

## QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

### III. PERSISTENCE AND VARIATIONS OF IDIOTYPIC SPECIFICITIES DURING THE COURSE OF IMMUNIZATION\*

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Evidence from two types of experiment indicates that structurally related antibody molecules may be found in the serum of an immunized rabbit over a long period of time. Hybrid recombinants of heavy and light chains, derived from antibenzoate antibodies purified from sera of a hyperimmunized animal taken at intervals of about 6 months, had specific activities comparable to those of the recombinants of chains derived from a single population of molecules (1). These results were interpreted as indicating that "memory" cells, resulting from stimulation by antigen, give rise directly or indirectly, upon subsequent challenge, to antibody molecules similar or identical in structure to those synthesized initially by that cell line.

The presence of individual antigenic ("idiotypic") specificities (2, 3) in antibody populations provided another method of approach to this question. Persistence of a given set of specificities was observed in a rabbit for at least 8 months (4), a period which greatly exceeds the half life of either an IgG molecule (5, 6) or an antibody-producing cell (7, 8). Recently, Oudin and Michel (9) reported the persistence for 29 months of related or identical idiotypic specificities in the anti-salmonella antibodies of one rabbit, as shown by double diffusion measurements in agar gel. In another rabbit they observed a loss of idiotypic specificities during the early stages of immunization. Idiotypic specificities were found to persist in two other rabbits during a period between 2 and 5 wk after the start of immunization.

Results to be presented here extend our quantitative studies of idiotypic specificities in antibenzoate antibodies from two rabbits, AZ1 and AZ5 (4, 10), and introduce data for a third rabbit. In each case, idiotypes present in the first sera of high titer were present for about 2 months and then were replaced, either gradually or abruptly, by a new set of specificities which persisted for a long period of time. Quantitative measurements, however, indicated that

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gradual changes of idiotypic specificities occurred continuously during the latter period, with the appearance of still a third set of specificities. Methods described previously (11, 12) were utilized for eliciting and quantifying precipitating and nonprecipitating idiotypic antibody populations.

### *Materials and Methods*

The following materials and methods have been described (11, 12): conjugates of *p*-azobenzoate with bovine  $\gamma$ -globulin and hemocyanin; rabbit IgG; fragments Fc and Fab of rabbit IgG and goat antisera specific for each type of fragment; isolation of specifically purified anti-*p*-azobenzoate antibodies of the IgG class; polymerization of such antibodies with glutaraldehyde for the purposes of immunization; iodination of proteins with  $^{125}\text{I}$ Cl (less than one atom of iodine per molecule of protein); tests of allotypic specificities; preparation of  $^{125}\text{I}$ -labeled  $\text{F}(\text{ab}')_2$  fragments of specifically purified anti-*p*-azobenzoate antibody; quantification of percentages of idiotypic anti-*p*-azobenzoate antibodies (D) directly precipitable by anti-D sera, or of  $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$  fragments, derived from D antibodies, that were precipitable by an indirect method utilizing goat antirabbit Fc to precipitate soluble complexes.

The  $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$  fragments were prepared by mixing  $^{125}\text{I}$ -labeled specifically purified anti-*p*-azobenzoate antibody (D) from an individual rabbit with a 20-fold excess of unlabeled non-specific IgG, prepared from the pooled sera of several rabbits, and treating the mixture with 2% by weight of pepsin for 8 hr at pH 4.3 and 37°C.  $\text{F}(\text{ab}')_2$  fragments were isolated by gel filtration over Sephadex G-150 and were characterized by double diffusion in agar gel and precipitin reactions with goat antirabbit Fc and goat antirabbit Fab. Positive reactions were obtained only with the latter reagent, and more than 85% of the radioactivity was brought down from each preparation in a single precipitation with excess anti-Fab antibody. This procedure permitted studies with a small amount of D antibody. It also ensured the presence of a large excess of unlabeled  $\text{F}(\text{ab}')_2$  from normal IgG to minimize nonspecific interactions.

Precipitations by the indirect method were carried out by incubating  $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$  fragments derived from D antibodies with excess anti-D serum for 1 hr at 37°C in the presence of 50  $\mu\text{g}$  of bovine serum albumin. The albumin was added to decrease the adherence of the labeled protein to glass (12). An excess of goat antirabbit Fc serum was then added to precipitate IgG and complexes of anti-D with  $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$ . After 1 day, precipitates were separated by centrifugation, washed three times, and dissolved in 1.5 ml of 0.04 N NaOH. Radioactivities of the combined supernatant fluids and the dissolved precipitate were determined. Preliminary experiments were carried out to ensure that both the anti-D and anti-Fc antibodies were present in excess. A typical experimental test comprised 0.5  $\mu\text{g}$   $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$ , 9.5  $\mu\text{g}$  unlabeled non-specific  $\text{F}(\text{ab}')_2$ , 10  $\mu\text{l}$  anti-D serum, and 0.5 ml goat anti-Fc. Nearly all experiments were carried out in triplicate. When another, unlabeled component was added to test for inhibition, the inhibitor and the  $^{125}\text{I}$ - $\text{F}(\text{ab}')_2$  fragments were mixed prior to the addition of anti-D serum. When whole serum was tested as inhibitor, the control tubes contained an equal volume of serum from a rabbit hyperimmunized to ovalbumin. The presence of antiovalbumin serum had a negligible effect in each case. Methods for carrying out tests of inhibition were described in detail previously (12).

Measurements of the hapten-binding capacity of anti-*p*-azobenzoate antibodies were carried out by the method of equilibrium dialysis, utilizing  $^{125}\text{I}$ -labeled *p*-iodobenzoate (13). Data were analyzed by use of the Sips distribution function (13, 14) to obtain an average binding constant ( $K_0$ ) and an index of heterogeneity,  $a$ . The parameters,  $K_0$  and  $a$ , for four preparations are shown in Table I.

*Preparation of Anti-*p*-Azobenzoate Antibodies.*—Anti-*p*-azobenzoate antibodies were elicited in three rabbits (AZ1, AZ5, and AZ11). Each was inoculated subcutaneously in

multiple sites in the footpads and back, with a total of 3 mg of bovine  $\gamma$ -globulin-*p*-azobenzoate conjugate incorporated in complete Freund's adjuvant. This was repeated after 3 wk and followed 1 wk later by an intravenous injection of 2 mg of protein. In each rabbit the first strong reaction with the hemocyanin-*p*-azobenzoate test antigen was obtained 1 wk after the intravenous inoculation. A pool of serum of high titer was collected from each rabbit by repeated bleedings over a period of 2-3 wk. Weekly intravenous injections were then resumed. Bleedings were taken periodically, 5-7 days after an injection.

