

## IMMUNOGLOBULIN ISOANTIGENS (ALLOTYPES) IN THE MOUSE

### I. GENETICS AND CROSS-REACTIONS OF THE 7S $\gamma_{2A}$ -ISOANTIGENS CONTROLLED BY ALLELES AT THE IG-1 LOCUS\*

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Isoantigenic differences in immunoglobulins have been demonstrated in man (Gm and Inv groups) (1-3), and rabbit (allotypes) (4). Recent studies in the mouse have described a similar polymorphism for immunoglobulin isoantigens (5-8), one of which occurs in a subclass of 7S gamma globulins,  $\gamma_{2a}$  (9), and the other in  $\gamma_{1A}$  ( $\beta_{2A}$ )-immunoglobulins (10). These antigens, which have been localized to the F fragments after papain digestion (11), and are therefore on the H (A) chain, are controlled by closely linked genetic loci (10). In this publication we will be concerned only with the locus which controls the isoantigens on 7S ( $\gamma_{2a}$ )-globulins. Four alleles at this locus have previously been described (10).

In order to further investigate the genetic control of these antigens, a highly sensitive immunochemical method was developed, based on the inhibition of precipitation of labeled  $\gamma$ -globulin (12). With this test the degrees of cross-reactivity of antigens of the individual strains could be readily detected, enabling the identification of eight distinct types.

The notation for the locus and its phenotypes used in this paper has previously been described (10) and follows the recommendations resulting from a discussion between members of the committee on Standardized Genetic Nomenclature For Mice (13) and some of those workers who have published on immunoglobulin isoantigens of mice. The locus discussed in this paper is designated *Ig-1* (Immunoglobulin-1) and eight alleles are described. The individual antigenic specificities determined by each of the alleles are defined and designated by a method analogous to that used to describe cross-reacting antigens in the Rh and H-2 blood group systems (14-16).

#### *Materials and Methods*

*Mice.*—Young adult mice of both sexes were obtained from inbred colonies maintained in various laboratories, as indicated by the substrain symbols (17). We are indebted to Dr. L. Russell, of Oak Ridge National Laboratories, and to the staff of the Roscoe B. Jackson Labora-

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ories, Bar Harbor, notably Dr. M. Green, for providing most of the mice used in linkage studies. The strains used, together with their substrain designation, are listed in Table X. In this paper the following abbreviations have been used: AKR/J, AKR; C3H/HeJ and C3H/Hz, C3H; C57BL/6J and C57BL/10Hz, C57BL; DBA/2J, DBA.

*Gamma Globulin Isolation.*—Samples of pooled normal whole serum were concentrated 3 to 4-fold by pressure dialysis and then subjected to electrophoresis in 1 per cent Ionagar in veronal buffer pH 8.2, on glass plates, using a wide central trough for 4 hours at 30 ma and 5 to 6 v/cm. Serial  $\frac{1}{2}$  centimeter strips were then cut perpendicular to the direction of the current flow from the cathodal end of the slide to the origin. Protein was eluted from the agar strips by soaking in phosphate-buffered saline (0.01 M phosphate, 0.85 per cent NaCl, pH 7.1) for 48 hours, followed by reconcentration by pressure dialysis. Samples of each fraction were individually tested for the presence of gamma globulin and other serum proteins by immunoelectrophoresis with a rabbit anti-mouse whole serum. Only the most cathodal fractions of gamma globulin were used for  $I^{125}$  labeling. A typical immunoelectrophoretic pattern of such a fraction is shown in Fig. 1.

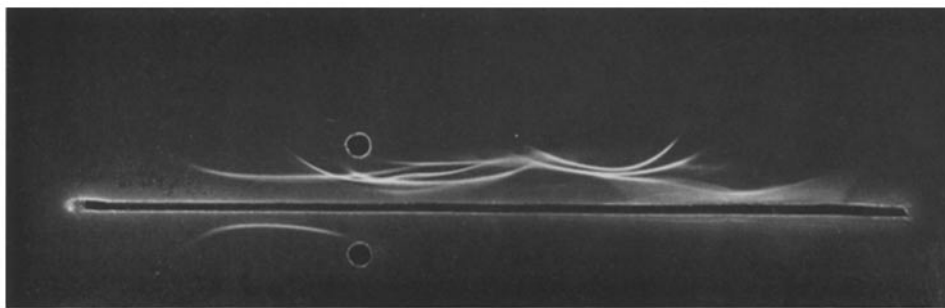


FIG. 1. Immunoelectrophoresis of a gamma globulin preparation from DBA/2 serum used for  $I^{125}$  labeling, compared to whole DBA/2 serum. The central trough contained a polyvalent rabbit anti-whole mouse serum.

*Iodination with  $I^{125}$ .*—Iodination of approximately 100  $\mu$ g portions of gamma globulin was performed by the method of Greenwood *et al.* (18) with an average labeling of 1 atom  $I^{125}$  per molecule. Passage through anion exchange resin columns followed by dialysis against saline was used to remove unbound iodide. Approximately 20 per cent of the added iodide became attached to the protein.

*Precipitation of Iodinated Gamma Globulin.*—All reactions employing labeled gamma globulin were carried out in 6 x 50 mm culture tubes. Labeled antigens were diluted in a 3 per cent bovine serum albumin (BSA) (Armour Pharmaceutical Company, Kankakee, Illinois) solution in 0.05 M tris buffer (final pH 7.6). All antisera used were diluted in serum diluent (S-dil): 0.05 M tris, containing 3 per cent BSA and 10 per cent normal rabbit serum, pH adjusted to 7.6.

For the reaction, 50  $\mu$ l of diluted labeled antigen was added to 50  $\mu$ l of varying dilutions of the antisera. The tubes were left at 37°C for 3 hours, chilled to 4°C and then spun cold at 10,000 g for 10 minutes; 50  $\mu$ l of the supernatant was carefully removed and placed in a 10 x 75 mm disposable tube containing approximately 0.5 ml of normal saline. These were then counted in a well type crystal gamma scintillation counter.

The maximum percentages of precipitation of the five labeled antigen preparations used for these studies by rabbit antisera, mouse isoantisera and trichloroacetic acid (TCA) are shown in Table I.

*Isoimmunization.*—A basic schedule of immunization was followed for preparing the majority of antisera used in this study. Minor changes in antigen dosage and timing have been made occasionally with little apparent affect. A primary subcutaneous injection of 10  $\mu$ l of

TABLE I  
*Precipitation of I<sup>125</sup>-Labeled Gamma Globulins*

Antiserum	I <sup>125</sup> -labeled gamma globulin preparation				
	C3H	C57BL	DBA/2	AKR	A/J
Trichloroacetic acid*	99‡	98	99	99	94
Rabbit anti-whole mouse serum§	96	88	83	90	70
Rabbit anti-mouse 7S $\gamma_1$ + 7S $\gamma_2$	95	79	75	87	69
Rabbit anti-mouse 7S $\gamma_2$	89	67	64	82	60
Specific mouse isoantiserum¶	76	55	55	72	61
Rabbit anti-mouse $\gamma_{1A}$ ( $\beta_{2A}$ )	5	2	0	0	2
Mouse anti-complement (Hc <sup>1</sup> )**	0	0	0	0	0

\* Final concentration of 7.5 per cent, reaction at 4°C.

