A STUDY OF RABBIT γ-GLOBULIN ALLOTYPY BY MEANS OF HETEROIMMUNIZATIONS*

By PAUL BORNSTEIN,‡ M.D., and JACQUES OUDIN, M.D.

(From the Service d'Immunochimie Analytique, Institut Pasteur, Paris, France)

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The concept of allotypy, *i.e.* that differences in antigenic specificity exist in a given protein within a species (1, 2) had its origin in the adequate interpretation of the results observed following isoimmunizations of rabbits with rabbit serum proteins. The same concept has since been extended to γ-globulin of other species by similar deliberate isoimmunizations. This example also prompted the search for isoimmunizations involuntarily realized in man after therapeutic transfusions and thus led to similar findings in human β-lipoprotein (3, 4).

Initially, in contrast to most other immunochemical investigations, heteroimmunizations were used solely to demonstrate that a common isotypic specificity (*i.e.* a specificity uniform within the animal species, reference 2) was carried by the molecules endowed with the allotypic specificities found in the rabbit (5). More recently, however, it was found that suitable heteroimmunizations elicited the formation of precipitating antibodies able to recognize allotypic specificities in the rabbit and even in man (6, 7). It seemed desirable to undertake a systematic study of the best understood kind of allotypy, that of rabbit γ-globulin, by means of heteroimmunizations (8) since this new perspective might provide useful confirmation of existing ideas and perhaps add new information. For this purpose a goat and a number of chicken antisera were employed.

Materials and Methods

Immunizations.—Rabbits, whose phenotypes had been determined as described previously (5), were immunized by repeated intravenous injections of 7 times recrystallized alum-precipitated chicken ovalbumin. Their antisera were precipitated by the addition of ovalbumin in slight antigen excess. The precipitates were allowed to stand at 0–4°C for 48 hours and then washed 10 times with cold 0.15 M NaCl. Subsequently, the precipitates were suspended in 0.15 M NaCl and emulsified with an equal volume of complete Freund's adjuvant (8.5 parts bayol, Esso Standard Oil Company, New York, 1.5 parts arlacel A and 1 mg of killed tubercle bacilli per 3 ml of bayol).

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‡ Postdoctoral Fellow of the Arthritis and Rheumatism Foundation. Present address: National Institutes of Health, Bethesda.

Male and female chickens of crossed Rhode Island and New Hampshire breeds, aged 5 to 6 months, were immunized intraperitoneally with the above preparations; the precipitate produced by the addition of 600 μg of ovalbumin to an appropriate volume of antiserum was incorporated in a volume of 3 ml and administered as the first injection. A second intraperitoneal injection containing twice this quantity of precipitate was administered 6 weeks later. Certain chickens received a third injection equivalent to 1800 μg of ovalbumin at 12 weeks. Bleedings were obtained from the wing vein at 6, 12, and 18 weeks.

Hyperimmune antiovalbumin sera from an individual rabbit were pooled (phenotype $Aa(1^+2^+3^-)b(4^+5^-6^-)$).[1] The γ-globulin was obtained by precipitation with 45 per cent saturated $(NH_4)_2SO_4$ and purified by DEAE (diethylaminoethyl) cellulose column chromatography. The specific precipitate from 0.64 mg of ovalbumin, prepared close to the equivalence ratio, was emulsified with complete Freund's adjuvant and injected intramuscularly into a goat in seven injections over a 4 month period.

All sera were filtered through Seitz filters, and stored at 4°C after the addition of 1/5000 merthiolate. Chicken sera were allowed to stand in the cold for 2 to 3 weeks before use in order to avoid non-specific precipitation (9).

Precipitin Reactions in Liquid Media.—The techniques used aimed at (*a*) detecting a precipitation at the interface (ring tests), using tubes of 2 or 3 mm in internal diameter, (*b*) determining the equivalence ratio between immune sera and non-immune sera, and (*c*) determining the amounts of nitrogen in the precipitates for the purpose of plotting curves. The ratio of rabbit serum to chicken antiserum necessary to produce antigen excess with regard to the isotypic specificity of rabbit γ-globulin was determined by precipitating a constant volume of antiserum with progressively larger quantities of a non-immune rabbit serum lacking all the allotypic specificities present in the original immunizing rabbit γ-globulin (such sera are designated below by S_{i8}). The presence of antibodies directed towards the allotypic specificities present in the immunizing material was indicated by the ability of supernatants in the zone of excess S_{i8} serum to give a precipitin reaction at the interface with rabbit sera containing these allotypic specificities individually.

The dependence of precipitating avian antibodies on salt concentration has been reported (10) although the nature of the additional protein precipitated at high salt concentrations is still under study (11). In our experiments, the quantity of precipitate obtained from chicken antisera increased in the presence of 8 per cent NaCl, but the equivalence ratios were usually unchanged from those obtained at physiological salt concentrations. Since primary interest in this portion of the work resided in a comparison of ratios, and since it appeared unlikely that chicken antibodies with different specificities differed in their behavior with respect to salt concentration, all ring tests were performed in 0.15 M NaCl.

The curves of Figs. 1 and 2 were obtained by the addition of increasing volumes of rabbit serum to a constant volume of antiserum: 0.2 ml for the chicken antisera (Fig. 2); 0.1 ml (curves I and II), and 0.15 or 0.25 (curves III and IV) for the goat antiserum (Fig. 1). Sera were incubated at 56°C for 30 minutes to inactivate complement, and NaCl was added to a final concentration of 8 per cent when chicken antisera were used. Errors due to dilution-dependent solubility of precipitates were avoided by maintaining the dilution of antisera constant (12). Precipitates were incubated at 37°C for 1 hour and then placed at 0–4°C for 72 hours with daily agitation. They were washed twice with 2-ml volumes of cold 0.15 M NaCl (1.5 M for precipitates from chicken antisera), air-dried, and dissolved in a known quantity of 0.1 N NaOH.

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[1] It may be useful to recall that the allotypic specificities controlled by alleles of locus *a* are designated by Aa1, Aa2, Aa3, and those controlled by alleles of locus *b* by Ab4, Ab5, Ab6 (2). The presence or absence of a given specificity in a serum or on a γ-globulin molecule is indicated by + or − in superscript.

Protein Determinations.—Protein content was measured with the use of the Folin-Ciocalteau reagent as described by Lowry *et al.* (13). Optical densities were read at 500 mμ in a Jobin-Yvon spectrophotometer. A solution of bovine γ-globulin purified by DEAE column chromatography was used as a standard. The nitrogen content of this solution was determined by the Conway modification of the microKjeldahl method (14).