After 6 months of immunization each rabbit was allowed to rest for 1 month. Rabbit AZ11

TABLE I  
Maximal Percentages of Donor Antibody Populations Precipitable by the Direct and Indirect (Antiglobulin) Methods\*

Donor antibody (D) <sup>†</sup> Rabbit	Recipient antiserum (Anti-D) Rabbit	Allotype of donor and recipient	Ouchterlony test	Amount precipitable		K <sub>0</sub> <sup>§</sup>	a <sup>  </sup>
				Direct precipitation	Indirect precipitation		
				%	%		
AZ1 (D <sub>2</sub> ) <sup>¶</sup>	RD8	1, 3, 4, 7, 21	0	1	34	9.1 × 10 <sup>5</sup>	0.8
	1-I		0	1	42		
AZ1 (D <sub>8</sub> )	9N	1, 3, 4, 7, 21	+	22	51	7.8 × 10 <sup>6</sup>	0.7
AZ5 (D <sub>2</sub> )	RD5	1, 3, 4, 7, 21	+	2	23	1.1 × 10 <sup>6</sup>	0.9
AZ5 (D <sub>8</sub> )	9Y	1, 3, 4, 7, 21	+	33	70	5.2 × 10 <sup>6</sup>	0.7
AZ11 (D <sub>2</sub> )	2X	1, 3, 4, 21	+	7	13		

\* <sup>125</sup>I-labeled specifically purified antibody (D) was used in the direct method; <sup>125</sup>I-F(ab')<sub>2</sub> fragments in the indirect technique.

<sup>†</sup> Specifically purified anti-*p*-azobenzoate antibody.

<sup>§</sup> Average binding affinity of the donor antibody for <sup>125</sup>I-*p*-iodobenzoate.

<sup>||</sup> Index of heterogeneity (14).

<sup>¶</sup> D<sub>2</sub> refers to antibenzoate antibodies isolated approximately 2 months after the start of immunization.

died at this time. Intravenous inoculations of the other two rabbits, at weekly or biweekly intervals, were resumed. After 17 months of immunization, rabbits AZ1 and AZ5 were still alive, and experiments were carried out with antibenzoate antibodies isolated from sera taken periodically up to that time.

All specifically purified antibenzoate antibodies were found by immunoelectrophoresis to be of the IgG class. This was expected, since a step in the procedure (11) is the passage of the protein solution through diethylaminoethyl (DEAE) cellulose at low ionic strength.

## RESULTS

*Preparation of Anti-Idiotypic (Anti-D) Antibodies.*—Anti-D antibodies were elicited by injection of specifically purified anti-*p*-azobenzoate antibodies into recipient rabbits whose allotype was matched to that of the donor with respect to the following specificities: a1, a2, a3, b4, b5, b6, b9, c7, and c21.

The first antibody preparation used for inoculation was specifically purified from pooled sera collected 5-8 wk after the start of immunization of each donor rabbit (AZ1, AZ5, or AZ11)

with bovine  $\gamma$ -globulin-*p*-azobenzoate. This will be designated  $D_2$ ; antibody from donor sera collected approximately 3 months after the start of immunization will be denoted  $D_8$ , etc.

$D$  antibodies from rabbits AZ1 and AZ5 were each injected into two recipients; one recipient rabbit was used for  $D_2$  antibodies of rabbit AZ11. Recipients were inoculated subcutaneously in the footpads and back with a total of 3 mg of  $D_2$  in complete Freund's adjuvant; this was repeated after 3 wk and was followed by one intravenous inoculation. No precipitating antibodies were detected at this time. Polymerized  $D_2$ , prepared by treatment with glutaraldehyde, was used as the antigen for subsequent inoculation. 3 mg portions in complete Freund's adjuvant were injected twice, 3 wk apart; this was followed after 2 wk by a single intravenous inoculation. 1 wk later, one of the two recipient rabbits (RD5), challenged with  $D$  antibody from rabbit AZ5, contained both precipitating and nonprecipitating antibodies; the serum of the second recipient was inactive. (Nonprecipitating antibodies were detected by the indirect method). Both recipients (1-I and RD8) of  $D_2$  from rabbit AZ1 yielded nonprecipitating antibodies, but precipitating antibodies were not detectable in either recipient serum. The recipient (rabbit 2X) of  $D_2$  from rabbit AZ11 had both precipitating and nonprecipitating antibodies. All tests of activity were carried out with monomeric antibenzoate ( $D$ ) antibody or its  $F(ab)_2$  fragments.

Percentages of molecules reactive with the homologous anti- $D$  sera, by the direct or indirect method, are listed in Table I.

Anti-idiotypic antisera were also prepared against specifically purified anti-*p*-azobenzoate antibodies ( $D_8$ ) isolated approximately 8 months after the start of immunization of rabbits AZ1 and AZ5. Two recipients were used for each donor antibody; each recipient was inoculated with polymerized  $D_8$  antibody. Two injections of 3 mg in complete Freund's adjuvant, spaced 3 wk apart, were followed by a single intravenous inoculation. All recipients responded with the formation of precipitating antibodies. Only the stronger recipient serum for each donor was studied quantitatively.

In each instance (Table I) a larger percentage of  $D$  molecules was precipitable by the indirect than by the direct procedure. Also, the percentage of  $D_8$  precipitable, by either procedure, was larger than the corresponding percentage of  $D_2$  precipitable from the same rabbit. The data for the  $D_2$  antibodies have already been presented (12) and are included here for the purpose of comparison.

*Specificity of Reactions of Anti-Idiotypic Sera.*—Data relating to the specificity of reactions of the  $D_2$  antibodies (early bleedings) of rabbits AZ1, AZ5, and AZ11 with their homologous anti- $D$  sera have been reported elsewhere (12). By the method of inhibition of indirect precipitation no cross-reactions comparable in strength to the homologous reactions were observed in that study, although two or three weak cross-reactions were noted. This method is very sensitive, since a 60-fold excess of inhibitor is employed. One strong cross-reaction was noted (12) in the direct interaction of antibenzoate antibodies from rabbit AZ11 with an anti- $D$  serum prepared against antibenzoate antibody from another rabbit. This is the only strong cross-reaction we have so far observed in our investigations.