‡ Numbers in table give the maximum per cent of I<sup>125</sup> radioactivity precipitated.

§ Polyvalent antiserum as used in Fig. 1.

|| Kindly provided by Dr. J. Fahey (27).

¶ Each antigen was precipitated by its homologous isoantiserum.

\*\* A DBA/2 anti-C3H anti-Hc<sup>1</sup> serum (19).

TABLE II  
*Comparison of Immunogenicity of Gamma Globulin of Normal and Immune Sera*

Donor strain	Type of donor serum	Recipient strain	No. of mice positive/total No. of mice*
C57BL	Normal	BALB/C	0/10
"	Anti-DBA/2 spleen‡	"	48/48
NZB	Normal	BALB/C and C57BL	0/12
"	Anti-Hc <sup>1</sup> §	" " "	8/9

\* Number of mice producing anti-gamma globulin isoantibodies as detected by precipitation of I<sup>125</sup>-labeled antigen/total number of mice injected.

‡ Antiserum prepared by immunizing C57BL mice with DBA/2 spleen cells (BALB/C and DBA/2 have the same H-2 allele).

§ Anti Hc<sup>1</sup> (a  $\beta$ -globulin component of complement) prepared by immunizing NZB mice with whole BALB/C serum.

whole serum per mouse in complete Freund's adjuvant was followed 3 weeks later by two weekly intraperitoneal injections each of 10  $\mu$ l. The mice were bled 1 week later and individual sera tested for the presence of isoantibodies. Mice failing to produce detectable antibodies were then alternately injected and bled at weekly intervals for about 1 month, and then discarded if still negative.

Although normal serum may be used as antigen, far better results have been obtained by

using as the antigen, serum from animals which contain antibodies directed to some tissue or protein component in the recipient strain. Two such examples of this are given in Table II. Whereas BALB/c mice immunized with normal (C57BL) serum failed to produce any detectable isoantibodies to C57BL gamma globulin, immunization of the same strain with a C57BL anti-DBA/2 spleen antiserum was quite successful (BALB/c and DBA/2 have the same H-2 antigen). Similarly, an antiserum to a complement component antigen, (Hc<sup>1</sup>) (19), was also effective as an antigenic serum.

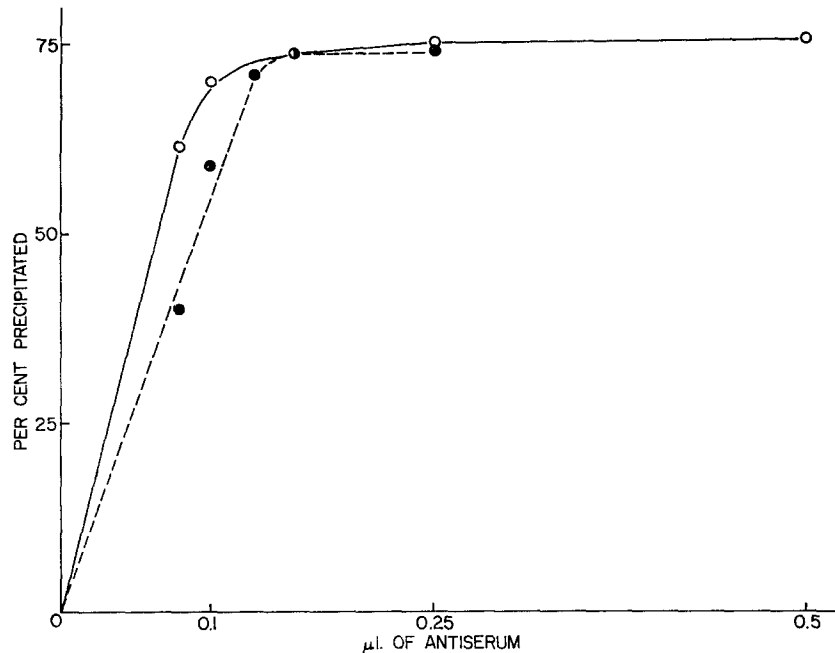


FIG. 2. Precipitation of I<sup>125</sup>-labeled C3H gamma globulin (0.01 µg) by two isoantisera. ○ C57BL anti-C3H; ● RIII anti-129. Total volume 0.1 ml.

*Testing of Antisera.—*

(a) *Precipitation in agar:* Individual samples of sera from immunized animals were tested for precipitation by the Ouchterlony technique (20) on microscope slides with normal sera of several strains and also with purified serum proteins. Those sera showing identical patterns of reactivity (and prepared in identical fashion), were then pooled. Immunoelectrophoresis (21) was used to determine the specificity of the antisera when several lines were observed in double diffusion with normal serum. Most of the precipitating isoantisera obtained react with immunoglobulins. Some were found that precipitated with other serum proteins as reported elsewhere (19, 22). Both Ouchterlony and immunoelectrophoretic tests were carried out in 0.05 M barbital buffer in 1 per cent Ionagar at pH 8.2.

(b) *Precipitation of labeled antigens:* Antisera from immunized mice were tested for ability to precipitate labeled gamma globulin of the immunizing strain type. These tests were performed as described above using approximately 0.01 µg labeled antigen per tube. This test was found to be consistently far more sensitive than direct precipitation in agar. Many sera

were found that did not show any precipitation in agar, but precipitated the labeled antigen even with antiserum dilutions of 1/1000.

*Inhibition of Precipitation of Isoantigens.*—An adaptation of a method of estimating gamma globulins (12) based on the inhibition of precipitation of labeled reference antigens was employed. Concentrations of labeled antigen and antisera were chosen as follows: A standard concentration of labeled gamma globulin, giving about 10,000 counts per minute per 50  $\mu$ l was chosen, and precipitated, as described above, with serial dilutions of isoantiserum. An amount of antiserum slightly below equivalence was then determined (*i.e.*, an amount which precipitates 80 to 90 per cent of the precipitable counts). A typical titration is shown in Fig. 2, in which two different antisera to C3H gamma globulin were titrated against a constant amount of labeled C3H gamma globulin.

The inhibition assay was performed in a fashion similar to the precipitation of labeled antigens. The order of addition of the reagents is quite critical. Fifty  $\mu$ l of a dilution of labeled antigen was placed in all tubes, followed by the addition of 5  $\mu$ l of serial dilutions (in S-dil) of test (inhibitor) whole sera. At the addition of 50  $\mu$ l of diluted isoantiserum each tube was immediately mixed on a vortex Jr. mixer (Scientific Industries, Springfield, Massachusetts). Two controls were included in each assay. One tube contained 55  $\mu$ l of the diluent (S-dil) with 50  $\mu$ l of labeled antigen and was the control of total radioactivity (*i.e.*, no precipitation and referred to as T). The other control tube determined maximum precipitation (*i.e.* no inhibition) and contained 50  $\mu$ l of antiserum, 5  $\mu$ l of S-dil, with 50  $\mu$ l of labeled antigen (referred to as A). Incubation, centrifugation, and sampling were carried out as described above.