Precipitation in Gel Tubes (Simple Diffusion).—Whenever chicken antisera were used, sufficient NaCl was added to both antigen and antibody layers to obtain a final concentration of 8 per cent. Dilutions of the antigen, when necessary, were performed with non-immune chicken or goat serum to maintain a constant protein content and thus avoid errors due to non-specific effects (15, 16). The tubes were allowed to stand in a constant temperature room at 20°C for 7 days and then photographed with a magnification of 2. Measurements were made on the photographic plates. Reproducible results could be obtained within the limits of the errors of measurement, 0.2 to 0.3 mm (the magnification of 2 taken into account).

When necessary, the relative content of γ-globulin in rabbit sera was determined by comparing the penetration, *h*, of the precipitation zones (*i.e.* the distance between the interface and their leading edge) in the reaction of these sera with a goat anti-human γ-globulin serum. This antiserum cross-reacted extensively with rabbit γ-globulin but did not distinguish between rabbit allotypes.

RESULTS

Reactions in Liquid Media.—

The recognition of the allotypic specificities by chicken antisera: Immunizing material furnished by six rabbits and including the six allotypic specificities Aa1 through Ab6, was used to inject 16 chickens. The chicken antisera so obtained were examined by precipitin reactions in liquid media after absorption with appropriate rabbit sera. The results are summarized in Table I.

Equal volumes of each of the chicken antisera were mixed with increasing volumes of a rabbit serum (designated above as S_{is}) which contained none of the allotypic specificities present in the immunizing preparation. Only antibodies directed towards the isotypic specificity of rabbit globulin are expected to be absorbed by this procedure. Following centrifugation of these mixtures, ring tests were performed with the supernatants and the absorbing rabbit serum S_{is}.

In column 5 of Table I are found the limiting ratios of S_{is} sera to chicken antisera necessary to inhibit further precipitation of the supernatants with S_{is} sera. These ratios are strictly comparable only amongst chicken antisera absorbed with the same S_{is} serum. This condition was always met for successive bleedings of the same chicken. Considerable variation in antibody titers can be seen from one animal to another and among bleedings of the same animal.

Those supernatants which did not precipitate with the rabbit serum used in the absorption were examined by means of ring tests with other rabbit sera, each of which contained one of the allotypic specificities present in the immunizing rabbit γ-globulin. The results of these reactions are listed in columns 6 to 11 of Table I. The symbol Ax will be used to indicate a given allotypic specificity found in the immunizing material. In a large number of cases, an excess of the absorbing rabbit serum was capable, even though Ax^-, of in-

TABLE I

Reactions of Antiallotype Chicken Sera with Rabbit Sera

1	2	3	4	5	6	7	8	9	10	11
Allotypic formula of the antiovalbumin serum used in the immunizing material	Allotypic formula of absorbing rabbit serum	Chicken No. and sex	Duration of immunization	Titer of antiisotypic antibodies*	Ratio of absorbing serum to chicken antiserum necessary to inhibit precipitation with sera carrying the specificities below‡					
					Locus *a*			Locus *b*		
					Aa1	Aa2	Aa3	Ab4	Ab5	Ab6
			wks.							
Aa(2,3)b(4,5)	Aa(1)b(6)	1-26 ♀	6	1/200–1/100		1	1	1	1	
			12	1/100–1/80		—	1	6–10	⩽2	
		1-38 ♂	6	1/40–1/20		1	1	1	1	
			12	1/20–1/10		1	⩽2	⩾20	10–20	
Aa(1,2)b(4,6)	Aa(3)b(5)	1-30 ♀	6	1/200	1	1		1		1
			12	1/40–1/20	1	1		⩽2		1
			18	1/60–1/50	⩽2	—		⩾10		3–5
		1-32 ♀	6	1/100–1/80	⩽2	—		3–5		⩽2
			12	1/10–1/5	1	—		⩾20		⩾20
			18	1/10–1/5	⩽2	—		⩾20		⩾20
		1-40 ♂	6	1/40–1/30	1	1		1		1
			12	1/60–1/50	1	1		1		1
Aa(1,3)b(5)	Aa(2)b(4)	1-36 ♀	6	1/80–1/60	1		1		1	
			12	1/30–1/20	1		1		3–5	
			18	1/50–1/40	1		1		⩾6	
		1-42 ♂	6	1/60–1 50	1		1		1	
			12	1/5–1/1	1		1		⩾10	
			18	1/5–1/1	⩽2		1		⩾20	
Aa(3)b(5)	Aa(2)b(4)	1-74 ♀	6	1/150–1/100			⩽2		⩾6	
			12	1/60			⩽2		⩾20	
		1-50 ♀	6	1/100			1		3–5	
			12	1/1–2/1			⩽2		3–5	
			18	1/1			⩽2		⩾20	
		1-44 ♀	6	1/200			1		1	
			12	1/100			1		1	
			18	1/80–1/60			⩽2		⩾20	

* Limiting ratios of absorbing rabbit serum to chicken antiserum necessary for antigen excess.

‡ The numerals indicate the ratios of absorbing serum to chicken antiserum expressed as multiples of the titers noted in column 5. —, suitable rabbit serum was not available.

TABLE I—*Concluded*

1	2	3	4	5	6	7	8	9	10	11
Allotypic formula of the antiovalbumin serum used in the immunizing material	Allotypic formula of absorbing rabbit serum	Chicken No. and sex	Duration of immunization	Titer of antiisotypic antibodies*	Ratio of absorbing serum to chicken antiserum necessary to inhibit precipitation with sera carrying the specificities below‡					
					Locus *a*			Locus *b*		
					Aa1	Aa2	Aa3	Ab4	Ab5	Ab6
			wks.							
Aa(2,3)b(4)	Aa(1)b(6)	1-52 ♀	6	1/40–1/30		1	1	⩾20		
			12	1/1–2/1		⩽2	1	⩾6		
			18	1/5–1/1		1	1	⩾20		
		1-54 ♀	6	1/5–1/1		1	1	⩾3		
			12	1/5–1/1		1	1	⩾20		
		1-58 ♂	6	1/10–1/5		1	1	⩾20		
			12	2/1–3/1		1	1	⩾6		
			18	1/1–2/1		1	1	⩾6		
Aa(2)b(4)	Aa(3)b(5)	1-56 ♀	6	1/20–1/10		1		⩾6		
			12	3/1–5/1		1		⩾3		
		1-78 ♀	6	1/20–1/10		3–5		10–20		
			12	1/10–1/5		10–20		⩾20		
			18	1/2–1/1		6–10		⩾6		
		1-60 ♂	6	1/100–1/80		1		⩾20		
			12	1/5–1/1		1		⩾20		
			18	1/5–1/1		1		⩾20		

hibiting the precipitin reaction of the resulting supernatant with an Ax^+ serum. The numbers in columns 6 to 11 of Table I, indicate ratios between that quantity of Ax^- serum necessary to inhibit this precipitin reaction and that quantity of Ax^- serum necessary to inhibit a precipitin reaction of the supernatant with the same Ax^- serum. The limits between which the latter value falls appear in column 5, Table I. In both instances the actual values used to calculate the reported ratios (of columns 6 to 11) are the means of experimentally determined limits. These ratios vary from 1 to more than 20. A ratio of 1 indicates that the antiserum fails to "recognize" the specificity. A ratio of 20 or greater indicates that a large excess of an Ax^- serum fails to inhibit the precipitation with an Ax^+ serum.