Data concerning the specificity of anti- $D$  sera prepared against  $D_8$  antibodies (later bleedings) of rabbits AZ1 and AZ5 are shown in Table II. By the method

TABLE II  
*Inhibition of the Indirect Precipitation of  $^{125}\text{I-F(ab')}_2$  Fragments from Donor Antibodies\**

Donor rabbit (D)	AZ1 (D <sub>8</sub> )	AZ5 (D <sub>8</sub> )	
Recipient rabbit (Anti-D)	9N	9Y	
Inhibitor (Rabbit)			Allotypes
AZ1 (D <sub>2</sub> ) ‡	103 (2)	101 (1)	1, 3, 4, 7, 21
AZ1 (D <sub>8</sub> )	<i>10 (2)</i>		
AZ5 (D <sub>2</sub> )	98 (3)	97 (<1)	1, 3, 4, 7, 21
AZ5 (D <sub>8</sub> )		<i>10 (&lt;1)</i>	
AZ3	98 (2)	101 (<1)	1, 3, 4, 7, 21
AZ4	100 (1)	100 (3)	3, 4, 7, 21
AZ8	102 (2)	96 (4)	3, 4, 7
AZ9	100 (2)	99 (1)	1, 3, 4, 7, 21
AZ11	80 (2)	91 (2)	1, 3, 4, 7, 21
AZ14	101 (3)	97 (1)	1, 3, 4, 5, 7
AZ15	103 (2)	99 (1)	1, 2, 4, 6, 7
A6A	99 (1)	93 (<1)	1, 3, 4, 7
A6B	99 (2)	110 (5)	1, 4, 7
A7	100 (2)	92 (1)	1, 4, 7, 21
V15	98 (5)	97 (2)	1, 4, 7
IgG (nonspecific) §	100 (1)	101 (<1)	1, 3, 4, 7, 21
Homologous serum (D <sub>8</sub> )	<i>11 (&lt;1)</i>	<i>25 (&lt;1)</i>	
Preimmune serum	111 (3)	98 (2)	
D <sub>8</sub> serum absorbed ¶ with hemo- cyanin azobenzoate	99 (8)	99 (1)	
D <sub>8</sub> serum absorbed** with hemo- cyanin	20 (1)	21 (<1)	

\* Inhibitors are specifically purified anti-*p*-azobenzoate antibodies from the rabbits specified in the first column, except for the last five inhibitors listed. Each experiment was carried out with 0.5  $\mu\text{g } ^{125}\text{I-F(ab')}_2$  and 30  $\mu\text{g}$  of the antibody or IgG as inhibitor. When serum was the inhibitor (last four rows) 10  $\mu\text{l}$  was used. Details are in the text. Data are given as the percentage of radioactivity precipitated, with the amount precipitated in the absence of inhibitor taken as 100%. Data for the homologous inhibitors are italicized. Experiments were carried out in triplicate; average deviations are given in parentheses.

‡ D<sub>2</sub> refers to antibody isolated from the donor serum approximately 2 months after the start of immunization.

§ IgG prepared from the pooled sera of several nonimmunized rabbits.

|| Serum taken from the homologous donor rabbit, AZ1 or AZ5, prior to immunization.

¶ Serum from the hyperimmunized donor rabbit (D<sub>8</sub>), absorbed with an optimal quantity of hemocyanin-*p*-azobenzoate.

\*\* As above, but absorbed with an amount of hemocyanin equal in weight to the hemocyanin-*p*-azobenzoate.

of inhibition of indirect precipitation, no cross-reactions comparable in strength to the homologous interaction were noted with any of the heterologous purified antibodies or with whole IgG present in 60-fold excess. A large degree of inhibition was observed only with each homologous antibody preparation.

Table II (last four rows) shows, first, that 10 microliters of the whole serum from which the D<sub>8</sub> antibodies were prepared strongly inhibited the binding of homologous <sup>125</sup>I-F(ab')<sub>2</sub> fragments by anti-D serum. This inhibitory capacity was entirely removed by prior absorption of the D<sub>8</sub> serum with hemocyanin-*p*-azobenzoate but not with the same weight of hemocyanin, indicating that the reactive component in the whole serum is antibenzoate antibody. The amount of hemocyanin-*p*-azobenzoate used was that which gave optimal precipitation; the precipitate was removed by centrifugation prior to testing the residual

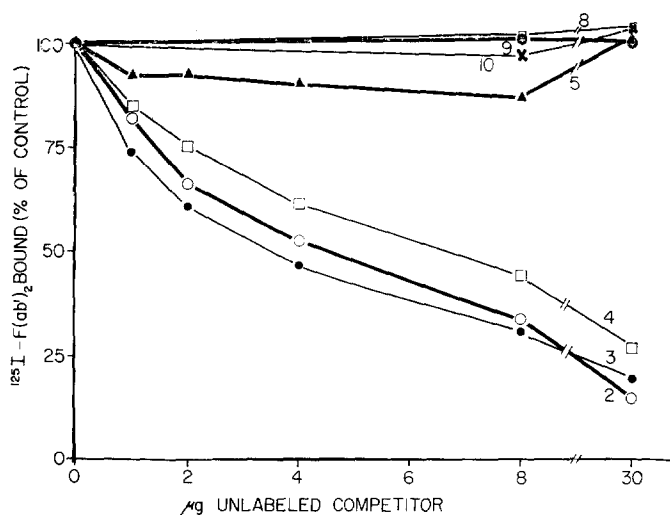


FIG. 1. Inhibition of binding of labeled F(ab')<sub>2</sub> fragments of anti-*p*-azobenzoate antibodies (D) from rabbit AZ1 to the homologous anti-D serum (from rabbit RD8). The D antibodies were isolated from sera taken approximately 2 months after the start of immunization of rabbit AZ1. Competitors are unlabeled, specifically purified antibenzoate antibodies prepared from sera of rabbit AZ1 at various times after the start of immunization; the approximate number of months is indicated by the numeral on each curve. Experiments were in triplicate with an overall average deviation from the mean, expressed as the per cent of control, of 1.8%.

serum for inhibitory capacity. It should be noted that the amount of antibenzoate antibody in each D<sub>8</sub> serum was less than 1 mg/ml; thus, depleting the antibody did not greatly reduce the total IgG content of the serum, and allotypic determinants present would have been expressed. In addition, serum taken from each rabbit prior to immunization failed to inhibit the reaction of <sup>25</sup>I-F(ab')<sub>2</sub> of D<sub>8</sub> antibodies with homologous anti-D serum (Table I).

These findings, together with the absence of any strong cross-reactions with antibodies from heterologous rabbits, demonstrate the specificity of the reactions and appear to eliminate allotypic specificities as the basis for the interaction between the D antibodies and anti-D sera.

*Persistence of Idiotypes during Continued Immunization of a Donor Rabbit.*—To analyze for the presence of a given idiootype, unlabeled antibodies were tested for their capacity to inhibit the binding of  $^{125}\text{I-F(ab')}_2$ , derived from the donor antibody, to its homologous anti-idiotypic antibody. The indirect method was used. Inhibitors were unlabeled, specifically purified antibenzoate antibodies of the IgG class isolated from the serum of the donor rabbit at various times during its course of immunization.