*Determination of Cross-Reaction.*—The usual methods of determining the presence of a cross-reaction in precipitating antigen-antibody systems, (spur formation in Ouchterlony and precipitin formation) are not sufficiently sensitive to detect many of the cross-reactions found in this study, particularly as some of our antisera do not even visibly precipitate in agar. A more sensitive method of determining cross-reactivity was therefore sought.

*Determination of percentage cross-reaction (CR):* The CR value is a measure of the ability of a test serum, present in excess, to inhibit labeled antigen precipitation. To determine this value, the inhibition assay was set up with a test serum using 5  $\mu$ l of a 1/10 dilution which previous tests have shown to contain an excess of the unlabeled antigen. The CR is calculated from the following formula, all values used being the counts per minute of 50  $\mu$ l of the supernatant.

$$CR(\text{per cent}) = \frac{I - A}{T - A} \times 100$$

where  $I$  is the count in the tube containing labeled antigen, antisera, and inhibitor (test serum);  $A$  is the count in the tube containing labeled antigen and antiserum;  $T$  is the count in the tube containing labeled antigen and S-dil; and  $T - A$  is the number of counts precipitated. When inhibition of precipitation of the labeled antigen is complete,  $I = T$  and  $CR = 100$ . When there is no inhibition of precipitation,  $I = A$  and  $CR = 0$ .

## RESULTS

*Ouchterlony Tests with Isoantisera.*—Five types of antisera produced by the methods described have been tested against normal whole sera from a large number of strains. Each antiserum reacted with its corresponding immunizing serum to form a single line of precipitation in Ouchterlony slides and a single arc of precipitation in immunoelectrophoresis corresponding to the gamma globulin arc seen with polyvalent rabbit anti-mouse whole sera. Previous stud-

TABLE III  
Precipitation of Normal Mouse Sera in Agar by Isoantibodies

Antigen strain	Antisera				
	C57BL anti-C3H (serum pool G-1L)	C57BL anti-C3H (serum pool G-1P)	C3H anti-C57BL	C57BL anti-DBA/2	C57BL anti-AKR
A/J	+	+	-	-	+
AKR/J	+	+	-	-	+
AL/N	+	+	-	-	+
B10.D2/Hz	-	-	+	-	-
BALB/cCrglGa	+	+	-	-	-
BDP/J	+	+	-	-	-
BRSUNT/N	+	+	-	nt	nt
BSL/Di	+	+	-	-	-
BUB/Bn	+	+	-	-	-
C3H/Hz	+	+	-	-	-
C3H.SW/Hz	+	+	-	-	-
C57BL/Ka	-	-	+	-	-
C57BL/6J	-	-	+	-	-
C57BL/10Sn	-	-	+	-	-
C57BR/CdJ	+	+	-	-	-
C57L/J	+	+	-	-	-
C58/J	+	+	-	-	-
CBA/J	+	+	-	-	-
CE/J	+	+	-	-	-
DA/Hu	-	+	-	+	-
DBA/1J	-	+	-	+	-
DBA/2J	-	+	-	+	-
DD/He	+	nt	-	-	nt
DE/J	+	+	-	-	-
FZ/Di	-	+	-	+	-
HG/Hu	-	-	+	-	-
JB/Di	-	+	-	+	-
LP/J	-	-	+	-	-
MA/J	+	+	-	-	-
MA/MyJ	+	+	-	nt	-
P/J	+	+	-	-	-
PL/J	+	+	-	-	-
RIII/J	-	+	-	+	-
RF/J	-	+	-	+	-
SEA/Gn	+	+	-	-	-
SEC/Gn	+	+	-	-	-
SJL/J	-	-	+	-	-
SM/J	-	-	+	-	-
ST/J	+	+	-	-	-
STR/N	+	+	-	nt	-
SWR/J	-	+	-	+	-
WB/Re	-	-	+	-	-
WC/Re	-	-	+	-	-
WH/Re	-	-	+	-	-
WK/Re	-	-	+	-	-
58/N	-	-	+	-	-
101/Rl	-	-	+	-	-
129/RrGa	+	+	-	-	-

+, positive reaction in agar gel diffusion with specified antiserum.  
-, negative reaction.  
nt, not tested.

ies have described in more detail the molecular class bearing the isoantigen to be 7S  $\gamma_{2a}$  (9). The reactions of these five antisera with 48 normal sera are given in Table III. Although two of the antisera were made with the same donor-recipient combination, each of the five has a different reaction pattern. The only difference between the two C57BL anti-C3H antisera is that serum pool G-1P reacts with some strains with which the serum pool G-1L does not. This group of strains is also the only one positive with the C57BL anti-DBA/2 antisera.

From the data in Table III, four groups of strains each with a unique pattern of reactions may be recognized. Evidence that the antigens carried by these strains are controlled by four alleles at a single locus is presented in the following section. The reactions of a reference (or type) strain for each group (allele) is listed in Table IV. Since two of the antisera react with strains in more than

TABLE IV  
*Precipitation of Reference Strain Sera in Agar by Isoantisera*

Allele	Type strain	Antisera				
		C57BL anti-C3H (G-1L)	C57BL anti-C3H (G-1P)	C3H anti-C57BL	C57BL anti-DBA/2	C57BL anti-AKR
Ig-1 <sup>a</sup>	C3H/HeJ	+	+	-	-	-
Ig-1 <sup>b</sup>	C57BL/10J	-	-	+	-	-
Ig-1 <sup>c</sup>	DBA/2J	-	+	-	+	-
Ig-1 <sup>d</sup>	AKR/J	+	+	-	-	+

one of the four antigen groups, it is evident that some of the alleles must determine several antigenic specificities. Further definition of antigenic specificities is made in a later section.

*Allelism.*—The results of testing reciprocal backcross and F2 progeny between C57BL and C3H with a C57BL anti-C3H gamma globulin and a C3H anti-C57BL gamma globulin antiserum (Table V, lines 1 to 3) indicate that these two antigens, Ig-1a and Ig-1b, are specified by genes at a single locus (or chromosome region), which has been named Ig-1 (Immunoglobulin-1) (10). The last two lines of Table V present the results of test crosses which show that *Ig-1<sup>a</sup>* and *Ig-1<sup>c</sup>*, and *Ig-1<sup>a</sup>* and *Ig-1<sup>d</sup>* are alleles. From these results, it must be concluded that all four antigens are determined at the same genetic locus and that there are four alleles.

