Chapter XV

GENETIC CONTROL OF MOUSE IMMUNOGLOBULINS*‡

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INTRODUCTION

The immunoglobulins pose an apparently unique and certainly fascinating problem for one interested in understanding the genetic control of protein structure. Included in the immunoglobulins are all proteins with antibody activity as well as structural and synthetically related proteins such as myeloma and Bence-Jones proteins which are produced in pathological conditions. One of the most striking characteristics of these proteins is their extreme heterogeneity (Fahey, 1962a). In each normal individual the immunogloblulin population is composed of thousands, if not millions, of different molecular species having different antibody specificities. Even within a population having similar antibody specificity there still remains extreme heterogeneity in various physicochemical properties. It does not appear within the power of present methods to isolate sufficient of one species to determine its complete structure. It is the heterogeneity which both challenges our imagination to devise genetic theories to explain and which at present defies a straight forward chemical approach culminating in a complete amino acid sequence and three dimensional model of "antibody." Nevertheless, considerable progress has been made in determining the general structure of the immunoglobulins and in elucidating some aspects of their gene control.

The current information on gene control of immunoglobulins is briefly summarized, in a rather stylized fashion, in Figure 1. The efforts of many workers reviewed recently by Cohen and Porter, 1964 and Fougereau and Edelman, 1965) have led to the general understanding that the unit structure of each Ig molecule has four polypeptide chains; two identical chains of approximately 25,000 molecular weight, the L (light) chains, and two identical chains of about 50,000 molecular weight, the H (heavy) chains. The immunoglobulins of all species studied in any detail are readily divided into several classes on the bases of molecular weight, antigenic, physiologic and other characteristics. We will not survey all this information but will, instead, use the mouse as illustrative of all the major points to be made.

In the mouse, five immunoglobulin classes have been defined (Fahey, *et al.*, 1964a, b). These classes differ in the type of H-chain in the molecules (represented by the larger bars of different colors on Fig. 1). The L-chains found in all classes are apparently the same and are indicated by the short grey bars on the figure. Molecules of γA and γM exist as polymers of the basic unit while those of the other classes exist as the basic four chain unit. The carbohydrate content (Fahey *et al.*, 1964a), catabolic rate (Fahey and Sell, 1965) and physiological activity of the different classes differ characteristically

*This investigation was supported by United States Public Health Service Research Grants GM-12075, CA-04681 and 5TI-GM295.

†Fellow of the Helen Hay Whitney Foundation.

‡With Appendix of special immunological methods prepared by Leonore A. Herzenberg.