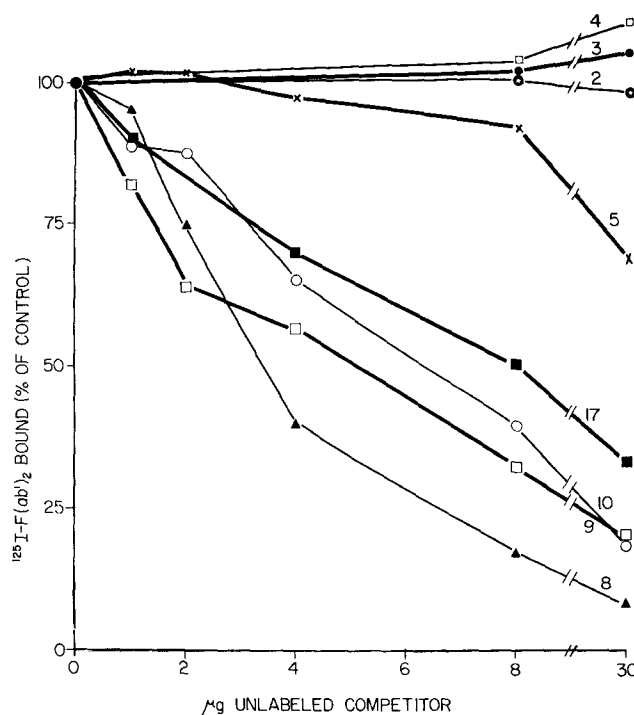


FIG. 2. The legend is the same as that of Fig. 1, except that the D antibodies from rabbit AZ1 were isolated approximately 8 months after the start of immunization; the recipient rabbit is 9N; and the overall average deviation is 1.7%.

Fig. 1 presents data on the inhibition of binding of  $^{125}\text{I}$ -labeled fragments of  $\text{D}_2$  (month 2) antibodies from rabbit AZ1 with homologous (anti- $\text{D}_2$ ) antiserum from rabbit RD8. The numbers on the curves represent the approximate number of months that elapsed between the start of immunization and the time at which the serum was drawn for isolation of the antibody used as inhibitor.

As expected, the homologous unlabeled antibody (month 2) inhibited the binding of  $^{125}\text{I-F(ab')}_2$  almost completely when present in large excess. In these

tests the weight ratio of unlabeled inhibitor to  $^{125}\text{I-F(ab')}_2$  fragments varied from 2:1 to 60:1. Failure to observe complete inhibition with a 60-fold excess of homologous inhibitor is attributable to the presence of an excess of anti-D antibody.

Antibody isolated from rabbit AZ1 at month 3 was approximately equivalent in its inhibitory capacity to the homologous unlabeled D<sub>2</sub> antibody. Some de-

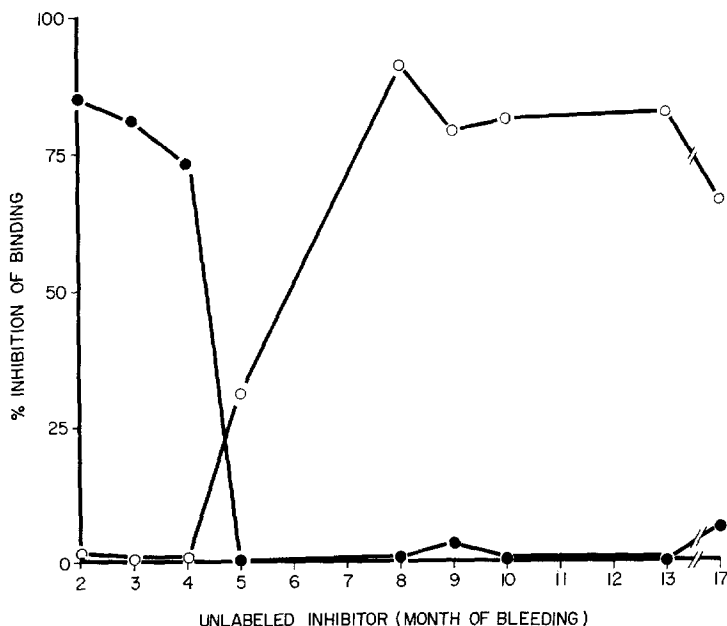


FIG. 3. Summary of data obtained with a 60-fold excess by weight of competitor (30  $\mu\text{g}$ , see Figs. 1 and 2). Solid circles, labeled donor antibodies from month 2 bleedings of rabbit AZ1 reacting with anti-D<sub>2</sub>. Open circles, labeled donor antibodies from month 8 antibodies reacting with anti-D<sub>8</sub>. Inhibitors are unlabeled antibodies from sera of rabbit AZ1 taken at the time specified on the abscissa. Note that the data are plotted as the per cent inhibition rather than the per cent of control.

crease in inhibitory capacity is noted for the antibody isolated at month 4. In contrast, antibodies isolated at months 5, 8, 9, and 10 interacted weakly, if at all, with anti-D<sub>2</sub> antibodies. Additional tests were carried out in triplicate with antibodies D<sub>13</sub> and D<sub>17</sub> at a single concentration (60-fold excess by weight of D<sub>2</sub> antibodies over  $^{125}\text{I-F(ab')}_2$ ). The amounts of inhibition of binding of D<sub>2</sub>, 0 and 6%, respectively, were not significant. Thus, molecules bearing similar idiotypic specificities were present over a period of about 2 months, from months 2 to 4. During the following month a marked change in idiotypic specificities took place, which was apparently irreversible.