Further subdivision of these four groups of strains was then attempted (a) by the production of different antisera and (b) by determining the degree of cross-reaction of normal sera from different strains in inhibition assays with different antigen-antibody systems.

*Precipitation of I<sup>125</sup>-Labeled Gamma Globulin by Isoantisera.*—Antisera were

prepared by immunization between mice with different Ig-1 alleles and with the same apparent allele as indicated by the patterns of reactivity in Table III. The ability of 10 of these antisera to precipitate labeled gamma globulin from five strains are listed in Table VI. Data from this table is used in a later section in determining antigenic specificities.

TABLE V  
*Segregation of Genes Determining Gamma Globulin Isoantigens (10)*

Cross		Progeny			
Parent strains	Parent <i>Ig-1</i> genotypes	Tested with	Result	Total tested	Progeny <i>Ig-1</i> genotype
(C3H x C57BL) <sub>F1</sub> x C57BL	a/b x b/b	Anti-Ig-1a*	+51 -39	90	a/b b/b
(C3H x C57BL) <sub>F1</sub> x C3H	a/b x a/a	Anti-Ig-1b†	+233 -209	442	b/a a/a
(C3H x C57BL) <sub>F1</sub> ⊗	a/b x a/b	(a) Anti-Ig-1b  (b) Anti-Ig-1b Anti-Ig-1a	+202§  - } 78   + }	280	b/a + b/b  a/a
(C3H x DBA/2) <sub>F1</sub> x B10.D2	a/c x b/b	(a) Anti-Ig-1a Anti-Ig-1c¶  (b) Anti-Ig-1a Anti-Ig-1c	+ } 78 - }  - } 71 + }	149	a/b  c/b
(AKR x DBA/2) <sub>F1</sub> x C57BL	d/c x b/b	(a) Anti-Ig-1c Anti-Ig-1d**  (b) Anti-Ig-1d Anti-Ig-1c	+ } 71 - }  + } 82 - }	153	c/b  d/d

\* Anti-Ig-1a, C57BL anti-C3H.

† Anti-Ig-1b, BALB/C anti-C57BL.

§ Not tested for segregation of Ig-1a.

|| Sera from only 51 of the 78 Ig-1b negative animals were available for testing with anti-Ig-1a. All were Ig-1a positive.

¶ Anti-Ig-1c, C57BL anti-DBA/2.

\*\* Anti-Ig-1d, DBA/2 anti-AKR.



Labeled C3H gamma globulin has been shown to be precipitated by many antisera produced in mice immunized with sera from strains apparently different from C3H. The actual percentage of C3H gamma globulin precipitated

TABLE VI  
*Precipitation of I<sup>125</sup>-Labeled Gamma Globulins by Mouse Isoantisera*

Antisera	I <sup>125</sup> -labeled gamma globulin				
	C3H	C57BL	DBA/2	AKR	A/J
C57BL anti-C3H	P*	N‡	P	P	P
BALB/C anti-C57BL	N	P	N	N	N
C57BL anti-DBA/2	P	N	P	P	P
DBA/2 anti-AKR	P	N	N	P	P
C57BL anti-AKR	P	N	N	P	P
BALB/C anti-NZB	N	N	N	P	P
C57BL anti-A/J	P	N	P	P	P
RIII anti-129	P	N	N	P	P
RIII anti-C57BL	N	P	N	N	N
CE anti-129	P	P	P	P	P

\* Percentage of precipitation shown in Table I.

‡ Less than 5 per cent precipitation.

TABLE VII  
*Precipitation of I<sup>125</sup>-Labeled Antigen by Homologous and Cross-Reacting Antisera*

Antiserum		Per cent of I <sup>125</sup> -labeled C3H/HeJ (Ig-1 <sup>a</sup> ) gamma globulin precipitated
Type	μl Added	
Ig-1 <sup>b</sup> (C57BL) anti-Ig-1 <sup>a</sup> (C3H)	1	75
	2	74
Ig-1 <sup>b</sup> (C57BL) anti-Ig-1 <sup>a</sup> (DBA/2)	1	75
	2	71
Both together	1 + 1	72
	2 + 2	72

Each tube contains 0.04 μg I<sup>125</sup>-labeled C3H gamma globulin and the indicated antiserum volume. Total volume per tube was 0.1 ml.

by antisera to two (C3H and DBA/2) of the four reference strains previously established is shown in Table VII. Since both of the antisera and a mixture of them, precipitate the same amount of labeled C3H antigen, each C3H molecule must have at least one antigenic specificity shared by DBA/2. However, these results do not preclude the possibility that different 7S γ-globulin molecules in a

serum may be carrying other antigenic specificities not detected with the anti-sera used in table VII.

*Sensitivity of Assay.*—Before describing the actual results used in the further elucidation of antigenic specificities, the data from a few inhibition assays will be given in order to demonstrate the ability of this technique to detect partial cross-reactions and also to indicate the sensitivity of the method for detecting gamma globulin. The results presented in Fig. 3 were obtained from four in-

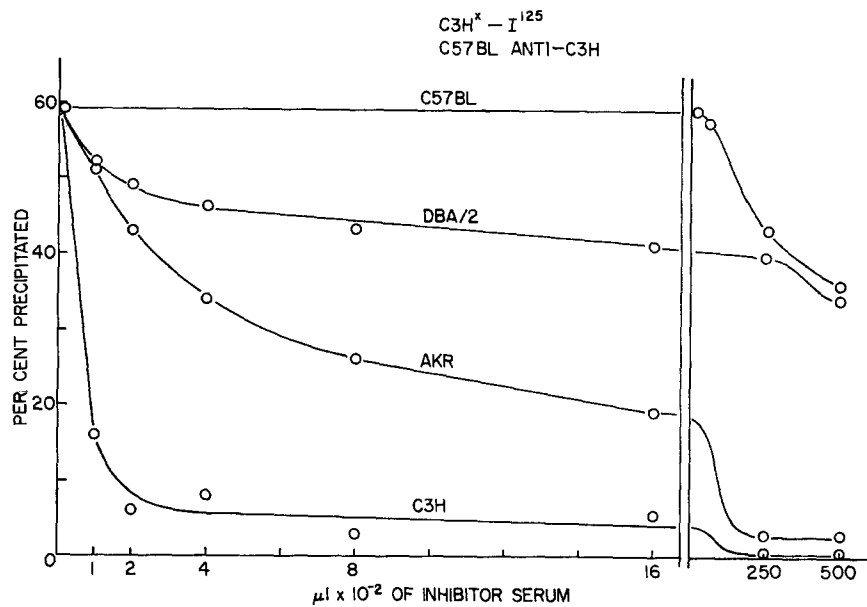


FIG. 3. Inhibition of precipitation of I<sup>125</sup>-labeled C3H gamma globulin by normal sera. Each tube contains 0.02 μg of labeled antigen and 0.25 μl of a C57BL anti-C3H isoantiserum in a total volume of 0.1 ml. The volumes of inhibitor serum added to each tube are shown on the abscissa. The scale is broken at the double vertical line.

hibition assays performed in the manner described in the methods section, using C3H-labeled antigen, a C57BL anti-C3H antiserum, and serial dilutions of four test sera, namely C3H, AKR, DBA/2, and C57BL. The results are plotted in Fig. 3 as the percent of radioactivity precipitated against the amount of inhibitor serum. C3H serum shows detectable inhibition of precipitation with 0.01 μl and complete inhibition is achieved with approximately 0.02 μl of whole serum. DBA/2 and AKR sera also show detectable inhibition with 0.01 μl of whole serum. However, complete inhibition is not achieved with up to 1.0 μl of DBA/2 and AKR sera, inferring that DBA/2 and AKR sera do not contain all the specificities on the reference (C3H)-labeled antigen to which antibodies are present. The greater degree of inhibition by AKR than DBA/2

could be interpreted to indicate a greater similarity of AKR with C3H than DBA/2 with C3H (see Discussion).