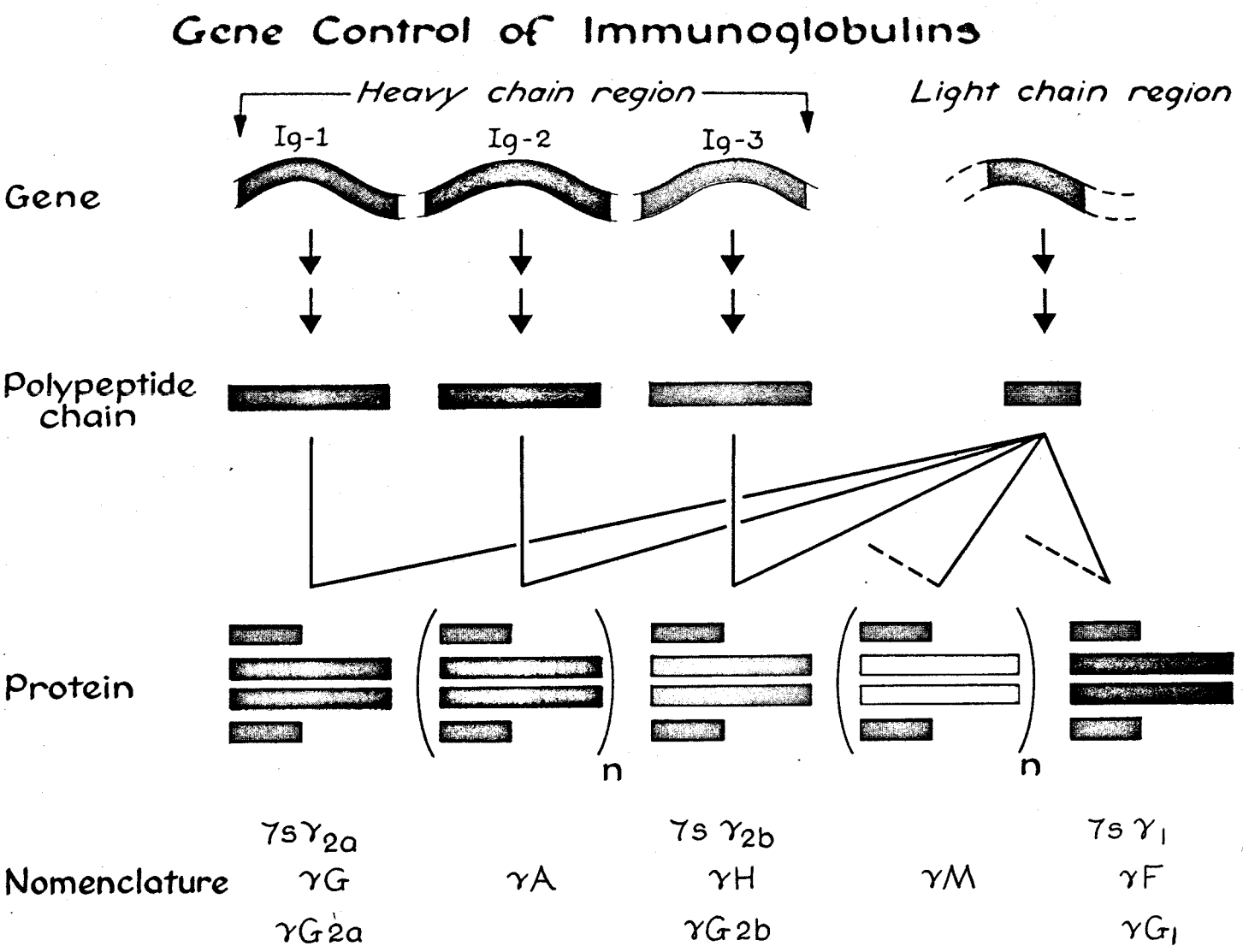

FIGURE 1. Gene control of immunoglobulins in the mouse. The five immunoglobulin classes of mice are depicted after the Porter model. The heavy polypeptide chains are shown to be determined by linked genes in a chromosome region. The L-chains, identical for all the classes, are (presumably) determined by genes in an unlinked region.

(Nussenzweig *et al.*, 1964; Ovary *et al.*, 1965).

The color and symbol scheme adopted in this figure will be used throughout this article. Long bars will represent H-chains, and short bars L-chains. A wavy bar will represent the DNA sequence of a particular locus. The color yellow will pertain to γG_{2a}, green to γA, pink to γG_{2b}, blue to γM, orange to γG_1 and grey to L-chains.

In accepting the simplification presented in the diagram, it must be borne in mind that a tremendous heterogeneity of amino acid sequences exists presumably associated with the antibody combining sites, among the polypeptide chains of each class (Koshland and Engelberger, 1963). More relevant for our purposes here, though, is that all H-chains of one class do have regions of common amino sequence(s) which are presumably responsible for the properties which allow us to group the immunoglobulins into classes (Potter *et al.*, 1966).

Each polypeptide chain type is determined by a separate gene locus. (We will return later to speculate as to the genetic basis for the antibody-site-associated heterogeneity within each group). The H-chain gene loci thus far identified, Ig-1, Ig-2 (Herzenberg, 1964), Ig-3 (Warner *et al.*, 1965) are in a single chromosome region (Herzenberg, 1964; Lieberman *et al.*, 1965). Since allotypes (genetic polymorphisms) in the H-chain classes γG_1 and γM, have not yet been found, these gene loci have not been specifically designated in the diagram, nevertheless we presume that they are in the same region. Similarly, no allotypes are yet known for L-chains in the mouse but by analogy with the rabbit and man (Oudin, 1960; Steinberg, 1962), we presume that there is a second chromosome region for L-chain determination (as pictured in Fig. 1).

All the above immunoglobulin classes are found in each normal individual. In humans, dysgammaglobulineamias are known (reviewed in Stiehm and Fudenberg, 1966), where one or more classes of immunoglobulins are missing or greatly diminished. In our studies with the mouse, we have found no example of an inbred strain missing an immunoglobulin class.

Much confusion concerning even the very existence of immunoglobulin classes may be traced to the early unitary conception of antibody or γ-globulin as basically a single protein which is somehow modified according to the antigenic stimulus (Breinl and Haurowitz, 1930; Pauling, 1940). It is now clear that in most immune responses, antibodies are evoked in many or all immunoglobulin classes (Fahey, 1962a). It is true, however, that different biological activities of antibodies are associated with particular immunoglobulin classes. The existence of a multiplicity of classes in most antibodies and in the total immunoglobulins of all individuals cannot be ignored in any genetic or chemical studies of the immunoglobulins.

Understanding of the structure and genetic control of immunoglobulins can be greatly facilitated by making use of the striking resemblance of many features of the immunoglobulins to the human hemoglobulins. To develop this analogy, first made by Fudenberg *et al.* (1963), to explain some pathological dysgammaglobulinemias some aspects of hemoglobin structure and genetics must be described.

Hemoglobin A (the major adult hemoglobin component) is composed of two α and two β-polypeptide chains. Hemoglobin F (fetal) also has two α-chains, but the β-chains are replaced by γ-chains. Similarly in hemoglobin A_2 and hemoglobin Gower I the β-chains are replaced respectively by δ and ϵ-chains. Thus the hemoglobin α-chains are analogous to the immunoglobulin L-chains, in being common to all the hemoglobins; and the β, γ δ, and ϵ-chains are analogous to various immunoglobulin H-chains.