A comparison of the results obtained with successive bleedings of the same animal reveals that the titer of antiisotypic antibodies frequently increases

considerably during the course of immunization (column 5, Table I). In addition, the sera of early bleedings (6 weeks) often recognize no allotypic specificity, whereas certain of these specificities are almost always recognized by later bleedings (12 to 18 weeks after the initiation of immunization).

It appears that systematic differences exist between the abilities of different allotypic specificities to be recognized by chicken antisera. Comparison of figures of columns 6 to 8 with those of columns 9 to 11 show that the specificities controlled by the genetic locus *a* are clearly less readily recognized than those controlled by the genetic locus *b*. A number of antisera were available whose reactions with $Ab4^+$, $Ab5^+$, or $Ab6^+$ serum could not be inhibited by a serum lacking this specificity. Such was almost always the case for later bleedings. On the contrary, the ratios listed in columns 6 to 8 of Table I, indicate that, in every antiserum tested, a precipitin reaction involving a specificity

TABLE II

Reactions of Chicken Serum 1-50 anti-Aa(3)b(5), Absorbed by an Aa(3^-)b(5^-) Serum, with Sera of Various Formulas

Reactions of the supernatants with rabbit sera of formula	Proportions of serum Aa(2)b(4) to chicken antiserum						
	1/1	2/1	3/1	4/1	6/1	10/1	15/1
Aa(2)b(4) = Aa(3^-)b(5^-)	+	0					
Aa(1,3)b(6) = Aa(3^+)b(5^-)	+	+	+	0			
Aa(2)b(4,5) = Aa(3^-)b(5^+)	+	+	+	+	+	0	
Aa(3)b(5) = Aa(3^+)b(5^+)	+	+	+	+	+	+	+

controlled by the *a* locus could be inhibited by a serum lacking this specificity. No significant differences were noted in the behavior of antibodies towards specificities controlled by the same genetic locus although chicken 1-78 recognized the Aa2 specificity to an exceptional extent. In this connection, it should be mentioned that the antiserum of another chicken, immunized for a different purpose with a specific precipitate in which the antigen was a pneumococcal polysaccharide, was also capable of recognizing the Aa2 specificity.

The particular case of certain reactions in which mixed allotypes are involved: A particular feature of inhibition of reactions is related to the presence of two allotypic patterns on the same molecule (a pattern of the *a* series and one of the *b* series, references 17 and 18). The example of the second bleeding (after 12 weeks) of chicken 1-50, immunized by Aa(3)b(5) material is summarized in Table II. It may be seen there, as in Table I, that the amount of serum S_{is} Aa(3^-)b(5^-) needed for the inhibition of the reactions of the supernatants with various rabbit sera varies according to the presence or absence of the Aa3 or Ab5 specificity in these sera. In addition, the reaction of the supernatant with

a serum containing both Aa3 and Ab5 specificities was not inhibited by an amount of serum Aa(3⁻)b(5⁻) much larger than that sufficient to inhibit the reaction of the supernatants with a serum containing either specificity alone.

Precipitin curves: Reactions of an anti-Ab4 goat serum and an anti-Ab4 chicken serum with an Ab4 homozygous and an Ab4⁻ rabbit serum were studied by means of nitrogen determinations in the precipitates. Although the immunizing materials possessed the Aa2 and Aa1 (or Aa3) specificities, these specificities were not recognized by the antisera. The results obtained with the goat antiserum are illustrated in Fig. 1. One notes that the maximum quantity of nitrogen precipitated by an Ab(4⁺5⁻6⁻) serum was 3.5 mg per ml of immune

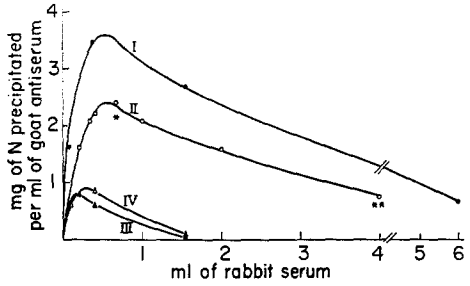

FIG. 1. Curves of nitrogen in the precipitates obtained with the antiserum of a goat immunized with a specific precipitate of rabbit antiovalbumin γ-globulin of allotypic formula Aa(1,2)b(4). Curve I: Precipitation with an Ab4⁺ rabbit serum. Curve II: Precipitation with an Ab4⁻ rabbit serum. Curve III: Supernatant of a precipitate at point * on curve II; precipitation with Ab4⁺ rabbit serum. Curve IV: Supernatant of a precipitate at point ** on curve II; precipitation with Ab4⁺ rabbit serum.

The γ-globulin contents of the two rabbit sera were compared by simple diffusion in gel tubes and adjustments were made in plotting so that equal volumes of serum denote equal quantities of γ-globulin. (See Materials and Methods.)

serum (curve I), whereas the maximum precipitate obtained with an Ab4⁻ serum was ⅔ this value (curve II). When the antiserum was absorbed at equivalence by the Ab4⁻ serum and subsequently precipitated with the Ab4⁺ serum (curve III), the maximum quantity of nitrogen so obtained approximated the difference between the two preceding values, indicating that the great majority of antibody molecules not precipitated by the Ab4⁻ serum was susceptible to precipitation by the Ab4⁺ serum. A comparison of curves III and IV reveals that a large excess of Ab4⁻ serum (6 times the equivalence volume) failed to inhibit the subsequent precipitation of these antibodies with Ab4⁺ serum. In this regard the goat antiserum resembles those anti-Ab4 chicken sera obtained late in the course of immunization.

The nitrogen contents of precipitates obtained with two anti-Ab4 sera from the same chicken, No. 1-54, are graphed in Fig. 2. Even the first bleeding ob-

tained 6 weeks following the commencement of immunization clearly produced more precipitate with an Ab4$^+$ rabbit serum (curve I) than with an Ab4$^-$ serum (curve II). A comparison of curves I and II with curves III and IV further demonstrates that both the total precipitate resulting from reaction with an Ab4$^+$ serum and the quantity of precipitate with an Ab4$^+$ relative to that with an Ab4$^-$ serum increase from the 6th to 12th week of immunization. If one assumes that the combining ratio at equivalence is the same in curves III and IV, it would appear that the anti-Ab4 antibodies in the later antiserum are more abundant than those directed towards the isotypic specificity.