Further inhibition was encountered with high concentrations of DBA/2, AKR, and normal C57BL serum (the strain in which the antibodies were made). The amount of serum required to cause this inhibition was about 100 to 1000-fold greater than that producing specific inhibition. This could be due to either very small amounts of C3H type gamma globulin specificities in the DBA/2, AKR, or C57BL serum causing inhibition by specifically competing for the antibody, or to the ability of high serum concentrations to non-specifically inhibit precipitation. Since this type of inhibition was not encountered with the concentrations of inhibitor sera used in the following sections, it does not

TABLE VIII  
*Antigenic Specificities Present in Ig-1 Alleles*

Allele	Type strain	Antigenic specificities							
		1	2	3	4	5	6	7	8
Ig-1 <sup>a</sup>	C3H/HeJ	1*	2	—†	—	—	6	7	8
Ig-1 <sup>b</sup>	C57BL/10J	—	—	—	4	—	—	7	—
Ig-1 <sup>c</sup>	DBA/2J	—	2	3	—	—	—	7	ND§
Ig-1 <sup>d</sup>	AKR/J	1	2	—	—	5	—	7	ND
Ig-1 <sup>e</sup>	A/J	1	2	—	—	5	6	7	8
Ig-1 <sup>f</sup>	CE/J	1	2	—	—	—	—	—	ND
Ig-1 <sup>g</sup>	RIII/J	—	2	3	—	—	—	—	ND
Ig-1 <sup>h</sup>	SEA/Gn	1	2	—	—	—	6	7	—

\* Number means specificity is present.

† Dash means specificity is absent.

§ Not done.

affect the conclusions to be made. However, further investigation is proceeding to determine the nature of this inhibition.

*Detection of More Alleles and Antigenic Specificities.*—At the present stage of this work, it is probable that as new antisera are produced further antigenic specificities will become apparent. However, sufficient data are available to commence to identify antigenic specificities for a number of alleles. The antigenic specificities defined by the available antisera for selected type strains are given in Table VIII. To assist in the interpretation of the data which was used to compile this chart, Table IX lists the antigenic specificities recognized by the antisera used in these tests. These in turn, were reasoned from the known reactions and cross-reactions of the various antisera and use the numbers for specificities listed in Table VIII.

It should be noted that the repeated production of an antiserum between

two strains does not of necessity yield antisera detectably reacting to the same determinants. In fact, such antisera have often been found to have different ratios of antibodies to the different specificities.

The absolute values of CR (cross-reactions, see Materials and Methods) depend on the particular pool of antiserum used as well as on its concentration, so while CR values in a given experiment are directly comparable, those in different sections are not. In all inhibition assays performed, complete inhibition was always obtained with sera of the immunizing strain and with sera from the same strain as that of the labeled gamma globulin used in the assay.

In the analysis of the antigenic specificities from the following data, several rules have been followed.

(a) A strain producing an isoantiserum has none of the specificities recognized by that antiserum.

TABLE IX

*Antigenic Specificities Detected by Isoantisera in Various Labeled Gamma Globulin Preparations*

Antiserum	Labeled gamma globulin	Antigenic specificities detectable
C57BL anti-C3H	C3H	1, 2, 6, 8
“ “	DBA/2	2
“ “	AKR	1, 2
“ “	A/J	1, 2, 6, 8
C57BL anti-DBA/2	DBA/2	2, 3
BALB/C anti-C57BL	C57BL	4
BALB/C anti-NZB	A/J	5
CE anti-129	C3H	6, 7
“ “	C57BL	7

(b) The immunizing strain has all the specificities recognized by the antiserum regardless of the type of antigen used to detect the antibodies present in the antiserum.

(c) However, the specificities detected in the immunizing strain by an antiserum made against it are not necessarily all of the antigenic specificities present in the immunizing strain (*i.e.*, antibodies may not be present to all the specificities).

(d) The specificities detected by an antiserum when used with a labeled antigen from a strain other than the immunizing strain, represent the specificities detected by the antiserum minus those specificities which are present in the immunizing strain and absent in the labeled antigen.

(e) A test serum which completely inhibits has all the specificities detected by the antiserum in that assay, while a test serum which does not inhibit at all has none.

(f) A test serum which partially inhibits a reaction has some but not all of the specificities detected in that assay.

(g) Two strains which each partially inhibit in a given reaction, need not share any specificities with each other, but each shares some specificities with the labeled antigen strain and the immunizing strain.

(h) The number of specificities is always a minimum estimation of the number compatible with the results.

The following notation is used:

An I<sup>26</sup>-labeled preparation of gamma globulin is indicated with an asterisk following the symbol of the strain from which it was prepared, (for example, C3H\*), while normal sera used in inhibition assays are listed by the strain symbols (*e.g.*, C3H). The symbols C3H\*-C57BL anti-C3H refer to the use of a labeled C3H gamma globulin preparation with a C57BL anti-C3H antiserum in an inhibition assay. The statement, "(C3H 1)" means C3H has specificity one. The statement, "(C57BL -1)" means C57BL does not have specificity one. The terms "partially inhibit" and "do not completely inhibit" are operationally synonymous but are used for semantic emphasis.

The data used to define the presence or absence of each specificity in each type strain is given below. The numerical designations of the various antigenic determinants are completely arbitrary.