TABLE I

ANALOGY BETWEEN HEMOGLOBLIN AND IMMUNOGLOBULIN PROTEINS

Hemoglobins			*Immunoglobulins*		
	Polypeptide			*Polypeptide*	
Protein	*Common*	*Specific*	*Protein*	*Common*	*Specific*
HbA	α	β*	γM	L‡	$H^{\gamma M}$
HbA_2	α	δ*	γA	L	$H^{\gamma A}$†
HbF	α	γ*	γG_1	L	$H^{\gamma G_1}$
Hb Gower-2	α	ϵ	γG_{2a}	L	$H^{\gamma G_{2a}}$†
			γG_{2b}	L	$H^{\gamma G_{2b}}$†

*Determined by closely linked genes.

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‡Two types of L-chains have been described in the mouse. Presumably both are common to all immunoglobulin classes.

The nomenclature for the specific chains is an ad hoc one for use in this table.

each being "class" defining. (See Table I) (reviewed by Huehns and Shooter, 1965).

The amino acid sequences of the β, γ, and δ-chains are quite similar, suggesting a genetic (evolutionary) relationship to each other. While the same detailed information is not yet available for the immunoglobulins, Potter's (Potter *et al.*, 1966) demonstration of the similarity of γG_{2a} and γG_{2b} H-chain peptide maps suggests a close relation between these two immunoglobulins classes. On the other hand, the fingerprints of γG_1 and γA H-chains have no marked similarity to each other, or to γG_2 H-chains (Potter *et al.*, 1964; Potter *et al.*, 1966). This is consistent with the known antigenic cross-reactions between γG_{2a} and γG_{2b} and lack of, or possible minor, cross-reaction between the H-chains of the other immunoglobulin classes (Fahey *et al.*, 1964a).

The genetic control of the immunoglobulins and the hemoglobins also display striking resemblance. The gene loci for β, γ and δ-chains are all closely linked, that is, are in one region of a chromosome. The α-chain locus is unlinked to this region (Heuhns and Shooter, 1965). Similarly, taking together the immunoglobulin data from mouse, rabbit and man, we find that loci determining H-chains are clustered in a chromosome region, that is, they are closely linked genetically, and that the L-chain loci are in a different chromosome region.

In neither hemoglobins nor immunoglobulins has direct evidence been found for genetic crossovers between the closely linked loci determining the β, γ, and δ-chains or between the loci determining H-chains. The reason for calling them separate loci is that, like the microbial cistron, they code for distinct polypeptide chains *and* are transmitted in the same haploid set. That is, each locus contains a length of DNA whose nucleotide sequence codes for a particular polypeptide chain, and each diploid individual has two chromosomes with this

locus. At each locus genetic variation is possible, the alternate forms of the DNA sequence being called alleles, and normal genetic segregation of these alleles is observed in both hemoglobin and immunoglobulins.

GENETIC VARIATION AT THE IG-1 LOCUS

Eight alleles at the mouse immunoglobulin locus Ig-1 have been recognized by immunogenetic techniques (Herzenberg *et al.*, 1965). Two of these, Ig-1^a and Ig-1^e, together with the proteins they determine, are shown in Figure 2. These alleles are found in the mouse inbred strains BALB/c and NZB, respectively, and (as is depicted by the yellow bar in the figure) determines antigens on γG_{2a} H-chains.

To indicate the alternate forms of the DNA sequence (i.e., alleles) at the Ig-1 locus, the wavy yellow bars are bordered with dark green for Ig-1^a and dark red for Ig-1^e. To indicate the protein products of these alleles, which are γG_{2a} H-chains distinguishable from each other allotypically(isoantigenically), the straight yellow bars are outlined bordered with the same respective dark colors.

Also depicted in Figure 2 is that in heterozygotes the γG_{2a} globulins produced are either of one or the other allelic type (allotypes). Ig-1^a and Ig-1^e molecules are found in the serum of an a/e heterozygote but no molecules with *both* a and e allotypes are detected. Although this point has not been well investigated in the mouse, the evidence available is entirely consistent with the well documented absence of a detectable population of hybrid molecules in allotype-heterozygous rabbits (Dray *et al.*, 1963). This absence is not due to chemical incompatibility of allotype carrying chains since hybrid molecules of rabbit immunoglobulins containing both allotypes can be made reassociate *in vitro* by chain dissociation and recombination (Mannik and Metzgar, 1965). A figure similar to Figure 2 presumably could be drawn for each of the immunoglobulin classes for which allotypes exist.

We should at this point pause to distinguish clearly between the two kinds of immunoglobulin variation under gene control which we have so far described. On the one hand there is the variation which arises because of the existence in one individual of several gene loci which allow the simultaneous production of related polypeptide chains. On the other hand, there is the variation which exists due to the alternate forms of the gene (e.g., mutants) which can exist at a given locus. That is, a class of immunoglobulins should be defined as those molecules having H-chains whose characteristics are determined at a single locus. Superimposed upon the variation from class to class within an individual is the variation between individuals (polymorphism) due to the variety of alleles which may exist at each locus. These latter are the allotypic differences. Thus an immunoglobulin will have one or more class defining specificities which identify it as the product of a particular locus, and one or more allotypic specificities which identify it as the product of a particular allele at that locus.