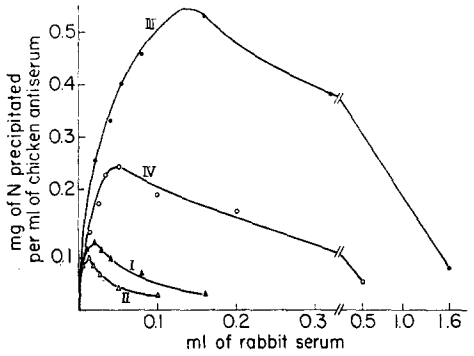

FIG. 2. Curves of nitrogen in the precipitates obtained with two antisera of chicken 1-54 immunized with a specific precipitate of an antiovalbumin rabbit serum of allotypic formula Aa(2,3)b(4). Curve I: Bleeding after 6 weeks of immunization; precipitation with an Ab4$^+$ rabbit serum. Curve II: Same bleeding; precipitation with an Ab4$^-$ rabbit serum. Curve III: Bleeding after 12 weeks of immunization; precipitation with an Ab4$^+$ rabbit serum. Curve IV: Same bleeding; precipitation with an Ab4$^-$ rabbit serum.

Reactions in Gels.—Two immune sera, an anti-Ab4 goat serum and an anti-Ab5 chicken serum, were similarly employed in precipitin reactions in gels.

Reactions of the goat anti-Ab4 serum: The goat antiserum had been shown to contain a large proportion of antibodies precipitable only by Ab4$^+$ sera (Fig. 1). This antiserum produced a single precipitation zone in gel tubes (simple diffusion) with Ab4$^-$ sera, whereas 2 zones were clearly visible with Ab4$^+$ heterozygous rabbit sera. The reaction of an antiserum which recognizes a given allotypic specificity, *e.g.* Ab4, with a rabbit serum which is heterozygous for this specificity is analogous to that of the complex precipitating systems described previously (19, 20) in which an anti-hen ovalbumin serum reacts with mixtures of hen and duck ovalbumins. In both instances, a fraction of the antibodies is able to precipitate all the antigens in the system. In the γ-globulin system, this fraction comprises the antibodies directed towards the isotypic specificity of rabbit γ-globulin (possessed by both Ab4$^+$ and Ab4$^-$ molecules)

and corresponds to those antibodies precipitable by duck ovalbumin. A second antibody fraction is capable of precipitating only part of the antigen molecules. The latter fraction is directed towards the Ab4 specificity and correspondingly, in the ovalbumin system, recognizes that antigenic specificity which characterizes hen ovalbumin. In these systems, $Ab4^+$ molecules and hen ovalbumin constitute homologous antigens since they are able to precipitate all the antibodies in their respective antisera. On the contrary, $Ab4^-$ molecules and duck ovalbumin are heterologous antigens. In the work that follows, $Ab4^-$ sera will be used as solutions of heterologous antigen. Ab4 homozygous sera might be used as solutions of homologous antigen deprived of the heterologous one if it might be assumed that, in such sera, all the molecules which carry the isotypic specificity of the γ-globulin also carry the Ab4 specificity. But it will be shown that this condition is never met completely.

When a complex system produces two precipitation zones, the first zone (that furthest from the interface) is necessarily due to the heterologous antigen (19), in this instance the $Ab4^-$ molecules, and the second zone is due to the homologous antigen, the $Ab4^+$ molecules. Confirmatory evidence exists in the observation that partial absorption of the antiserum by an $Ab4^-$ rabbit serum results in a reduction in the density of the first zone and an increase in its penetration. When absorption is complete, the first zone disappears. Partially absorbed antisera were used in the procedures to be described since an increase in distance between the two zones was technically desirable.

As a result of a quantitative study of a complex precipitating system by means of simple diffusion in one dimension, it could be shown that the distance, d, between the two zones is a function of the relative concentrations of the two antigens; if their ratio is maintained constant, variation of absolute values, within certain limits, has little influence on d (20). In order to be certain that variations in the total concentration of γ-globulin among rabbit sera did not significantly influence the value of d, varying proportions of an Ab4 homozygous and an $Ab4^-$ serum were mixed (from 20 to 90 per cent of $Ab4^+$ serum in the mixtures). These mixtures, undiluted and diluted 1/2 and 1/4 in non-immune chicken serum, were reacted with the goat antiserum in gels under the same conditions used to measure d. It was seen that, when the penetrations of the resulting zones varied within the same limits as those of the sera to be studied, variations of d for a constant C_4/C_T did not significantly exceed the experimental errors of measurements. (C_4 symbolizes the concentration of Ab4 molecules and C_T the total concentration of molecules precipitable by the antiserum.)

The sera of 58 rabbits were studied and the distance d correlated with the phenotypic expression of the b locus (Table III). The results lend themselves to three initial observations: (*a*) Two precipitation zones were observed in reactions with all $Ab4^+$ sera including those of phenotype $Ab(4^+5^-6^-)$. The distance, d, in the latter sera was often of the order of 1 mm or more. This finding indicates that the sera of $Ab(4^+5^-6^-)$ rabbits contain an appreciable proportion of $Ab4^-$ molecules even though it is logical to consider these rabbits as homozygous for the A_b^4 allele. (*b*) d is distinctly greater among rabbits heterozygous

at the *b* locus indicating, as might be expected, that in these sera $Ab4^-$ molecules are present in higher relative concentration. (*c*) The precise phenotype influences *d* since this value was significantly greater in Ab(4,6) heterozygotes than in Ab(4,5) heterozygotes. Representative reactions in gels are illustrated in Fig. 3.

We endeavored to derive quantitative information regarding the relative

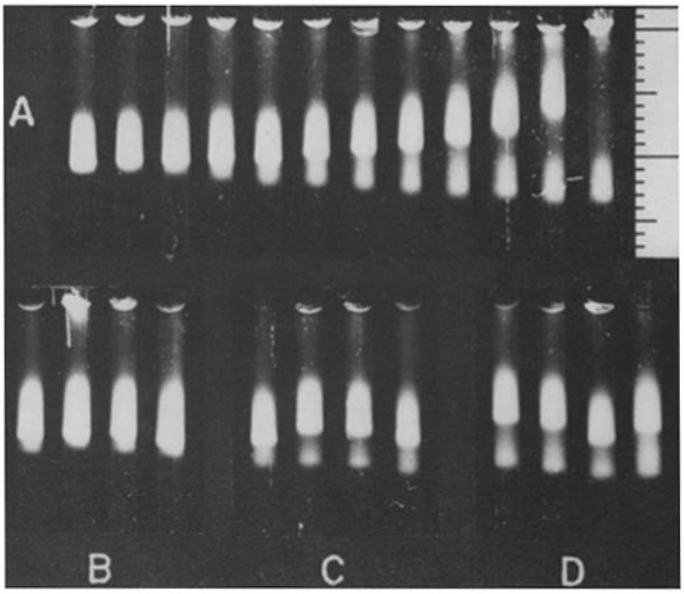

FIG. 3. *A*, Gel tubes used in establishing calibration curve for $Ab4^+$ sera. Reaction of partially absorbed goat antiserum with mixtures of $Ab4^+$ and $Ab4^-$ rabbit sera in the proportions from left to right: 100/0, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 0/100. *B*, Example of 4 tubes in which the upper layers are made of 4 sera of rabbits supposedly Ab4 homozygotes. *C*, The upper layers are sera of 4 heterozygous Ab(4,5) rabbits. *D*, The upper layers are sera of 4 heterozygous Ab(4,6) rabbits.