- i C3H\*-C57BL anti-C3H precipitation is not completely inhibited by DBA (CR 21). C3H therefore has at least one specificity not present in DBA (C3H 1; C57BL -1; DBA -1).
- ii DBA\*-C57BL anti-C3H precipitation is completely inhibited by AKR. Therefore C3H, DBA, and AKR share at least one specificity, 2 (C3H 2; C57BL -2; DBA 2; AKR 2).
- iii AKR\*-C57BL anti-C3H precipitation is partially inhibited by DBA (CR 10). Therefore AKR and C3H must share at least one specificity that is not present in DBA (AKR 1).
- iv C3H\*-C57BL anti-C3H precipitation is not completely inhibited by AKR (CR 81). C3H therefore has at least one specificity not present in AKR (C3H 6; C57BL -6; AKR -6).
- v Since C57BL anti-C3H recognizes specificity 6, statement ii proves that DBA does not have 6, (DBA -6).
- vi DBA\*-C57BL anti-DBA precipitation is not completely inhibited by C3H or AKR (CR respectively 81, 66). Therefore DBA has at least one specificity not present in either of these two strains, (C3H -3; C57BL -3; DBA 3; AKR -3).
- vii C3H anti-C57BL precipitates C57BL\*, but not C3H\*, DBA\*, AKR\*, or A/J\*. C57BL therefore has at least one specificity not present in the latter four strains, (C3H -4; C57BL 4; DBA -4; AKR -4; A/J -4).
- viii C3H\*-C57BL anti-C3H precipitation is completely inhibited by A/J. Therefore A/J has all specificities previously assigned to C3H, (A/J 1, 2, and 6).

- ix DBA\*-C57BL anti-C3H precipitation is completely inhibited by CE. Therefore CE has specificity 2, (CE 2).
- x CE anti-129 precipitates C57BL\*. Therefore C3H and C57BL share at least one specificity not previously described, (C3H 7; C57BL 7, CE-7).
- xi C3H\*-CE anti-129 precipitation is completely inhibited by A/J. Therefore A/J must have 7, (A/J 7).
- xii C57BL\*-CE anti-129 precipitation is completely inhibited by DBA and AKR. DBA and AKR must also have specificity 7, (DBA 7; AKR 7).
- xiii AKR\*-C57BL anti-C3H precipitation is completely inhibited by CE. Therefore CE has 1, (CE 1).
- xiv BALB/C anti-NZB precipitates AKR\* and A/J\*. Therefore these 2 strains must have one specificity not previously recognized. This precipitation is not inhibited by CE or C3H. (AKR 5; A/J 5; C3H-5; Ce-5.)
- xv A/J\*-C57BL anti-C3H precipitation is not completely inhibited by CE (CR 56). CE therefore does not have 6, (CE -6).
- xvi DBA\*-C57BL anti-DBA precipitation is completely inhibited by RIII. Therefore RIII has specificities 2 and 3, (RIII 2, 3).
- xvii AKR\*-C57BL anti-C3H precipitation is partially inhibited by RIII (CR 41). RIII therefore does not have 1, (RIII -1).
- xviii C3H\*-CE anti-129 precipitation is not inhibited by RIII. Therefore RIII does not have 6 or 7, (RIII -6, -7).
- xix DBA\*-C57BL anti-DBA precipitation is not completely inhibited by CE or A/J (CR respectively 67, 80). Therefore these latter two strains do not have specificity 3, (A/J -3; CE -3).
- xx C57BL\*-C3H anti-C57BL precipitation is not inhibited by CE or RIII. Neither of these strains therefore has 4, (CE-4; RIII -4).
- xxi A/J\*-BALB/C anti-NZB precipitation is not inhibited by C57BL, RIII or DBA. Therefore these three strains do not have specificity 5, (C57BL -5; DBA -5; RIII -5).
- xxii AKR\*-C57BL anti-C3H precipitation is completely inhibited by SEA. Therefore SEA has 1, (SEA 1).
- xxiii DBA\*-C57BL anti-C3H precipitation is completely inhibited by SEA. Therefore SEA has 2, (SEA 2).
- xxiv C3H\*-CE anti-129 precipitation is completely inhibited by SEA. Therefore SEA has specificities 6 and 7, (SEA 6 and 7).
- xxv C3H\*-C57BL anti-C3H precipitation is not completely inhibited by SEA, (CR 87). Since SEA has so far been assigned all the C3H specificities, a new specificity must be given to C3H, which is not present in C57BL or in SEA, (C3H 8; C57BL -8; SEA -8).
- xxvi All strains previously shown to completely inhibit in C3H\*-C57BL anti-C3H, must also have specificity 8, (A/J 8).

xxvii Since SEA behaves exactly like CE in sections xiv, xix, and xx, SEA does not have specificities 3, 4, or 5, (SEA -3, -4, -5).

The definition of each antigenic specificity has been made with a single given antiserum, a total of five antisera being used to define the eight specificities. Other isoantisera have also been produced whose reactions are consistent with the specificities already defined. However in the reactions with these antisera listed below, it is also possible that they are reacting with other undefined specificities whose distributions are identical with one of the previously defined eight specificities.

(a) C57BL anti-AKR and C57BL anti-A/J both precipitate all labeled antigens in Table VI except C57BL. This is consistent with both antisera having antibodies to specificity two and with all these strains having this specificity, although other antibodies are also probably present.

(b) RIII anti-129 precipitates C3H\*, AKR\*, and A/J\*, but does not precipitate C57BL\* or DBA\*. This serum therefore does not have antibodies to specificity 7, but could have antibodies to specificities 1 and 6.

(c) C3H\*-RIII anti-129 precipitation is completely inhibited by C3H, AKR, A/J, CE, and SEA. Since all these test sera contain specificity 1, but only some contain 6, the antiserum RIII anti-129 does not contain antibodies to specificity 6, but has antibodies to specificity 1, or a new specificity whose distribution is identical with 1.

(d) In many inhibition assays, test sera were found that partially inhibited precipitation to different degrees. Comparisons of CR values in a given assay have not been used in the derivation of specificities, although they are consistent with the data (see Discussion). One example of this type of finding is as follows: C3H\*-C57BL anti-C3H precipitation is partially inhibited by AKR and DBA (CR respectively 81 and 21). This would be consistent with Table VIII, since AKR has at least one more specificity in common with C3H than DBA/2 (see Discussion).

*Allelic Types of Inbred Mouse Strains.*—Sixty-eight mouse strains have been tested with several antisera both by agar precipitation and the inhibition assay. All the strains could be grouped into classes corresponding to the eight type strains in Table VIII. The results are given in Table X.

*Linkage of Ig-1.*—Ig-1 has been found not to be closely linked to the following gene loci (a minimum of 50 segregating offspring being tested): *Agouti* (*A*), brown (*b*), albino (*c*), dilute short ear (*d se*), hemolytic complement (*Hc*), histocompatibility-2 (*H-2*), pinkeye-dilution (*p*), piebald (*s*), the X or Y chromosome, caracul (*Ca*), and steel (*sl*).

#### DISCUSSION

The Ig-1 locus, controlling isoantigens on mouse 7S  $\gamma_{2a}$ -immunoglobulins, is highly polymorphic with eight alleles recognized. Each antigen serologically





cross-reacts with the others and thus shows multiple antigenic specificities. These are inherited as a single unit and therefore constitute a phenogroup (23). The present data identifies a minimum of eight such antigenic specificities.

In the course of this work, a very sensitive method for detecting 7S  $\gamma_{2a}$ -globulins of a specific isoantigenic type was developed.