Fahey *et al.* (1964a) defined four immunoglobulin classes in the mouse, γA, γM, γG_1 and γG_2. They then found that the γG_2-globulins could be subdivided into two groups which have specificities in common as well as unique specificities (Fahey *et al.*, 1964b). The two groups were called the γG_{2a} and γG_{2b} subclasses of γG_2. Potter confirmed Fahey's protein classification by showing, as mentioned earlier, that γG_{2a} and γG_{2b} Fc fragments have some peptides in common with one another and some different, whereas γG_1 proteins differ consid

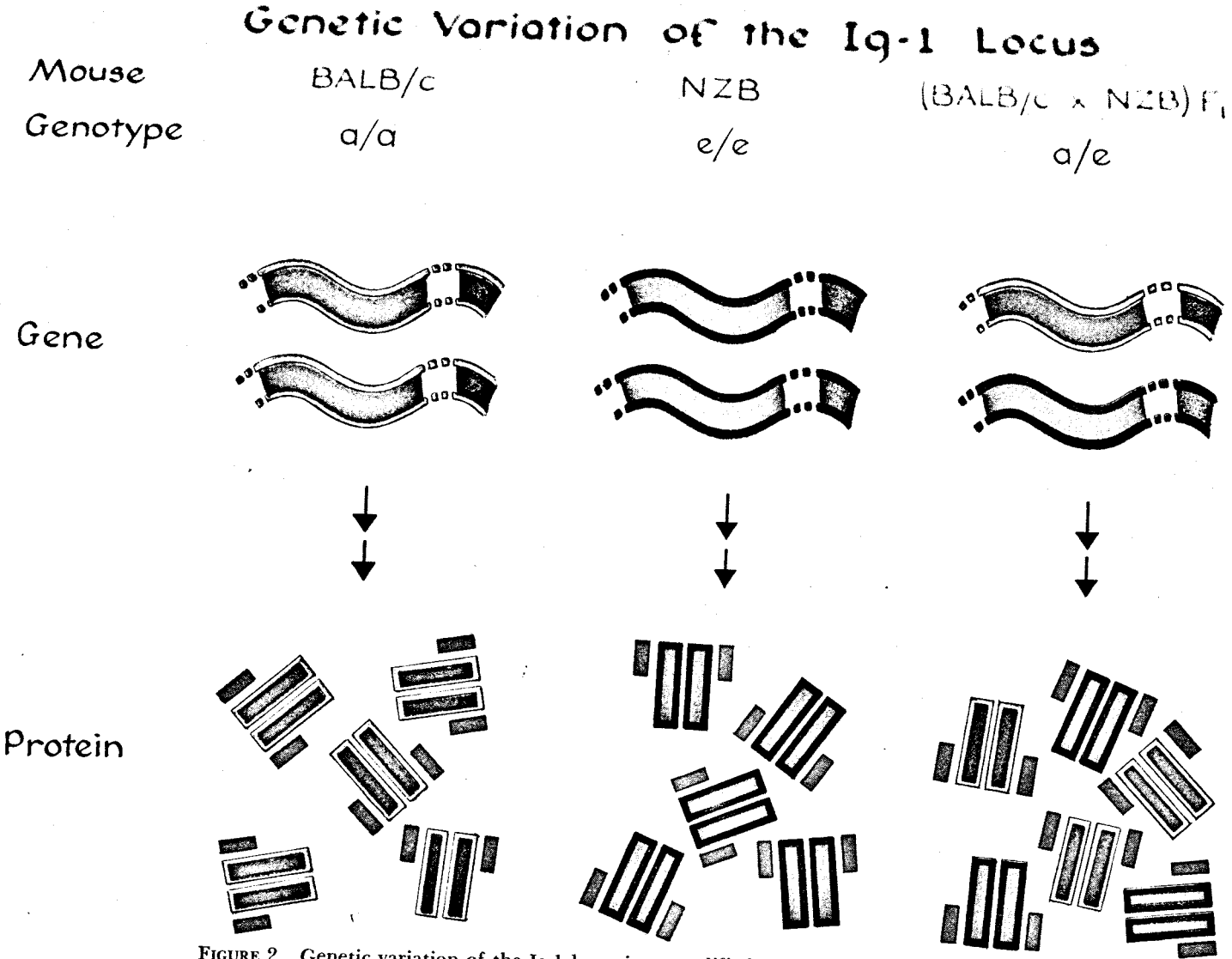


FIGURE 2. Genetic variation of the Ig-1 locus is exemplified for two inbred mouse strains BALB/c and NZB. The allotypic variation is indicated by a red or blue outline over the Ig-1 gene or protein. In heterozygous mice individual molecules carry heavy chains of only one allotypic type.