concentrations of the different allotypic molecular species from the observed values of *d*. Towards this end a reference curve was established by plotting values of *d* as a function of the proportion of an $Ab(4^+5^-6^-)$ serum in mixtures with an $Ab4^-$ serum. Several corrections were necessary in order to permit the desired quantitative information to be drawn from the use of this curve.

1. An adjustment was required to take into account the fact that the total concentration of γ-globulin in the two sera used to construct the reference curve was not the same. This was achieved by the use of an anti-human γ-globulin goat serum which cross-reacted strongly with rabbit sera but reacted indiscriminately with respect to their allotypic specificities. Neighboring reactions of this antiserum and the anti-Ab4 goat serum with rabbit sera in a cell with

parallel walls (double diffusion) resulted in continuous precipitation zones. The rabbit sera used as standards with the anti-Ab4 goat serum and the anti-Ab5 chicken serum to construct reference curves (see below) were reacted in gel tubes with this anti-human γ-globulin serum. The rabbit serum producing the largest penetration was subjected to five twofold dilutions and the penetrations, h, in the reaction of these dilutions in gel tubes containing the goat antiserum, served to create a plot of h against the logarithm of the reciprocal of the dilution. The relative concentrations of γ-globulin in the other rabbit sera could then be obtained from this plot (an almost perfect straight line) by comparing their penetrations, h. The dotted curve in Fig. 4 represents values of d plotted as a function of C_4/C_T after this initial correction.

2. The Ab($4^+5^-6^-$) serum chosen to construct the reference curve produced, in gel tubes with anti-Ab4 goat serum, one of the smallest values of d encountered in testing homozygous rabbits. Nevertheless the finding of two zones demonstrated the presence of a probably small,

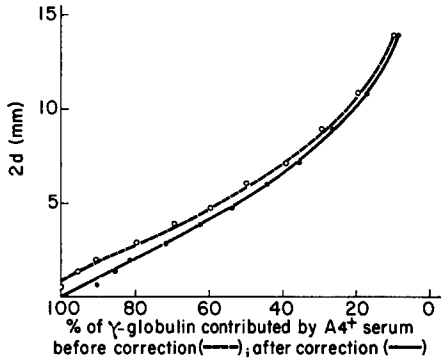

FIG. 4. Curves used for the estimation of the proportions of $Ab4^+$ and $Ab4^-$ molecules in $Ab4^+$ rabbit sera. $2d$ represents twice the distance between the leading edges of the two precipitation zones in gel tubes. Dashed curve was obtained with varying proportions of an $Ab4^+$ homozygous and an $Ab4^-$ rabbit sera (corrected for difference in γ-globulin content). The abscissae of the solid curve were further corrected, as stated in the text, on the assumption that 10 per cent of the γ-globulins in the $Ab4^+$ serum behaved as those of the $Ab4^-$ serum.

but unknown, percentage of $Ab4^-$ molecules. If one assumes that, in this serum, 10 per cent of the molecules reacting with the goat antiserum were $Ab4^-$ and therefore in establishing the solid curve in Fig. 4, one replaces the ratio $\frac{\text{volume of Ab4}^+\text{ serum}}{\text{total rabbit serum}}$ (already corrected as in the preceding paragraph) by $\frac{0.9\text{ (volume of Ab4}^+\text{ serum)}}{\text{total rabbit serum}}$, the resulting extrapolated curve passes through the origin as though the reaction at this point produced only a single precipitation zone. The reference curve used was the latter. It should be pointed out that the second correction is theoretically imperfect since a study of the complex system involving chicken and duck ovalbumin has demonstrated that mixtures of the two ovalbumins containing a small proportion of heterologous antigen will, below a certain limit of the latter, produce a single precipitation zone. Hence the solid curve in Fig. 4 may represent a somewhat higher percentage of $Ab4^+$ molecules, plotted as a function of d, than in reality exists. The resulting error, if any, is probably slight.

Reactions of the chicken anti-Ab5 serum: The sera of 28 Ab5$^+$ rabbits were studied using an antiserum from chicken 1-42. A part of the antibodies in this antiserum were precipitable only by Ab5$^+$ molecules. After partial absorption by an Ab5$^-$ serum in order to increase, in gel tubes, the distance between the precipitation zones attributable to Ab5$^+$ and Ab5$^-$ molecules, results analogous to those with the anti-Ab4 goat serum were obtained:

1. All Ab5$^+$ sera, including those from Ab(4$^-$5$^+$6$^-$) rabbits produced two precipitation zones in gel tubes indicating that even in supposedly homozygous animals an appreciable proportion of molecules lacked an allotypic specificity controlled by the *b* locus.

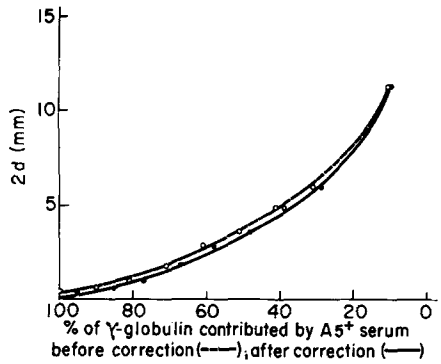

FIG. 5. Curves used for the estimation of the proportions of Ab5$^+$ and Ab5$^-$ molecules in Ab5$^+$ rabbit sera. 2*d* represents twice the distance between the leading edges of the two precipitation zones in gel tubes. Dashed curve was obtained with varying proportions of an Ab5$^+$ homozygous and an Ab5$^-$ rabbit sera (corrected for difference in γ-globulin content). The abscissae of the solid curve were further corrected as stated in the text, on the assumption that 5 per cent of the γ-globulins in the Ab5$^+$ serum behaved as those of the Ab5$^-$ serum.