Before discussing the genetics of the antigens, several points on the production of the isoantibodies and the precipitation of labeled antigens warrant further discussion. An antigen which was itself an antibody directed to some protein or tissue component in the host was much more immunogenic than gamma globulin from normal sera in all cases where a direct comparison was made. Several workers have specifically studied the immunogenicity of antigen-antibody complexes and found them to be far more effective as antigens than the antigen alone (24, 25). It is quite probable that a similar phenomenon is occurring here, the complex being formed in the recipient animal.

Several points indicate that the antibodies produced are highly avid. The precipitation of a trace-labeled antigen is as complete at approximately 0.01  $\mu\text{g}$  antigen per tube (or 0.1  $\mu\text{g}/\text{ml}$ ) as at the concentrations, 100 or more times greater, commonly employed in precipitation assays. A single precipitation of the labeled mouse  $\gamma$ -globulin without cold antigen is maximal. In contrast, some rabbit isoantisera require two or more sequential precipitations with carrier unlabeled antigen to reach maximal precipitation (26).

The various conditions of precipitation described in Materials and Methods (time and temperature of incubation, force of centrifugation, etc.), have all been tested and found to be sufficient for maximal precipitation. The use of S-dil and 3 per cent BSA as diluents probably serves two purposes: (a) to provide a high protein concentration to reduce adsorption of the small amounts of antigens to the glass surfaces and (b) some component of rabbit serum is advantageous in obtaining maximal precipitation.

The precipitation of trace-labeled preparations of  $\gamma$ -globulin by specific isoantisera was never greater than 75 per cent. Immunoelectrophoresis of the labeled antigens with polyvalent rabbit antisera showed the preparations to contain a single  $\gamma$ -globulin arc, although occasionally a line which may be due to  $\gamma\text{X}$  (27) is also present. The lack of contamination with  $\beta$ -globulins is confirmed by the failure of specific anti- $\beta_{2A}$  and anti- $\text{Hc}^1$  (a  $\beta$ -globulin  $\text{Hc}^1$  component) sera to precipitate any significant amount of label. However, the preparations do contain 7S  $\gamma_1$  as well as 7S  $\gamma_2$  (Table I). The slightly higher precipitation by rabbit anti-mouse 7S  $\gamma_2$  than by the specific isoantisera is presumably due to the content of 7S  $\gamma_{2b}$  as well as 7S  $\gamma_{2a}$ . The differences between the various preparations in the amounts precipitated by isoantisera could therefore represent different proportions of  $\gamma_2$ -subclasses a and b. Since the precipitation by polyvalent rabbit antisera did not reach the level of trichloroacetic acid precipitation, it is possible that the method of trace labeling causes some partial

denaturation of protein and/or that there is some protein not recognized by the antisera.

Antigenic specificities for a given antigen are assigned with data derived from the precipitation of labeled antigens and inhibition assays. The inhibition of precipitation of an  $I^{125}$ -labeled antigen by unlabeled antigens is a sensitive and powerful method of detecting cross-reactions (CR). In the order of 0.01  $\mu$ g of labeled antigen was used in this work with at least 100 times as much of the test or inhibiting antigen. This latter need not be purified but can be added in the form of whole serum. The inhibition of precipitation is due to competition between the labeled and unlabeled antigens for antibody. However, as only that fraction of the antibody population which combines with both antigens is competed for, cross-reactions and identities can be distinguished. The principle is analogous to that involved in absorption of an antiserum by an antigen and then using the absorbed antiserum to react with a second antigen (14–16, 23). If a particular antigen present in excess partially (not completely) inhibits the precipitation of a labeled antigen by an antiserum, this indicates that (a) the antiserum must contain antibodies to  $n$  antigenic specificities in the labeled antigen, where  $n \geq 2$ , and (b) the test antigen contains from 1 to  $n - 1$  of these specificities.

In this work a given antigenic specificity has always been defined with a single antiserum. The assignment of a numbered specificity to two strains means that they do share at least one specificity. Further analysis with other strains may show that a specificity can be divided into two or more. Table VIII gives the minimum number of specificities compatible with the data. Although only one antiserum has been used to define a given antigenic specificity, antisera produced in other strain combinations have been shown to have patterns of reactions consistent with the definition of that specificity. While positive results of this type are consistent with the assigned specificities, these antisera may be reacting to a second specificity which is coincident in its distribution among the tested strains. Such a cryptic specificity could only be revealed if a strain could be found in which it is absent.

Comparisons of degrees of partial inhibition (CR values between 0 and 100) have not been used in the definition of any of the antigenic specificities. However there are many cases where the assignment of specificities is supported by this type of data. There are two possible interpretations of differing intermediate CR values. For example, if two test sera A and B gave CR values respectively of 70 and 30 in a given inhibition assay, it could indicate that: (a) the antiserum reacts with at least three antigenic specificities in the labeled antigen, and that test serum A has two of these three, while test serum B has only one of the three; or, (b) the antiserum may only recognize two specificities in the labeled antigen, but the serum titers of the two antibodies are markedly different. If serum A inhibited one of these antibodies, and B the other, the

residual amount of total antibody to the labeled antigen would be different, and would therefore precipitate different amounts. In some cases, we can infer that the antibody titers are similar and hence, the former explanation can be used as supportive evidence.

Further expansion of the table of specificities will depend upon the production of new isoantisera. The most satisfactory combinations would be ones in which according to Table VIII, only a unispecific antiserum should be produced. An example of this would be to immunize RIII with DBA/2 serum in an attempt to produce an antiserum that would only react with specificity 7. If the precipitation of labeled antigen of the immunizing strain by such an antiserum could be partially, but not completely, inhibited by any test serum, it could only mean that the antiserum was reacting to more than one antigenic specificity; *i.e.*, was not unispecific. This would then define a new specificity. By this means, the specificities assigned to a given allele would be confirmed or more would be added. Similar tests with all strains currently grouped with the same type strains would be necessary in order to detect any new alleles. If a strain were found to be different from its respective type strain, it would become a new type strain for a new allele.

Each individual antigenic specificity, designated by a number, indicates that all antigens having this number have structural features which allow them to react with a common fraction of the antibody population. The chemical nature of the antigenic specificities is however at present unknown. Although it is not formally ruled out that these antigens are carbohydrate, it is likely that they result from protein structural differences, in view of the large number of cross-reactions observed.

The relationship between the defined antigenic specificities and the structure of the whole antigen has not been completely understood in any cross-reacting antigen system. Individual specificities may represent single haptenic groups or combinations of them, and cross-reactions would be a reflection of the distribution of the haptenic groups among different antigens. Or, as proposed by Owen; "The symbols (specificities) actually stand for fractions of antibody in a heterogeneous molecular population" (28). In this case, antibody molecules in a given antiserum are all produced in response to a single antigen and have varying affinities for related antigens. Thus, the related antigen could absorb some, but not all, of the antibody molecules in the antiserum. These two explanations are not mutually exclusive and are both formally consistent with the serological analysis carried out in this paper.