This change in idiotypic specificities after month 4 is confirmed by data obtained with anti-D antibodies prepared against specifically purified antibenzoate antibodies isolated from the same rabbit, AZ1, at month 8 (Fig. 2). Antibodies D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> did not interact effectively with the anti-D<sub>8</sub> antiserum. However, some activity was noted in D<sub>5</sub>, indicating that a transition was occurring during the 5th month. As expected, unlabeled D<sub>8</sub> was a potent inhibitor of the homologous reaction (Fig. 2). Antibodies isolated at months 9, 10, 13, and 17 also

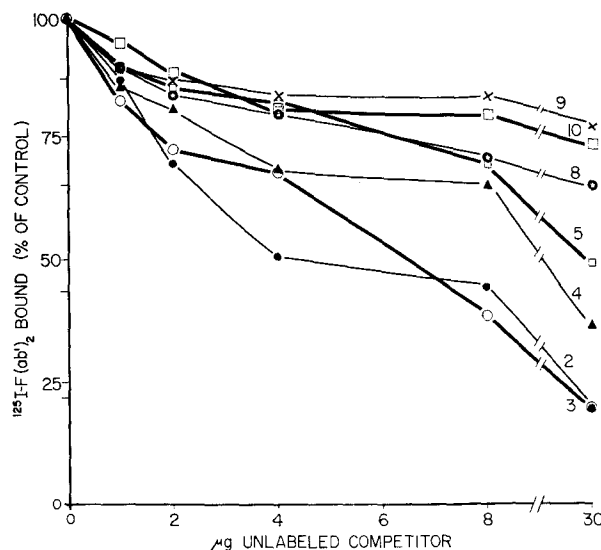


FIG. 4. Inhibition of binding of labeled F(ab')<sub>2</sub> fragments of anti-*p*-azobenzoate antibodies (D) from rabbit AZ5 to anti-D serum from rabbit RD5. The D antibodies were isolated from sera taken approximately 2 months after the start of immunization of rabbit AZ5. Competitors are unlabeled, specifically purified antibenzoate antibodies prepared from sera of rabbit AZ5 taken at various times after the start of immunization; the approximate number of months is indicated by the numeral on each curve. Experiments were in triplicate with an overall average deviation from the mean, expressed as the per cent of control, of 3.3%.

reacted strongly with anti-D<sub>8</sub>. (The inhibition curve for D<sub>13</sub> is almost superimposable on the curve for D<sub>10</sub> in Fig. 2 and is not shown.) Thus, a shift in idiotypic specificities occurred after month 4 which was essentially complete by month 8. This new set of idiotypic specificities persisted in large part through month 17. Similar idiotypic specificities were present from months 5 through 17, but gradual changes occurred almost continuously, as shown by the fact that the inhibitory capacity of the homologous D<sub>8</sub> was greater than that of any of the other antibody preparations, and by the gradual decrease in the slopes of the inhibition curves with antibodies from later bleedings.

Fig. 3 summarizes data on inhibition obtained with the highest concentration of antibody from each bleeding of rabbit AZ1 (Figs. 1 and 2). In contrast to the results in Figs. 1 and 2, the data in Fig. 3 are presented as the per cent inhibition of binding.

Data obtained with donor antibodies from rabbit AZ5 are shown in Figs. 4-7. The results in Fig. 4 were obtained with anti-D<sub>2</sub> serum reacting with its homologous <sup>125</sup>I-F(ab')<sub>2</sub> fragments. Persistence of idiotype between months 2

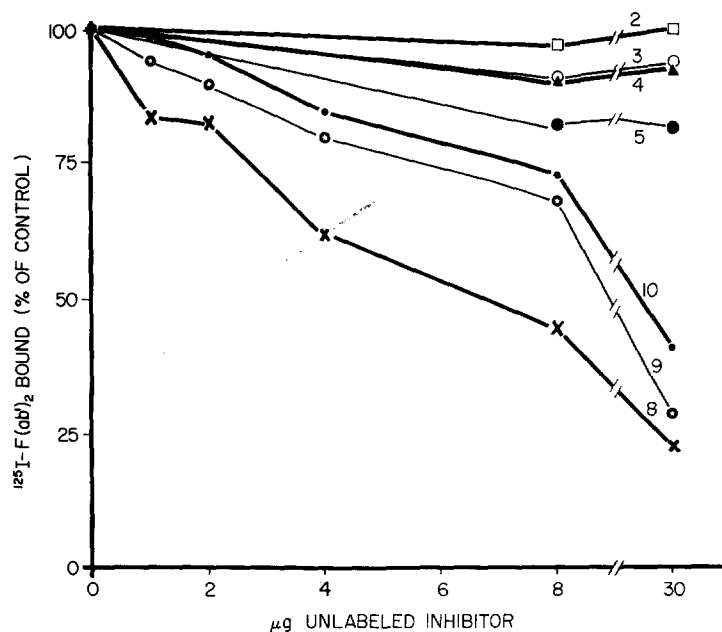


FIG. 5. The legend is the same as that of Fig. 4, except that the D antibodies from rabbit AZ5 were isolated approximately 8 months after the start of immunization; the recipient rabbit is 9Y; and the overall average deviation is 1.6%.

and 4 and a change after that time is noted. In contrast to the D antibodies of rabbit AZ1, the shift in idiotype after month 4 is gradual rather than abrupt, and some antibodies reactive with anti-D<sub>2</sub> were still present at month 10.

Fig. 5 presents data obtained with anti-D antibodies specific for D<sub>8</sub> of rabbit AZ5. The strongest inhibition was again observed with the homologous unlabeled antibody preparation (D<sub>8</sub>). However, antibodies with idiotypes corresponding to those of the month 8 antibodies were also present in D<sub>5</sub>, D<sub>9</sub>, and D<sub>10</sub>. Antibodies D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> did not interact appreciably with anti-D<sub>8</sub> (Fig. 5), despite the fact that D<sub>8</sub> did react with anti-D<sub>2</sub> antibodies (Fig. 4). This may be explained on the basis that there are antibodies in D<sub>8</sub>, similar or identical to

those present in  $D_2$ , which were not immunogenic in the rabbit used as recipient for  $D_8$  antibodies.

The investigation of antibodies from later bleedings of rabbit AZ5 was complicated by the exhaustion of the  $D_8$  antibodies used in the test system. For this reason tests of inhibition were set up with  $^{125}\text{I-F(ab')}_2$  fragments of  $D_{10}$  antibodies and the same anti- $D_8$  antiserum (from rabbit 9Y) used in the experi-