TABLE IIa

ANTIGENIC SPECIFICITIES CONTROLLED BY Ig-1 ALLELES ON γG_{2a} IMMUNOGLOBULINS

Allele	*Type Strain*	*Antigenic Specificities*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Ig-1a	BALB/cJ	1*	2	–†	–	–	6	7	8	9	10
Ig-1b	C57BL/10J	–	–	–	4	–	–	7	–	9	–
Ig-1c	DBA/2J	–	2	3	–	–	–	7	–	9	–
Ig-1d	AKR/J	1	2	–	–	5	–	7	–	–	–
Ig-1e	A/J	1	2	–	–	5	6	7	8	–	–
Ig-1f	CE/J	1	2	–	–	–	–	–	8	9	–
Ig-1g	RIII/J	–	2	3	–	–	–	–	–	9	–
Ig-1h	SEA/Gn	1	2	–	–	–	6	7	–	9	10

*Number indicates specificity is present.

†Dash indicates specificity is absent.

TABLE IIb

ANTIGENIC SPECIFICITIES CONTROLLED BY Ig-3 ALLELES ON γG_{2b} IMMUNOGLOBULINS

*Allele**	*Type Strain*	*Antigenic Specificities*			
		1	*2*	*3*	
Ig-3a	BALB/c	1	2	–	
Ig-3b	C57BL/10J	–	–	–	
Ig-3e	A/J	1	–	3	

*The alleles are lettered to correspond with the Ig-1 alleles.

erably from both (Potter *et al.*, 1966). However, since each H-chain is the product of a single locus, we would prefer to consider γG_{2a} and γG_{2b} simply as two classes of immunoglobulins, thus making at present five immunoglobulin classes. γG_{2a} is determined at the Ig-1 locus and γG_{2b} is determined at the Ig-3 locus.

To return to consideration of the genetic variation at the Ig-1 locus: In Figure 2 we depicted two alleles at this locus and their corresponding allotypes. Eight such alleles are known. The allotypes determined by each of these alleles are comprised of multiple antigenic specificities inherited as a phenogroup. Ten such specificities have been defined (Warner *et al.*, 1966).

In Table IIa the Ig-1 alleles are listed with the type strain in which they are found and the set of specificities which they determine. Table IIb lists the isospecificities presently detected for Ig-3. The specificities are symbolic representations of the cross-reactions of the antigens determined by each allele. These cross-reactions have been studied by the use of precipitation and inhibition of precipitation of I^{125} labelled myeloma proteins and immunoglobulins isolated from normal sera (see Appendix). The presence of a particular number (specificity) in two allelic types (in Table IIa) signifies that these strains have some structural site in common which is absent from the γG_{2a} globulins in mice not having that specificity.

The chemical nature of this structural site is unknown. It might represent a particular conformational arrangement of a few amino acids on the surface of the polypeptide chain. Two specificities present on the same molecule could be interpreted as being two physically separate determinants or a single determinant eliciting a population of antibodies only part of which reacts with a second antigen. The participation of a carbohydrate moiety in the antigenic determinant has not been eliminated.

In spite of the lack of information regarding the chemical nature of the antigenic specificities, they are very useful as markers of the immunoglobulin genes at both the cellular (Herzenberg and Cole, 1966; Warner *et al.*, 1965) and molecular level.

Recent work with some newly produced mouse myeloma proteins, utilizing these specificities as gene markers, has led to further understanding of the genetic control of immunoglobulins (Warner *et al.*, 1966;

PRODUCTION OF PLASMA CELL TUMORS IN MICE

The induction of plasma cell tumors in mice by injection of mineral oil, adjuvants, and by other means has been reported by several groups (Rask-Nielsen and Ebbesen, 1965; Merwin and Algire, 1959; Potter and Robertson, 1960). After injection of mineral oil 68 per cent of BALB/c mice developed plasma cell tumors (Potter and Robertson, 1960), whereas in another laboratory using the same procedure very few DBA/2 or CBA mice developed plasma cell tumors (Rask-Nielsen and Ebbesen, 1965). A large incidence (around 40 to 50 per cent) of various types of reticulum cell sarcomas, however, did occur in these latter strains through this induction procedure. The genetic constitution of the mouse may therefore be of importance in the nature of the tumor developed.

In recent studies (Goldstein *et al.*, 1966) twenty-eight hybrid mice from the cross NZB X BALB/c were injected three times with medicinal paraffin and, by fifteen months after injection, fourteen of these mice (50 per cent) had developed peritoneal tumors. Of these tumors, nine were plasma cell tumors producing a myeloma protein (Table III). Eight of these nine are being successfully maintained (now up to the 5-6th passage) in (NZB X BALB/c)F_1 mice. One of these tumors, although still passaging, no longer produces myeloma protein. In this case, it is possible that a nonplasmacytic tumor also arose in the original mouse and it is this tumor which is now being maintained.

