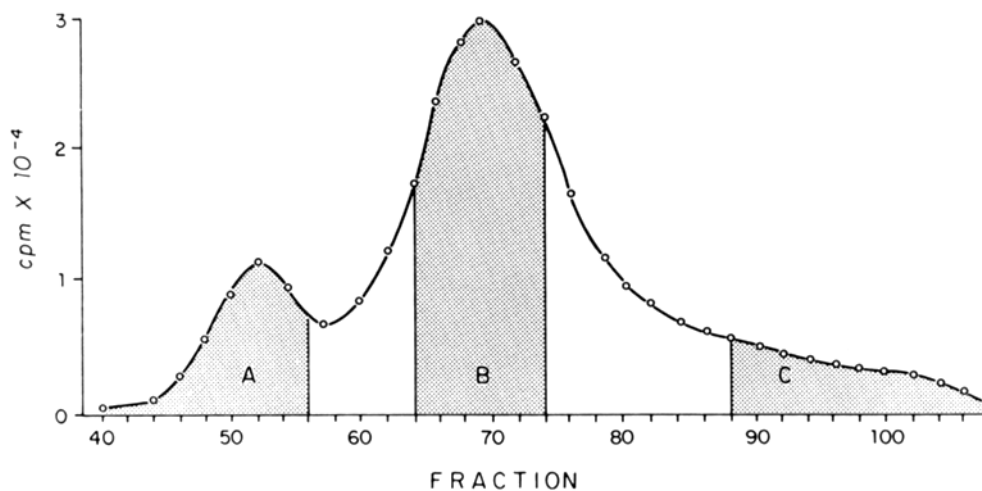


FIG. 3. Gel filtration of antibody from rabbit R27-11. A 3 ml sample containing 2.7 mg of radioiodinated ( $^{125}\text{I}$ ) antibody isolated by electrophoresis (6) was applied to a column ( $90 \times 2.5$  cm) of Sephadex G-200 in 0.1 M pH 6.8 potassium phosphate buffer. Fraction size was 5 ml.

TABLE III  
Precipitation of Peaks of Fig. 3

Peak	Amount precipitated by	
	Anti-a1	Polysaccharide*
	%	%
A	50	33
B	0	99
C	0.5	90
Sample‡	10	81

\* Group C streptococcal carbohydrate. This precipitation was carried out in the presence of carrier antibody from R27-11.

‡ Electrophoretically isolated antibody from rabbit R27-11.

the effluent was essentially the same (Fig. 3). In this case the fractions were pooled as indicated in Fig. 3 and concentrated by pervaporation. The contents of allotype a1 and the precipitating reactivity to Group C carbohydrate were determined on the pools A, B, and C. As indicated in Table III, peaks B and C contain most of the antibody activity and are essentially free of allotypic specificity a1. As expected from the ability of fractions B and C to bind to

Sephadex, these peaks are essentially pure antibody. Unlabeled antiserum was used as a carrier after initial experiments in which somewhat lower values were obtained in its absence. These initial results are attributed to the difficulty of handling the polysaccharide precipitates quantitatively at this high dilution. The antibody in peak C is presumed to be a variant with a slightly higher binding affinity.

Not all antibodies to Group C streptococcal carbohydrate show this cross-reactivity with Sephadex. As shown in Fig. 4, Sephadex gel filtration of anti-

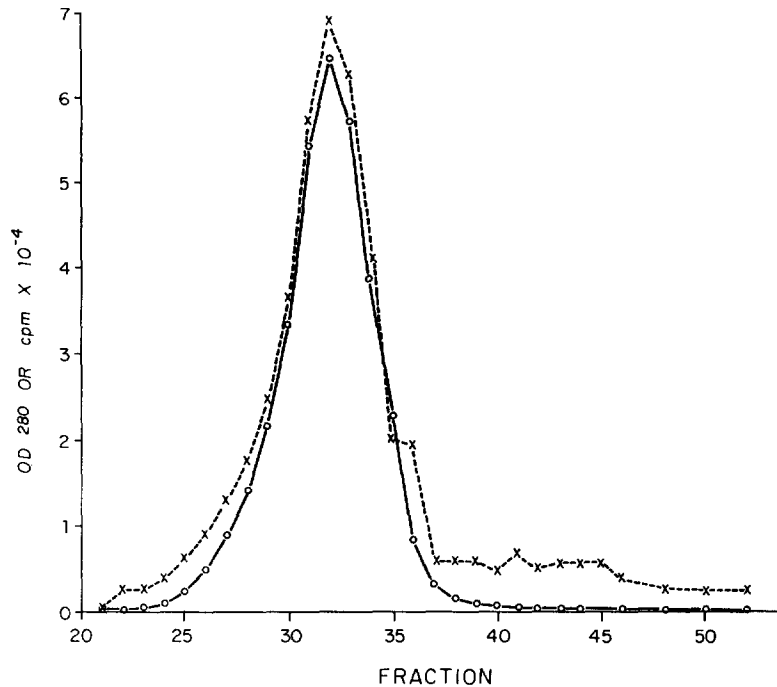


FIG. 4. Gel filtration of antibody from rabbit R14-81. A 5.0 ml sample containing 0.075 mg of radioiodinated (<sup>125</sup>I) antibody isolated by electrophoresis (6) and 21 mg of normal IgG was applied to a column (90 × 2.5 cm) of Sephadex G-200 in 0.1 M pH 6.8 potassium phosphate buffer. Fraction size was 8.0 ml. X---X, OD 280; O—O, radioactivity.