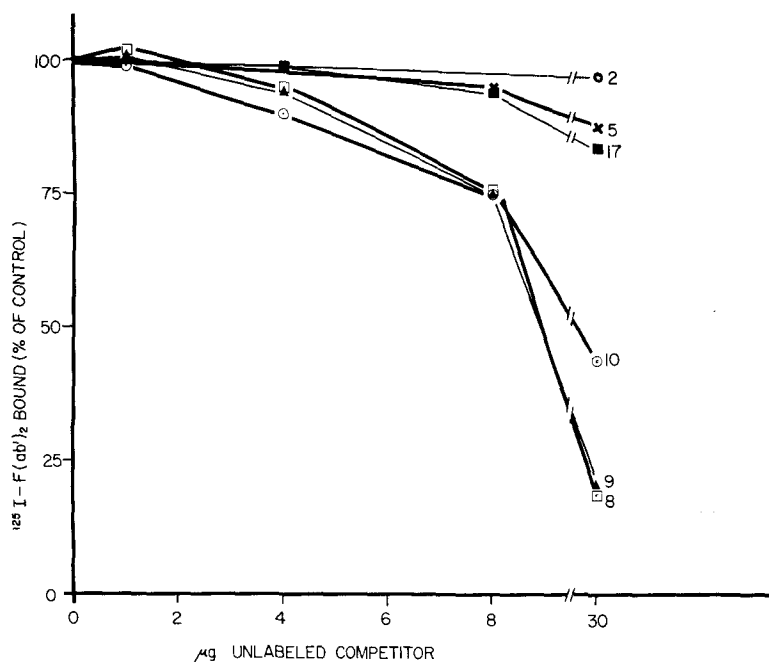


FIG. 6. In this figure the donor antibodies from which the  $^{125}\text{I-F(ab')}_2$  fragments were isolated were  $D_{10}$  antibodies of rabbit AZ5 (month 10 bleedings), but the anti-D serum was prepared against  $D_8$  antibodies in rabbit 9Y (see text). Competitors are unlabeled D antibodies isolated from sera of rabbit AZ5 at various times after the start of immunization; the number of months is specified on each curve. Experiments were in triplicate with an overall average deviation from the mean, expressed as the per cent of control, of 1.1%.

ments of Figs. 4 and 5. The results are shown in Fig. 6. Antibodies  $D_8$ ,  $D_9$ , and  $D_{10}$  competed effectively with  $^{125}\text{I-F(ab')}_2$  fragments of  $D_{10}$  for combination with anti- $D_8$  antibodies.  $D_8$  and  $D_9$  were the most effective inhibitors.  $D_{17}$  antibodies, at the highest concentration tested, inhibited the binding to an appreciable extent (16%), but much less strongly than  $D_{10}$ . Thus, as in the case of rabbit AZ1, prolonged persistence of idiotypic specificities was observed, but the changes in specificities after month 8 in rabbit AZ5 were much more pronounced.

A summary of the data obtained with the highest concentration of each inhibitor tested (Figs. 4-6) is shown in Fig. 7.

Fig. 8 presents data obtained with anti-idiotypic antibodies prepared against  $D_2$  from rabbit AZ11. These data are very similar to those obtained with  $D_2$  from rabbit AZ1 (Fig. 1), in that molecules of the same idio type persisted be-

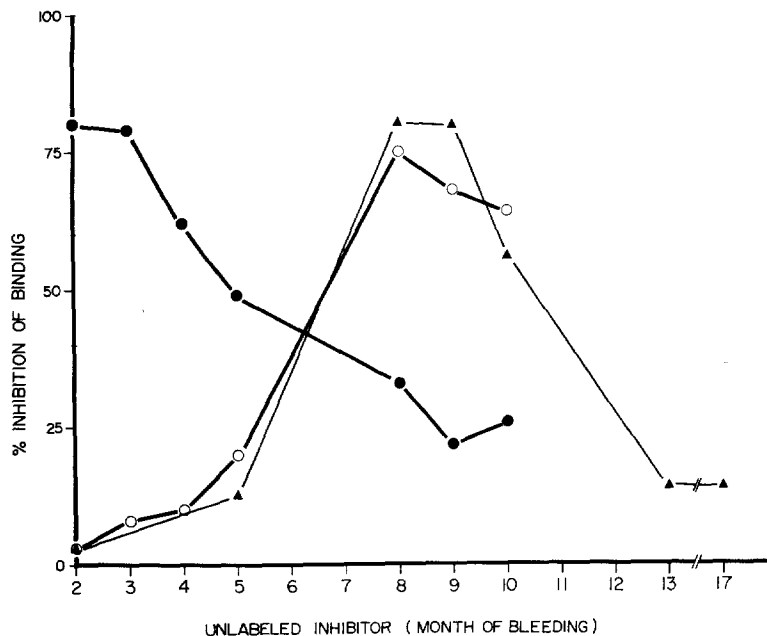


FIG. 7. Summary of data (see Figs. 4-6) obtained with the highest concentration of inhibitor tested ( $30 \mu\text{g}$ , or a 60-fold excess by weight over the  $^{125}\text{I-F(ab')}_2$  fragments from the donor antibody (from rabbit AZ5)). Solid circles, donor  $^{125}\text{I-F(ab')}_2$  fragments from month 2 bleedings reacting with homologous antiserum, anti- $D_2$ ; open circles,  $^{125}\text{I-F(ab')}_2$  fragments from antibody of month 8 bleedings reacting with homologous antiserum, anti- $D_8$ ; closed triangles,  $^{125}\text{I-F(ab')}_2$  fragments from  $D_{10}$  bleedings reacting with anti- $D_8$ . Inhibitors are unlabeled specifically purified anti-*p*-azobenzoate antibodies from bleedings of rabbit AZ5 taken during the month after start of immunization specified on the abscissa.

tween months 2 and 4 and were then replaced abruptly by molecules lacking that idiotypic specificity. Rabbit AZ11 died shortly after the month 5 bleedings.

#### DISCUSSION

The data presented relate to the following points: (a) changes in idiotypic specificities during the early stages of immunization, with the emergence of new populations of molecules having unrelated determinants; (b) the prolonged presence of antibodies bearing the new sets of idiotypic specificities; and (c)

continual quantitative changes in the distribution of idiotypes after hyper-immunization. The word "idiotype" is used here with the understanding that a population of specifically purified antibodies from a single rabbit may comprise a number of unrelated idiotypic populations, and that a single molecule may possess more than one idiotype determinant.

Anti-idiotypic sera were prepared by injection into rabbits of polymerized,

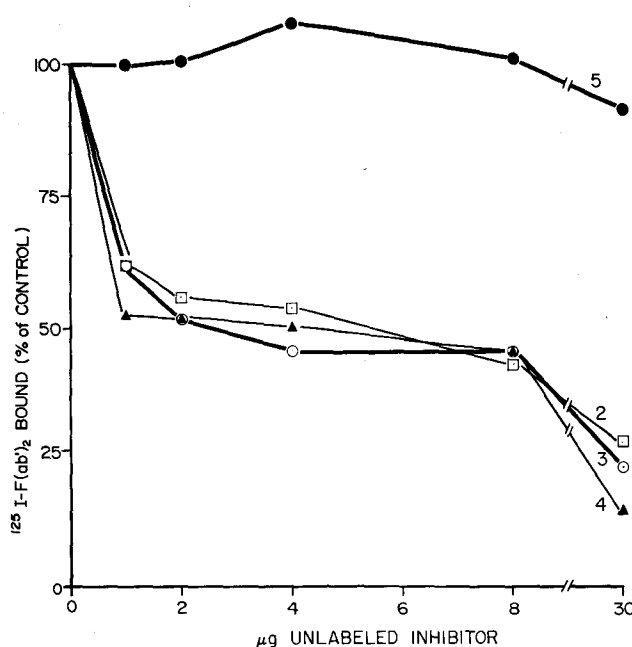


FIG. 8. Inhibition of binding of  $F(ab')_2$  fragments of anti-*p*-azobenzoate antibodies (D) from rabbit AZ11 to anti-D serum from rabbit 2X. The D antibodies were isolated from sera taken approximately 2 months after the start of immunization of rabbit AZ11. Corapetitors are unlabeled, specifically purified antibenzoate antibodies prepared from sera of rabbit AZ11 taken at various times after the start of immunization; the approximate number of months is indicated by the numeral on each curve. Experiments were in triplicate with an overall average deviation from the mean, expressed as the per cent of control, of 2.5%.