The relationship between the antigenic analysis and the structure of the antigens depends on whether the antigenic specificities are all on the same molecules or on separate molecules. The data in Table VII indicates that some of the shared specificities are on the same molecules. The tendency to reach the same maximum per cent precipitation of a given labeled antigen preparation

with all antisera reacting with the preparation, supports this interpretation. However, in view of the great heterogeneity of gamma globulins (29), these results are also consistent with a statistical sharing of specificities. That is, there may be many subpopulations of molecules each sharing some but not all "subspecificities" 1, 1', 1'', 1''' . . . , 2, 2', 2'', 2''' . . . present in that strain so that all the molecules would be precipitated by any antiserum with anti 1 and anti 2 activity as defined above. Subspecificities may be thought of as slight differences in association constants between antigen and antibody molecules.

The conclusion that each of the eight antigens are controlled by alleles at one locus is based upon genetic and molecular evidence. The first four antigens identified Ig-1a through Ig-1d were shown to be controlled by alleles by direct genetic test. That the remaining four Ig-1 antigens are controlled by alleles at the same locus requires a statement of the meaning of the term "locus" in a mammal. "Locus" in higher organisms has come to be synonymous with "cistron" in microbial genetic terminology, that is, the region of chromosome coding for the structure of a polypeptide chain. The original definition of locus as a region not separable by crossing over has been neglected whenever it became inconsistent with the newer structural definition (30, 31). Thus, for our purposes here, the association of the various Ig-1 antigens with a single polypeptide would be the most cogent evidence for terming Ig-1 a locus. It has been shown that some, if not all, of the Ig-1 antigenic specificities are on the same class of protein molecules. Furthermore, it has previously been demonstrated that they are associated with only one constituent polypeptide chain of these molecules (11). Thus, except for the reservations concerning some of the antigenic specificities, we conclude that the eight antigens are all controlled by alleles at the Ig-1 locus.

The evolutionary mechanism of development of a complex multiallelic locus such as Ig-1 may have been based on crossovers within the locus giving new combinations of antigenic specificities. Although no evidence of crossing-over in approximately 150 mice tested has been obtained, this number of animals still allows for a maximum crossover distance between specificities of approximately 2.5 per cent, which is considerably greater than the known value for the interlocus distance for the genes dilute and short ear (32). This proposed mechanism is similar to observations in the H-2 system where new alleles have been actually known to arise by crossing-over (15). Different specificities may also have arisen by mutation.

Of all the strains so far examined in detail, only one of the alleles (Ig-1<sup>b</sup>) determines an antigenic specificity (No. 4) that is not present in any other antigen. Gamma globulin of this type would therefore be antigenic for a recipient strain of any other allele. The other gamma globulins differ by virtue of the combinations of antigenic specificities composing the antigen.

The knowledge of the antigenic specificities in a certain strain could be of

assistance in tracing the lineage of that strain. Several examples are known of strains of a common origin showing the common specificities (CE, DE; SEA, SEC; the C57BL strains; and the NZ strains).

A notation for mouse immunoglobulin loci and their phenotypes has not been generally adopted and several notations have been published (5-8, 10, 11). It would obviously be desirable to find one which is generally acceptable. The revised nomenclature recently proposed for the Rh blood group system in man and the histocompatibility genes and antigens in the mouse (16, 33), provides a model for the notation used in this paper (13). In view of the cross-reactions between the strains so far studied, the designation of a type strain for each allele is advantageous.

The antigens recognized by an antiserum in one laboratory may be presumed to be identical with those in another laboratory if sufficient evidence exists of consistency of reactions of the antiserum with a large number of inbred strains. It is likely, that the antigens  $\gamma^{BA}(5)$ ,  $Asa^1(34)$ , and  $MuA1(7)$  refer to the same allele designated in this paper as  $Ig-1^a$ ;  $MuA2(7)$ ,  $Asa^2(34)$  probably are equivalent to  $Ig-1^b$ ;  $Asa^3(34)$  to  $Ig-1^c$ ;  $Asa^4(34)$  to  $Ig-1^d$ ; and  $Asa^5(34)$  to  $Ig-1^f$ . The notations  $Gg$  and  $Iga$  previously used in this laboratory are replaced by  $Ig-1$  system.

The usefulness of the immunoglobulin isoantigens as markers in many systems is readily apparent. Donor type antibody production in rabbits by transferred donor lymphoid cells (35), and donor type gamma globulin synthesis in mouse radiation chimeras (36, 37) exemplify their application in cell transfer systems. Further use as markers has been made in studies on the structure (38-42) and timing of synthesis of gamma globulins (43).

A second immunoglobulin locus,  $Ig-2$ , controlling antigens on mouse  $\gamma_{1A}$  ( $\beta_{2A}$ )-globulin has been found which is closely linked to  $Ig-1$  (10). Further investigation is in progress to detect loci controlling isoantigens on the other subclasses of murine immunoglobulins.

#### SUMMARY

Eight antigens of 7S  $\gamma_2$ -immunoglobulins controlled by alleles at a single locus  $Ig-1$ , have been identified in mice. This locus has previously been shown to determine antigenic specificities on the F fragments of 7S  $\gamma_{2a}$ -globulins. The reactions of these antigens with various isoantisera have shown that the antigens all cross react with one another.

New methods for the analysis of antigenic specificities of soluble proteins are presented in detail. A sensitive method for detecting in the order of 0.01  $\mu g$  of these isoantigens has been developed, based on the quantitative inhibition of precipitation of  $I^{125}$ -labeled antigen.

Cross-reactions of the antigens were analysed in inhibition assays and the data is compatible with the existence of a minimum of eight antigenic specifi-

ties. Each of the antigens is composed of different combinations of these specificities, with only one antigen having a specificity not present in any other.

Sixty-eight mouse strains have been tested with specific isoantisera, and on the basis of the results, have been placed into the eight allele groups.

Evidence for close genetic linkage of the Ig-1 locus and 11 chromosome markers has been sought and not found.

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*Addendum.*—After submission of this report, the article by R. Lieberman and S. Dray (Five allelic genes of the Asa locus which control  $\gamma$ -globulin allotypic specificities in mice, *J. Immunol.*, 1964, 93, 584) referred to in the text as reference 34 was published. Several of their allele groups have here been subdivided. Ig-1<sup>a</sup> and Ig-1<sup>h</sup> are in the Asa<sup>1</sup> group, and Ig-1<sup>c</sup> and Ig-a<sup>e</sup> are in the Asa<sup>3</sup> group.

The system of defining antigenic components (specificities) used by Lieberman and Dray does not meet the necessary criteria for assigning the minimum number of specificities compatible with observed cross reactions. For example, different antigens may differ because they have different combinations of specificities. Each antigen need not have a unique specificity. Thus, no direct comparison is possible between our Table VIII and their specificity assignments.