TABLE III

INDUCTION OF TUMORS IN (NZB × BALB/c)F_1 MICE WITH MEDICINAL PARAFFIN

Tumor	*Morphological Type*	*When Tumor Developed*	*Myeloma Protein Class*	*Transplantation*
GPC-1	P. C. Tumor	11 months	γG_1	Yes—But no myeloma
GPC-2	P. C. Tumor	11 months	γA	Yes
GPC-3	P. C. Tumor	10 months	γA	Yes
GPC-4	P. C. Tumor	9 months	γA	Yes
GPC-5	P. C. Tumor	11 months	γG_{2b}	Yes
GPC-6	P. C. Tumor	13 months	γA	Yes
GPC-7	P. C. Tumor	14 months	γG_{2a}	Yes
GPC-8	P. C. Tumor	14 months	γG_{2a}	Yes
GPC-9	Not classified		No myeloma detected	No successful passage
GPC-10	P. C. Tumor		γA	Yes
GPC-11	Not classified		No myeloma detected	No successful passage
GPC-12	Not classified		No myeloma detected	Yes
GPC-13	Not classified		No myeloma detected	Yes
GPC-14	Not classified		No myeloma detected	No successful passage

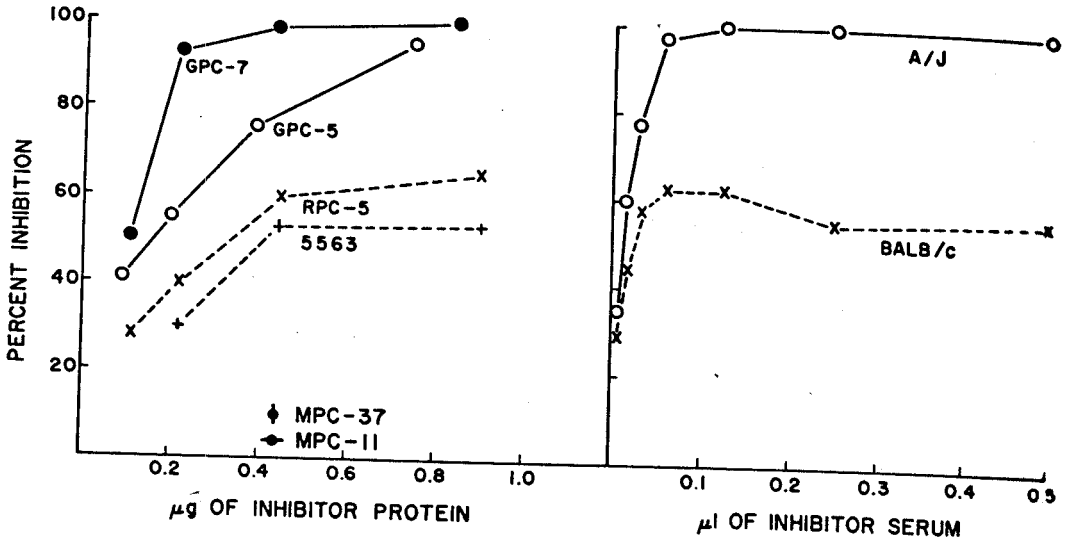


FIGURE 3. Inhibition of precipitation of I^{125} labelled GPC-5 myeloma protein by various purified myeloma proteins or normal sera. Each tube contains constant amounts of labelled GPC-5 protein and of a rabbit antiserum to GPC-7 myeloma protein. The antiserum was previously absorbed with PAPS-conjugate MPC-11 myeloma protein. The amounts of inhibitors are as given in the figure, precipitation conditions as in the appendix.

The other five original neoplasms, which do not produce myeloma proteins, are probably not plasma cell tumors, but have not yet been rigorously classified histologically.

The immunoglobulin classes of each of the myeloma proteins produced by the GPC tumors have been determined using specific inhibition assays. Each myeloma protein, with the exception of GPC-5 (see later section), inhibited in only one class specific assay. The immunoglobulin classes found are listed in Table III. A detailed allotypic analysis of the three γG_2 myelomas is presented in the following sections.

ANTIGENIC ANALYSIS OF γG_{2a} MYELOMA PROTEINS FROM (NZB X BALB/c)F_1 MICE

Two of the myelomas described in Table III, GPC-7 and GPC-8 have been found to be γG_{2a} immunoglobulins by both rabbit and mouse antiserum typing. Since BALB/c and NZB Ig-1 allelic products are antigenically distinguishable it was possible to determine whether one or both alleles determine characters of the myeloma protein. GPC-8 carries all the BALB/c Ig-1 specificities and GPC-7 all the NZB Ig-1 specificities (Table IV). Thus each of these two γG_{2a} myeloma proteins carries the antigenic specificities determined by only one or the other of the Ig-1 alleles present in the hybrid mouse in which the tumor arose.