2. As expected, the distance, *d*, was clearly greater among heterozygous than among homozygous animals.

3. Sera from Ab(4,5) animals gave rise to a greater value of *d* than did sera from Ab(5,6) animals.

A reference curve (Fig. 5) was established under the same conditions as that of Fig. 4 and subjected to similar corrections: (*a*) the ratio of total γ-globulin concentration of the two rabbit sera used to establish the reference curve was determined at the same time as that for the standard sera of Fig. 4 and taken into account in plotting the dotted curve of Fig. 5. (*b*) When the assumption was made that, in the Ab5$^+$ serum used, 5 per cent of globulin molecules defined by their isotypic specificity behaved antigenically as those of the Ab5$^-$ serum, the resulting plot (solid curve, Fig. 5) closely approached the origin. As stated above for the Ab4$^+$ molecules, the actual values of the ratios of the

$Ab5^+$ molecules to the total γ-globulin are presumed slightly smaller than or equal to those evaluated from the curve.

Theoretically, the previously observed cross-reaction between anti-Ab5 and anti-Ab6 rabbit sera (5), were it sufficiently intense, might have introduced a cause of error by increasing the apparent concentration of $Ab5^+$ molecules in Ab(5,6) heterozygous sera. Such a weak cross-reaction was noted with the

TABLE III

Measurements of d and Derived Relative Concentrations of Molecules Carrying Ab4 and Ab5

	Assayed with goat serum anti-Ab4			Assayed with chicken serum anti-Ab5		
Genotype	$A_b^4A_b^4$	$A_b^4A_b^5$	$A_b^4A_b^6$	$A_b^5A_b^5$	$A_b^5A_b^4$	$A_b^5A_b^6$
No. of rabbits examined	25	18	15	10	14	4
Range of 2d, *mm**	0.4–3.0	3.8–5.5	5.2–7.4	0.2–2.0	5.0–6.3	2.4–3.1
2*d* (mean) ± SD*	1.7 ± 0.69	5.1 ± 0.42	6.5 ± 0.69	0.7 ± 0.53	5.6 ± 0.38	2.8 ± 0.29
Range $\frac{C_4}{C_T} \times 100$‡, *per cent*	70–95	48–62	35-50			
$\frac{C_4}{C_T} \times 100$ (mean) ± SD	83 ± 7 per cent	52 ± 4 per cent§	40 ± 5 per cent			
Range $\frac{C_5}{C_T} \times 100$‡, *per cent*				65–95	28–36	52–60
$\frac{C_5}{C_T} \times 100$ (mean) ± SD				84 ± 9 per cent	32 ± 3 per cent	55 ± 3 per cent
$\frac{C_T-(C_4+C_5)}{C_T} \times 100$, *per cent*	17			16		

* 2*d* is the distance, measured on photographic plates, between the leading edges of the precipitation zones; *i.e.*, twice the actual distance in gel tubes. SD, standard deviation.

‡ $\frac{C_4}{C_T}$, $\frac{C_5}{C_T}$ are the ratios of concentrations of molecules carrying A4 and A5 specificities to the total concentration of molecules with which the antiserum gives a precipitin reaction.

§ The mean value of $\frac{C_4}{C_T} \times 100$ for the 14 sera assayed with the chicken antiserum was 53 ± 4 per cent.

immune serum of another chicken, No. 1-32, but not with the chicken serum actually used.

The results of reactions of the anti-Ab4 goat serum compared with those of the anti-Ab5 chicken serum: The values of *d*, and the numerical results derived therefrom by means of the curves in Figs. 4 and 5, are summarized in Table III. It can be seen that both mean values and extreme limits of *d* varied significantly in accordance with the phenotypes of the sera under study. A slight overlap between two phenotypes was noted in only one instance, $Ab(4^+5^+6^-)$ and $Ab(4^+5^-6^+)$. Thus it appears that these reactions in gel tubes enable the determination of the phenotypic expression of the *b* locus by quantitative means with a high probability of accuracy. In addition, new confirmation is

provided for the rather well established allelism of the genes controlling the Ab4, Ab5, and Ab6 specificities.

The data in Table III indicate that the molecules carrying each of the two specificities found in heterozygous sera must be present in widely different concentrations in the same serum. It would be unlikely that the difference in C_5/C_T between, for example, Ab(4,5) and Ab(5,6) sera can be attributed solely to different concentrations of molecules lacking all specificities controlled by the *b* locus (b^- molecules). On the contrary, b^- molecules have been found, on the average, in similar proportions in the sera of several phenotypes. Experimental data, part of which is summarized in Table III, permit the estimation of the percentage of b^- molecules in Ab($4^+5^+6^-$) sera. Fourteen of these sera were studied both with the anti-Ab4 goat serum and the anti-Ab5 chicken serum. The mean value of C_4/C_T for these 14 sera was 0.53 ± 0.04 (as compared to the value of 0.52 ± 0.04 for the 18 sera from which they were selected), whereas the mean value for C_5/C_T was 0.32 ± 0.03. The sum of these two ratios is 0.85 indicating that 15 per cent of the γ-globulin molecules in Ab($4^+5^+6^-$) sera carry neither of these specificities. Despite the corrections and criticisms stated above for the derivation of these figures, the similarity in the percentage of b^- molecules in Ab($4^+5^+6^-$) sera (15 per cent) with that in Ab($4^+5^-6^-$) sera (17 per cent) and in Ab($4^-5^+6^-$) sera (16 per cent) can hardly be considered a mere coincidence.

DISCUSSION

The antisera usually employed in the study of allotypy of rabbit γ-globulin have been rabbit antisera. Such antisera naturally lack antibodies against those antigenic specificities which they themselves possess and are therefore incapable of recognizing molecules which do not carry at least a common allotypic specificity or cross-reacting specificities. Chickens or goats, on the contrary, elaborate antibodies which are able to recognize the isotypic as well as the allotypic specificities of rabbit γ-globulins. One is therefore justified in considering the reaction of γ-globulins in rabbit serum Ax^- (which lacks a given allotypic specificity Ax) with a chicken or goat anti-Ax serum as a cross-reaction, analogous to the cross-reaction of rabbit anti-chicken ovalbumin serum with duck ovalbumin. One difference between these two sets of cross-reactions lies in the fact that, if Ax is a specificity of the *b* series, the Ax^+ serum which acts as the solution of homologous antigen apparently contains, even in homozygous animals, a small quantity of heterologous antigen; *i.e.*, Ax^- molecules with the same isotypic specificity. The same is probably true if Ax is a specificity of the *a* series. In addition, it is known that the proportion, in whole serum, of the molecules which differ in their allotypic specificities does not always reflect the proportion of the same molecules in a given antibody preparation (21, 22).

It is therefore possible that the proportion of b^- molecules (*i.e.* heterologous antigen) differs in the immunizing material and in the serum from which this material was derived.