specifically purified rabbit anti-*p*-azobenzoate antibodies, in some instances preceded by injections of the monomeric antibody. All tests of activity in antisera were carried out with the monomeric purified antibody or its fragments. The use of purified antibody tends to minimize complications such as the formation of anti-bacterial antibodies upon injection of antibody-bacteria complexes; when testing such anti-idiotypic sera, it is necessary to ensure the absence of bacterial antigens from the serum used as test antigen. Specific purification of the antibenzoate antibodies was carried out at neutral pH; this could prove

to be important in preserving idiotypic determinants. Allotypes of each donor and recipient were matched with respect to nine specificities for which anti-sera were available.

The antibodies studied were evidently directed to idiotypic and not to allotypic determinants. Evidence for this includes the reactivity of anti-idiotypic sera with the hyperimmune donor serum but not with serum of the donor taken prior to immunization (Table II); the removal of reactivity from the donor serum by absorption with a hemocyanin-*p*-azobenzoate conjugate but not by hemocyanin; and the failure to observe any strong cross-reactions with anti-benzoate antibodies from a number of other rabbits or with pooled nonspecific IgG. The infrequency of cross-reactions is somewhat surprising in view of the homology of sequences even in the variable regions of immunoglobulin polypeptide chains.

The use of the indirect method of precipitation in the present experiments could conceivably detect allotypes, such as a11 (15) or a14 (16), or buried determinants in  $F(ab')_2$  fragments (17-19) which do not ordinarily elicit precipitating antibodies. As indicated above, the absence of reactivity in donor serum taken prior to immunization, or in hyperimmune serum absorbed with the antigen, appears to eliminate this possibility. Such anti-allotypic antibodies could, however, have been present in one or more of our sera, but have been undetected because of absorption by the large excess of nonspecific  $F(ab')_2$  fragments present in the test system.

It may be noted that each of the hyperimmune anti-benzoate (D) sera also contained large amounts of precipitating antibody to the protein carrier, bovine  $\gamma$ -globulin. Since removal of anti-benzoate antibodies eliminated reactivity with anti-D sera, the idiotypic specificities were not present on antibody molecules directed to bovine  $\gamma$ -globulin.

Our previous quantitative investigations (11, 12) have shown that the fraction of a population of specifically purified antibody from an individual rabbit that is reactive with its homologous anti-D serum may vary greatly in magnitude. Thus, positive precipitin reactions in agar gel were obtained with donor antibodies of which 2-56% of the molecules were precipitable. By the indirect method of precipitation, using an antiglobulin reagent, larger fractions were almost invariably precipitated, and in two instances substantial proportions of molecules were precipitated by the indirect method from donor preparations which failed to form precipitates directly with anti-D serum (12). One of these preparations was the antibody purified from the early serum of rabbit AZ1, used in the present study. The present results extend these findings to the D<sub>8</sub> (month 8) antibodies of rabbits AZ1 and AZ5; larger fractions of each were precipitable by the indirect technique (Table I). The increased extent of precipitation by the indirect method may be attributable (12) to the presence of a small number of combining sites on the antigen molecules.

An observation which may be significant is the increase in the fraction of the antibody population precipitable when derived from the later, as compared to earlier bleedings (Table I). This is true whether the comparison is made of data obtained by the direct or indirect methods of precipitation. It is relevant to consider first the reason for the failure of anti-idiotypic serum to precipitate all of the D molecules. We suggested previously that the nonimmunogenic portion of the D population may comprise a large number of different idiotypes, so that the concentrations of many individual subpopulations are insufficient to elicit anti-antibodies (11). On this basis the increase in the fraction precipitable from later bleedings could be explained by a decrease in the number of idiotypic populations present, i.e., a decrease in heterogeneity of the antibody. This type of change would not necessarily be reflected by the Sips index of heterogeneity,  $a$ . For example, a small number of populations differing greatly in their  $K$  values would yield a lower value of  $a$  than a large number of populations with a narrow range of equilibrium constants.

*Changes in Idiotypic Specificities during Immunization.*—The first antisera with a sufficient concentration of antibenzoate antibodies for specific purification and subsequent use as an immunogen were taken 5–8 wk after the start of immunization, and are denoted as “month 2 antibodies” from the donor rabbit, or  $D_2$ . In each of the three rabbits investigated, idiotypic specificities present in  $D_2$  were also found in  $D_3$  and  $D_4$  of the same rabbit, as shown by the capacity of the unlabeled purified antibodies,  $D_3$  and  $D_4$ , to inhibit the binding of homologous  $^{125}\text{I-F(ab')}_2$  fragments by anti- $D_2$  antibodies.

Antibodies  $D_3$  and  $D_4$  from rabbits AZ1 or AZ11 competed about as effectively as  $D_2$  from the same rabbit for sites on the homologous anti- $D_2$  antibodies (Figs. 1, 3, and 8). Thus, the idiotypes in  $D_2$  antibodies were present in comparable concentration 2 months later. In both rabbits there was an abrupt change in idio type after the 4th month; in each case  $D_5$  failed to compete effectively with  $D_2$  of the same rabbit for sites on anti- $D_2$  antibodies.

Somewhat different results were obtained with anti- $D_2$  homologous to donor antibenzoate antibodies from rabbit AZ5. Antibodies  $D_2$  and  $D_3$  again interacted about equally well with anti- $D_2$  (Figs. 4 and 6). After month 3, however, there was a gradual shift in idiotypic specificities, which continued through month 10, in contrast to the abrupt change noted after month 4 in rabbits AZ1 and AZ11. Even the  $D_{10}$  antibodies of rabbit AZ5 interacted to a significant extent with anti- $D_2$ .