TABLE IV

ANTIGENIC SPECIFICITIES PRESENT ON GPC-7 AND GPC-8 MYELOMA PROTEINS AND NORMAL γG_{2a} GLOBULINS

NZB/Bl γG_{2a}	Ig-1.1, 2, 5, 6, 7, 8
BALB/c γG_{2a}	Ig-1.1, 2, 6, 7, 8, 9, 10
GPC-7	Ig-1.1, 2, 5, 6, 7, 8
GPC-8	Ig-1.1, 2, 6, 7, 8, 9, 10

Immunoglobulin molecules contain two H-polypeptide chains, the structure of which is determined at the same locus (or

(BALB/c x NZB) F_1 Hybrid

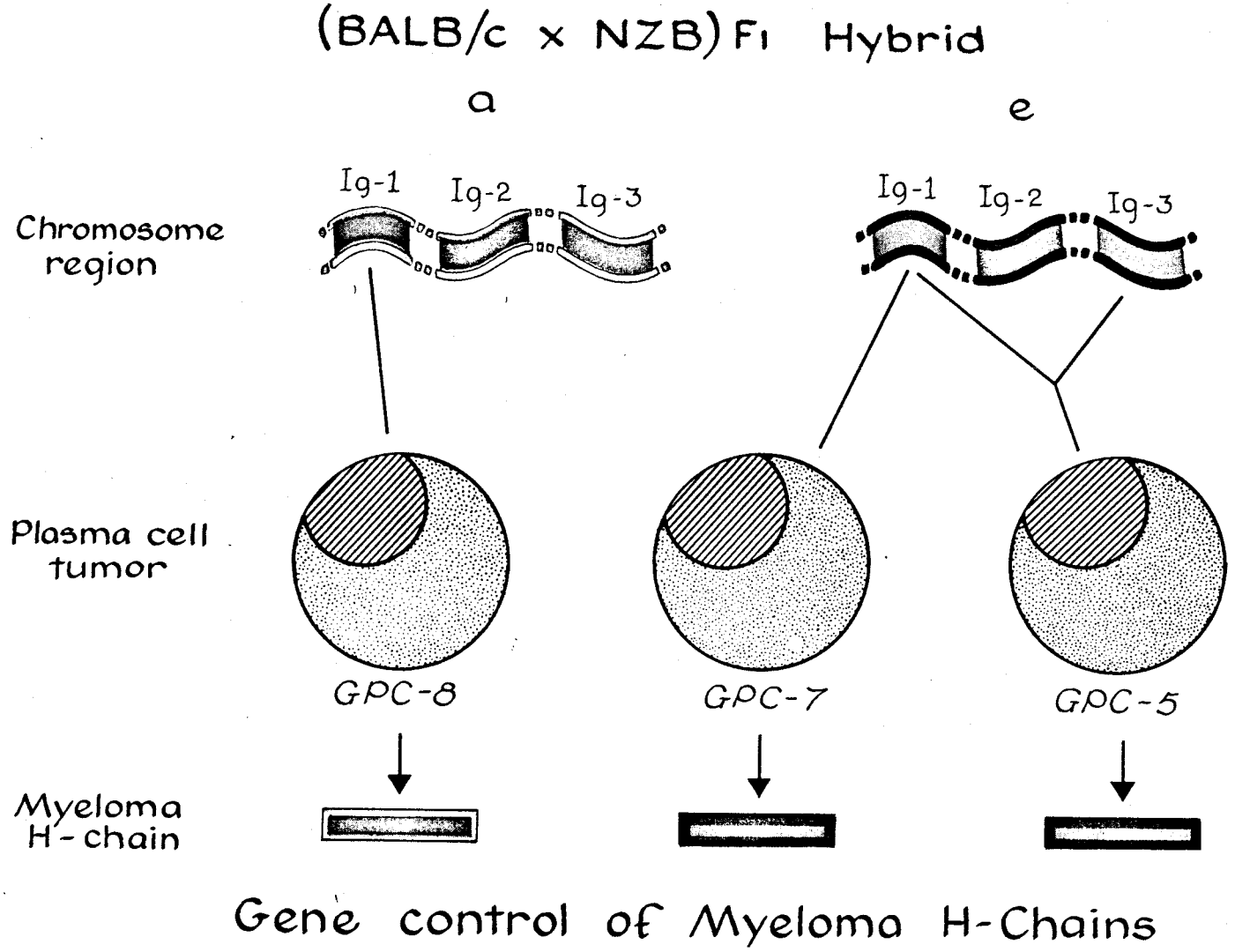


FIGURE 4. Schematic representation of the genetic determination of the H-chains of three γG_2 myeloma proteins produced by plasma cell tumors arising in (BALB/c x NZB) F_1 hybrid mice. The Ig-1^a allele determines GPC-8, H-chains Ig-1^e GPC-7 H-chain and parts of both Ig-1^e and Ig-3^e are involved in the GPC-5 determination of the H-chain.