The Inhibition of Precipitation Reactions and Its Relation to the Duration of Immunization.—Reactions in liquid media have demonstrated that antibodies against an allotypic specificity are variably inhibited by a rabbit serum lacking this specificity. Almost every intermediate from those reactions which are incapable of being inhibited by even a large excess of heterologous antigen to those which are inhibited by a very small excess of this antigen can be found among the reactions listed in Table I. The latter reactions do not distinguish the homologous antigen (which carries a given allotypic specificity) from the heterologous antigen (which lacks this allotypic specificity but carries the identical isotypic specificity). The former are examples of classical cross-reactions.

It was noticed many years ago by several authors, among them Hooker and Boyd (23), that antibodies became more cross-reactive as the course of immunization progressed. The antisera obtained by Hooker and Boyd from rabbits after the injection of either chicken or duck ovalbumin were at first very specific and did not react with the heterologous ovalbumin. Subsequent bleedings reacted with both ovalbumins. In another work by the same authors (24) a similar conclusion resulted from the finding that a given hapten, related to the immunizing antigen, became more and more capable of inhibiting the reaction of this antigen with its antiserum as immunization was prolonged.

The above conclusions do not seem to apply to the behavior of cross-reactions studied in the present work since (*a*) the reaction, for example, of a chicken anti-Ab4 serum was more often inhibited by an $Ab4^-$ serum when the antiserum was obtained from an early rather than from a late bleeding. This indicates that those antibodies directed against the homologous antigen were able to combine with the heterologous antigen more readily in the early than in the late bleedings. (*b*) The proportion of antibody precipitable by an $Ab4^-$ (heterologous) serum decreases as immunization progresses (Fig. 2) although its absolute quantity may increase.

Our results are more nearly in agreement with those of Landsteiner and Van der Scheer (25). These authors, in studying inhibition by heterologous antigen in cross-reactions involving ovalbumins, pointed out that such inhibition was encountered under certain conditions, namely "when the precipitin reactions were not too strong and the ratio of antibody to antigen not high." Thus it may be presumed that later bleedings, in which the antibody content is generally greater, were less susceptible to inhibition. It should be noted that the techniques employed in the present work, in the performance of immunizations and in the study of antibodies, differed from those used by the authors cited.

The Apparent Difference in Immunogenicity between Allotypic Specificities Controlled by the a and b Loci.—The behavior of antiallotype antibodies varies with the specificities against which they are directed. Inhibition by a heterologous antigen has always been possible when a specificity controlled by the *a* locus was concerned, but frequently impossible, especially later in the course of immunization, when *b* locus specificities were involved. This difference might be due to a greater similarity of the allotypic patterns controlled by the *a* locus to either (*a*) antigenic determinants present in the immunized animals, (*b*) the rest of the γ-globulin molecule, or (*c*) among themselves, than is true for those patterns controlled by the *b* locus. On the other hand one might consider that the specificities controlled by the *b* locus are intrinsically more immunogenic than those controlled by the *a* locus. The apparent disparity in immunogenicity, which is clearly independent of the physical properties of γ-globulin molecules as a whole, could result from causes of a quantitative as well as a qualitative nature.

The presence of molecules carrying only one allotypic specificity has been observed previously (5, 17). The present work, while confirming the general existence of b^- molecules in rabbit sera, provides data regarding their proportions. We do not possess similar information with respect to a^- molecules. However, Dray, Young, and Nisonoff (26) have recently reported the percentage of a^- molecules in the serum of one rabbit, determined by means of I^{131} labeling, to be 34 per cent. If, in the absence of more extensive information, this figure is taken as representing the general order of magnitude of the proportion of a^- molecules, the difference in concentration of a^+ and b^+ molecules would not appear sufficient to explain a marked difference in their immunogenicity. One would then be inclined to incriminate a qualitative difference in the nature of the allotypic specificities controlled by the two genetic loci. In a previous work (18) one of the present authors was able to distinguish the participation, in a single precipitation reaction, of two different specific groups both comprising a part of the same allotypic pattern.[2] One is therefore led to inquire whether the differences in structure thought to be responsible for the differing immunogenicity of the patterns controlled by the two loci might be related to the number of their specific groups.

The Proportion of Different Types of Molecules as Judged by Reactions in Gels.—The absence of overlapping in the values of *d* for different phenotypes, with the single exception to a minor degree in the case of phenotypes Ab(4,5) and Ab(4,6), demonstrates the extent to which these differences are significant and permits the prediction, with a high degree of accuracy, of whether a given serum is derived from an $A^4_bA^4_b$, $A^4_bA^5_b$, or $A^4_bA^6_b$ rabbit. Nevertheless there exists an appreciable spread in values of *d* within the same phenotype. Among homo-

[2] This could be demonstrated by the observation that two anti-Aa1 sera, both in themselves non-precipitating, became precipitating with respect to all $Aa1^+$ sera tested when mixed.

zygotes this is accounted for by the variability in the proportion of b^- molecules to b^+ molecules; in heterozygotes an additional cause of variation in the value of d may well lie in the difference in relative concentrations of molecules each carrying one of the two allelic specificities.

Our results indicate a significant disproportion among heterozygotes in the ratio of concentrations of molecules carrying the two allelic specificities. The ratio of the concentration of Ab4$^+$ molecules in a heterozygous $A^4_bA^5_b$ serum to that in an Ab4$^+$ homozygous serum (63/100), found by Leskowitz by entirely different techniques (27), is in remarkably good agreement with a similar ratio calculated by means of the data in Table II (62.7/100). Similarly, the value of 36/100 for the proportion of Ab5$^+$ molecules in a heterozygous $A^4_bA^5_b$ serum to that in a homozygous Ab5$^+$ serum, found by Leskowitz, is close to the ratio of 38/100 derived from Table II. Such agreement is undoubtedly in part haphazard in view of the considerable spread in values of individual sera used to calculate the averages listed in Table II. The agreement with the results of Dray and Nisonoff (28), obtained from the sera of two $A^4_bA^5_b$ heterozygous rabbits by means of I^{131} labeling, is less perfect. Nevertheless the values derived by the latter authors fall between the limits of the percentages which may be calculated from the data in lines 5 and 7 of Table III, provided the assumption is made that the percentage of b^- molecules is the same in heterozygous and homozygous sera. This assumption appears justified since experimentally the average percentage of b^- molecules was 15 per cent in 14 heterozygous $A^4_bA^5_b$ sera, 17 per cent in 25 homozygous $A^4_bA^4_b$ sera and 16 per cent in 10 homozygous $A^5_bA^5_b$ sera. This percentage is in agreement with the limits of 10 and 20 per cent for b^- molecules derived by Dray and Nisonoff from the study of 2 Ab4 homozygous, 2 Ab5 homozygous, and 2 Ab(4,5) heterozygous rabbits (28). The concordance between our results and those of Dray and Nisonoff, while mutually confirmatory, also indicates that the inability of the latter authors' anti-Ab4 and anti-Ab5 sera to precipitate 100 per cent of the labeled γ-globulins was not due to soluble complexes or impurities in the γ-globulin preparations.