bodies from rabbit R14-81 are not retarded on the column and no additional separation from other minor  $\gamma$ -globulin components is achieved. At least one other antibody tested in this way behaved in a fashion similar to antibody R27-11. The applicability of this method to purify antibodies to other carbohydrates is being examined.

#### DISCUSSION

These results provide further evidence for the similarity between myeloma proteins and antibodies to streptococcal polysaccharides. They also add to the



growing body of evidence that, despite the normal heterogeneity of the immune response, it is possible for certain antigens to elicit a highly selective response.

Gell and Kelus (17) examined antibodies from several rabbits against a series of antigens for the presence of allotypic markers by Ouchterlony diffusion. In one case, they failed to obtain a reaction with an antiserum directed against a specificity present in the rabbit from which the antibody was derived, but concluded that the test system was inadequate to demonstrate complete elimination of this specificity. Rieder and Oudin (18) used quantitative diffusion techniques to demonstrate variations in the concentrations of allotypic specificities in antibodies from the same rabbit but directed against different antigens. No deletions of allotypes were found. Lark, Eisen, and Dray (19) obtained similar results in a study of rabbit antibody against two haptens. Nisonoff, Zappacosta, and Jureziz (20) have reported on the absence of allotype a2 in a crystalline anti-*p*-azobenzoate antibody from a rabbit possessing both a1 and a2 allotypes.

Kunkel, Mannik, and Williams (21) have shown that a number of human antibodies to different carbohydrates possess the individual antigenic specificity characteristic of myeloma proteins (22). While in a number of instances these antibodies showed wide variations in the ratio of  $\kappa$  to  $\lambda$  chains (23), in certain cases only one or the other type of light chain was present. Not uncommonly, deletions of Gm and Inv specificities also were observed (24).

In the present study, one antibody (R24-35) has shown a slight shift to higher concentrations of both its group a and group b specificities. A second antibody (R14-81), heterozygous for both group a and group b specificities, has shown a very marked increase in one from each group with a marked depletion of the second. It is believed likely that the small amounts of residual a3 and b5 specificities in this case represent contaminating immunoglobulin not directed to the streptococcal carbohydrate. In the third case (R27-11), it was possible to show that this residual specificity was eliminated from the antibody preparation by a purification procedure based on the fortunate observation that this antibody binds weakly with Sephadex.

Oudin (25) has shown that rabbits normally contain some  $\gamma$ -globulin molecules lacking the group a allotypic specificities. David and Todd (9) have shown that it is possible to obtain rabbits devoid of immunoglobulin with the group a allotypes by embryo transfer to does that produce antibody to the allotypic specificities genetically present in the transferred embryo. The absence of group a allotypic specificities in these uniform antibodies from R27-11 suggests that it is possible to obtain specific antibodies selected from this population, just as individual myeloma proteins are believed to result from enhanced production of an otherwise normal  $\gamma$ -globulin molecule.

The reason for the binding properties of Group C antibody R27-11 for Sephadex remains unexplained. Because the antibody to the Group C carbohydrate which was eluted emerged in a symmetrical second peak followed by a long

shoulder, it could be separated from the nonantibody IgG in the first minor peak. It would seem that only a low binding affinity would be required to effect this retardation in the sequential adsorption-desorption processes occurring during passage through the column. However, the normal symmetrical shape of the peak would indicate that this affinity must be highly uniform for the antibody within it.

Richards and Haber (26) have described antibody to a D,L-alanine copolymer carrying dinitrophenyl groups with a uniform binding affinity. Similar results have been obtained with antibodies to angiotensin (27). Brenneman and Singer (28) have obtained electrophoretically homogeneous antibodies in rabbits by immunization with a dinitrophenyl lysyl haptenic groups attached to the single mercapto group present on papain, but the concentration of antibodies in all these cases has been disappointingly low. In the case of the antibodies to streptococcal polysaccharide, the combination of high antibody concentration attained within 4 wk of immunization and their uniform properties makes them especially attractive for the type of studies described here. It is yet to be learned by what mechanism such a high concentration of uniform antibody is so quickly achieved.

#### SUMMARY

Rabbit antibodies to streptococcal polysaccharide are described which show selectivity of expression of the allotypic specificities on both the heavy (H) and light (L) chains. One of these antibodies binds weakly to Sephadex. A purification method based on this binding has yielded antibody completely lacking any group an allotypic marker on its H chains.

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