Further evidence for the observed change in idio type after month 4 was obtained with anti-idiotypic antibodies prepared against specifically purified antibenzoate antibodies isolated from the sera of rabbits AZ1 and AZ5 approximately 8 months after the start of immunization ( $D_8$ ). Results obtained with these two anti- $D_8$  antisera (Figs. 2, 3, 5, and 6) confirm the finding, made with anti- $D_2$  sera, that changes in idio type occurred during the course of immuniza-

tion.  $D_2$ ,  $D_3$ , and  $D_4$  of rabbit AZ1 did not interact appreciably with anti- $D_8$ .  $D_5$  combined to a significant extent, but not as effectively as the homologous  $D_8$  (Figs. 2 and 3). Thus, the idiotypic population present at month 8 in rabbit AZ1 was already represented in part at month 5, but not to a significant extent at months 2, 3, or 4.

A loss of idiotypic specificities during immunization has also been reported by Oudin and Michel (9). An anti-idiotypic antiserum prepared against *Salmonella typhi* antibody taken from a rabbit 34–38 days after the start of immunization failed to form precipitates in agar gel with antibody isolated at 13–17 days. However, four other anti-idiotypic antisera prepared against the same donor antibody did recognize determinants common to antibodies present in both sets of bleedings, although differences in the patterns of reactions were noted in each case.

Subsequent to the appearance of a new set of specificities in each rabbit after month 4, somewhat different results were obtained with the sera of rabbits AZ1 and AZ5. Idiotypic specificities recognized by anti- $D_8$  of rabbit AZ1 were already present, although in lower concentrations, at month 5 (Figs. 2 and 3). Subsequent to month 8, there was a gradual change in the pattern of idiotypic specificities, as shown by quantitative inhibition measurements of the reaction of  $^{125}\text{I-F(ab')}_2$  of  $D_8$  with anti- $D_8$  antibodies. Unlabeled antibenzoate antibodies from month 17, when present in large excess, caused 65% inhibition of the reaction. On a weight basis, about twice as much  $D_{17}$  as the homologous  $D_8$  was required to give 50% inhibition of the reaction. Thus, changes in idio type occurred continuously but very gradually in rabbit AZ1. These changes after month 8 did not result in a reversion to the idiotypes present early in immunization;  $D_{17}$  of rabbit AZ1 did not interact detectably with anti- $D_2$  (Fig. 3). This indicates that a third set of idiotypic specificities had been established in that rabbit.

In the case of rabbit AZ5, the changes after month 8 were more rapid. Although  $D_{10}$  antibodies still reacted effectively with anti- $D_8$ , the reactions of  $D_{13}$  and  $D_{17}$  were much weaker, although still significant (Fig. 7). Because of exhaustion of material, inhibition tests of anti- $D_2$  were not carried out with  $D_{13}$  and  $D_{17}$  of rabbit AZ5.

In rabbit AZ1, molecules reactive with anti- $D_8$  were present for at least 1 yr, from months 5 to 17. This extends our previous work (4, 10) and confirms the prolonged persistence of closely related molecules, inferred from studies of mixed recombinants of heavy and light chains of antibodies derived from different bleedings of the same rabbit (1).

Oudin and Michel (9) recently reported the presence of the same idiotypic specificities in samples of anti-salmonella antibodies taken from a rabbit with an interval of 29 months between bleedings. The rabbit was challenged with antigen shortly before each bleeding but not during the 29-month period. In



contrast, except for a 1-month rest period during month 6, rabbits AZ1 and AZ5 were inoculated on a weekly or a biweekly basis. On the assumption that a given idio type is the product of a single cell line, this would mean that during the period of persistence, antigen molecules are utilized in large part to stimulate cells of an established cell line, rather than to initiate new clones. This is true whether there is a long delay between challenges with antigen (9), or, as in our experiments, frequent inoculations.

It should be of interest to determine the conditions that favor the initiation of new clones. That new cell lines can indeed be established is indicated by the observation of continual quantitative changes in idiotypic specificities. Immunization schedules and dosages of antigen may well be important in determining these transitions.

It is relevant that the half life of a rabbit antibody molecule of the IgG class is about 6 days (5, 6), and that the lifetime of an antibody-producing cell is also of the order of a few days (7, 8). It would appear then that the presence of molecules bearing a particular idio type over a long period of time must reflect the continued production of new cells synthesizing molecules of the same structure.

The changes in idio type we observed after month 4 in each of three rabbits may be explained by a hypothesis similar to that employed by Eisen and Siskind (20) to account for the increase in combining affinity of rabbit antihapten antibodies over a period of time after immunization. They postulated that, as the concentration of immunogen in the rabbit decreases, cells with receptors of high affinity compete most effectively for the limited amount of immunogen remaining, with the result that the average combining affinity of the antibody population increases. (A corollary assumption is that the receptor is a representative of the antibody that will eventually be produced by that cell line.) Our results are analogous to theirs, if it is assumed that the antibodies of high and low affinity possess different idiotypic specificities. In this connection it is significant that the average combining affinity ( $K_0$ ) of each of our  $D_8$  antibodies was higher by a factor of five to eight than  $D_2$  antibodies of the same rabbit (Table I). The increase in the average binding constant observed here is smaller than that noted by Eisen and Siskind; however, antibenzoate antibodies do not, in general, attain binding affinities comparable in magnitude to those of the high-affinity anti-dinitrophenyl antibodies ( $10^8$  or greater) utilized in their investigations.

#### SUMMARY

Changes and persistence of idiotypic specificities of specifically purified rabbit anti-*p*-azobenzoate antibodies were studied by quantitative methods. In each rabbit idio types identified 2 months after the start of immunization were still present in comparable concentrations 2 months later. After month 4, they were

replaced by new and unrelated specificities; the changes were abrupt in two rabbits and gradual in the third, and were associated with an increase in the average affinity for specific haptens. In two surviving rabbits the new sets of specificities persisted in part for at least 1 yr. Quantitative changes occurred during this period, and the antibody preparation used as immunogen reacted most effectively with the homologous anti-D serum. The antibody population present at month 17 ( $D_{17}$ ) in one rabbit was deficient in idiotypic specificities present in  $D_8$  and lacked specificities present in  $D_2$ , indicating the presence in  $D_{17}$  of a third group of specificities. The percentage of the antibody population from each rabbit reactive with homologous anti-idiotypic serum was greater at month 8 than at month 2, suggesting a decrease in heterogeneity.

Since the donor rabbits were challenged repeatedly with antigen, it appears that, after month 8, a portion of the antigen was utilized to stimulate existing cell lines and a portion to initiate new clones.

Precipitation of anti-*p*-azobenzoate antibodies removed idiotypic specificities, indicating that they were not present on the anti-bovine  $\gamma$ -globulin antibodies in the same sera.

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