The Significance of b^- Molecules.—The existence of b^- molecules may be explained in several ways. 1. These molecules may carry an as yet undiscovered allotypic specificity controlled by an allelic gene at the b locus. This explanation can be considered only in those animals which carry a single known specificity controlled by the b locus unless the allelism of the A^4_b and A^5_b genes is reopened to question. Even in this case, the consideration is improbable since it would require that individuals homozygous for A^4_b be rare, although Ab4 is found in approximately 95 per cent of the population studied.

2. b^- molecules may result from a splitting of γ-globulin molecules into subunits, one of which may be deprived of a specificity controlled by the b locus. The progressive cleavage of human γ-globulin during the conservation

of sera has been the subject of observations by 2 workers (17, 29–31). Were rabbit γ-globulin subject to a similar cleavage, it is possible that one of the products may lack the allotypic specificities controlled by the *b* locus but yet retain the essentials of the isotypic specificity of the parent molecule. A somewhat analogous situation apparently exists in the digestion of γ-globulin by papain since only one of the three fragments produced (fragment III) was precipitable by a goat anti–γ-globulin serum (32) whereas the allotypic specificities controlled by the *a* and *b* loci were found by Kelus, Marrack, and Richards (33) and by Stemke (34) on fragments I and II. However, if such was the origin of b^- molecules, one might expect to find these molecules in a relatively higher concentration (resulting in a greater value of *d*) among those sera stored the longest period of time. This was not confirmed. The oldest sera among the 25 Ab($4^+5^-6^-$) studied gave the following values of 2*d* (in millimeters; the year during which the blood was drawn is indicated in parentheses): 1.0 (1949), 1.2 (1953), 1.0 (1955), 2.6 (1956), 1.2 (1957), and 1.4 (1958), with an average of 1.37 ± 0.55 mm as compared to an average of 1.7 ± 0.7 for the entire group of 25 Ab($4^+5^-6^-$) sera (Table III). On the other hand, two sera collected less than 24 hours before their use produced values of 2*d* of 1.8 and 2.6 mm.

3. Finally, it may be suggested that a minority of γ-globulin molecules lack the specificities controlled by the *b* locus without prior splitting. The B chain obtained as described by Fleischman, Pain, and Porter (35) by means of reduction with mercaptoethanol appears to carry the *b* locus specificities to the exclusion of specificities controlled by the *a* locus.[3] Thus if it is hypothesized that the synthesis of the B chain is regulated by the *b* locus, one is led to conclude that b^- molecules lack the B chain, at least in the form in which this chain is present in b^+ molecules. The complete absence of a B chain or a similar structure would result in a difference in molecular weight and other physical properties. It is conceivable and perhaps more likely that the B chain of b^+ molecules is replaced in b^- molecules by a more or less analogous structure controlled by another locus lacking known allelic forms.

The Heterogeneity of γ-Globulin Based on Its Allotypic Properties.—The absence of allotypic specificities controlled by a given locus in a fraction of γ-globulin molecules in all rabbit sera studied represents a heterogeneity which, although based on allotypy, is not peculiar to certain members, or groups of members, of the species. This heterogeneity is thus distinguished from that which results from the coexistence of several allotypes among heterozygotes as well as that which may be attributed, even in homozygotes, to the subdivision

[3] A and B chains were prepared from a rabbit serum of phenotype Aa(1)b(4,5). The Ab4 and Ab5 specificities could be detected in the preparation of B chains whereas the Aa1 specificity could not be detected. In the A chain preparation the Aa1 but also the Ab4 and Ab5 specificities were found (36). Similar results have been obtained by Kelus (37).

of an allotypic specificity into families carried by different molecules (5, 38). On the other hand, this heterogeneity resembles that which results from the classification of γ-globulin molecules according to their physicochemical properties or isotypic specificities. In recent years several examples of such heterogeneity have been described (39–43). It is conceivable that, for example, b^+ and b^- molecules may be susceptible to differentiation on the basis of their physical properties. It does not appear, however, that there is any connection between b^+ and b^- or a^+ and a^- γ-globulin and the categories I and II described by Palmer, Mandy, and Nisonoff (40) since even if their proportions had been comparable, the specificities controlled by the *a* and *b* loci are found on both fragments I and II derived by papain digestion from the corresponding two categories of γ-globulin (27, 33, 34).

SUMMARY

Representatives of two species, the chicken and the goat, were immunized by injection with Freund's adjuvant, of specific precipitates prepared from the antisera of rabbits with various allotypic specificities. The precipitation reactions of the resulting antisera with normal rabbit sera were studied in liquid and in gellified media. In these reactions the isotypic specificity and often one or the other of the allotypic specificities of rabbit γ-globulin were involved.

It was always easier to obtain antibodies against the specificities controlled by the alleles of locus *b* (Ab4, Ab5, Ab6) than of locus *a* (Aa1, Aa2, Aa3). The precipitation reaction of antibodies against a specificity of the *a* series could always be inhibited by an excess (nearly always moderate) of a serum lacking this specificity. A similar inhibition of the antibodies against the specificities of the *b* series was often impossible, especially if the antiserum had been collected after a second injection of immunizing material.

The reactions of 58 $Ab4^+$ sera with a goat anti-Ab4 serum and of 28 $Ab5^+$ sera with a chicken anti-Ab5 serum were studied in gel tubes (simple diffusion). The constant observation of two precipitation zones due respectively to two types of molecules, $Ab4^+$ and $Ab4^-$ (or $Ab5^+$ and $Ab5^-$), indicated the existence of an appreciable number of $Ab4^-$ or $Ab5^-$ molecules, even in supposed homozygotes. In the latter rabbits $Ab4^-$ or $Ab5^-$ molecules are b^- molecules; *i.e.*, molecules lacking the specificities of the *b* series. Measurements of the distances between the two zones in the reaction of 72 sera revealed significant differences between sera of individuals of the same phenotype, as well as systematic differences not only between sera of homozygotes and of heterozygotes, but also between heterozygotes of different genotypes. These measurements were used to evaluate the ratio of concentrations of the two types of molecules detected by each of the two antisera.

The occurrence of b^- molecules in every one of the fairly large number of sera studied indicates a new aspect of the heterogeneity of γ-globulin. Al-

though detected by means of allotypy, this heterogeneity is not dependent upon factors which vary with individuals.

These results are discussed in connection with what is known of the structure of γ-globulin.

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