TABLE V

EXPRESSION OF ALLELES AT IMMUNOGLOBULIN LOCI IN HETEROZYGOTES

Author	*Species*	*Immunoglobulin Locus*	*Expression at Cellular or Protein Level*
Harboe *et al.*, 1962	Human	Gm	Myeloma protein carries no more than one parental allelically controlled antigen
Martensson, 1964	Human	Gm	
Kunkel *et al.*, 1964	Human	Gm	
Dray *et al.*, 1963	Rabbit	Ab	Two allelically controlled antigens on different normal immunoglobulin molecules
Warner *et al.*, 1966	Mouse	Ig-1	
Pernis *et al.*, 1965	Rabbit	Ab	Two allelically controlled normal immunoglobulins always synthesized in different plasma cells
Weiler, 1965	Mouse	Ig-1	Two allelically controlled antibodies synthesized in different cells

Fig. 2). Furthermore, in heterozygous animals, both of these chains have been shown to be determined by the same allele at this locus. This selective expression has been demonstrated for human γG myeloma proteins, rabbit γG immunoglobulins, and mouse immunoglobulins (references in Table V). Similarly at the cellular level, in heterozygous animals, only one allele at one locus is expressed in each cell at any one time. A number of examples are given in Table V.

The results obtained with GPC-7 and GPC-8 are therefore in agreement with the general observation that both normal and malignant cells of the plasmacytic series express only one H-chain or L-chain allele at a given locus.

This brings us to the first major point of divergence between immunoglobulins and hemoglobins. In all previously analyzed autosomal systems including the hemoglobins, both alleles of a given locus are expressed in each cell. For example, in sickle cell heterozygotes (genotype *HbA/HbS*) all erythrocytes partially sickle when the oxygen tension is lowered rather than some remaining normal and some sickling (Neel and Schull, 1954). Recently, electrophoresis of single red cells has directly demonstrated the presence of hemoglobin A and hemoglobin C in the same cells (Matioli and Niewisch, 1965). To take another case, it is well established that for blood group antigens (Neel and Schull, 1954) and transplantation antigens, both alleles are expressed in each cell.

In fact, it was generally accepted that for all systems both alleles function at all loci until Lyon (1962) first suggested that some X-linked genes differed in this regard. The immunoglobulin loci which are not X-linked, constitute the first autosomal exception to the general rule.

Since only one allelic type of L or H-chain is synthesized per cell, the absence of hybrid immunoglobulin molecules in hybrid individuals is not surprising. This obviates the necessity of invoking preferential associations of like chains during synthesis within the cell. It is tempting to speculate that the outstanding exception of the immunoglobulin producing cells in having only one allele functioning is of importance to antibody synthesis or function.

ANALYSIS OF A γG_2 MYELOMA CARRYING γG_{2a} AND γG_{2b} SPECIFICITIES

GPC-5 is the third γG_2 producing plasmacytoma arising in (NZB X BALB/c)F_1 mice (Table III). Using rabbit antisera to normal mouse immunoglobulins (from pooled C3H and C57BL/6) and to the BALB/c γG myeloma proteins, GPC-5 myeloma was typed unambiguously as a γG_{2a} protein. That is, no inhibition was observed with GPC-5 in specific γM, γA, γG_1 or γG_{2a} class assays using BALB/c myelomas as labelled antigens whereas in every specific γG_{2b} assay inhibition was always observed.

Antigenic analysis of GPC-5 was then made with mouse isoantisera. Because the protein was typed as γG_{2b} we would have expected to find all the Ig-3 specificities of either BALB/c or all the Ig-3 specificities of NZB and no Ig-1 specificities of either type. As shown in Table IV, all the specificities found on GPC-5 are NZB specificities. Quite surprisingly, however, one of these specificities is an Ig-3 specificity (Ig 3.3) and another is an Ig-1 specificity (Ig 1.5). The rest of the Ig-1 and Ig-3 specificities of NZB type are missing from this myeloma protein.

TABLE VI

ANTIGENIC SPECIFICITIES PRESENT ON GPC-5 MYELOMA PROTEIN AND NORMAL IMMUNOGLOBULINS

BALB/c γG_{2a}	Ig-1.1, 2, 6, 7, 8, 9, 10	
NZB γG_{2a}	Ig-1.1, 2, 5, 6, 7, 8	
BALB/c γG_{2b}		Ig-3.1, 2, 4
NZB γG_{2b}		Ig-3.1, 3
GPC-5	Ig-1.5 and Ig-3.3	

Since we have found isospecificities normally associated with two different classes on GPC-5, it is important to be sure that we are dealing with a single myeloma protein which carries both specificities. More than 95 per cent of GPC-5 molecules carry the Ig-1.5 and more than 95 per cent carry Ig-3.3 as determined by retardation of migration on acrylamide gel of purified labelled GPC-5 protein in the presence of specific isoantisera (see Methods Appendix).

We therefore conclude that more than 90 per cent of GPC-5 molecules must carry both specificities and hence that GPC-5 is a single myeloma protein. In view of finding on GPC-5 a γG_{2a} specificity (Ig-1.5), we attempted to see if a rabbit antiserum could also detect some γG_{2a} antigenic specificity on GPC-5. A rabbit was immunized with GPC-7 myeloma protein (NZB γG_{2a} which also carries Ig-1.5) and tested against both GPC-5 and GPC-7. This antiserum contained a high titer of "myeloma specific antibody," which obscures all cross-reacting systems when the antiserum is used with its homologous antigen. To avoid this complication, we used the antisera (rabbit anti GPC-7) with the nonhomologous labelled myeloma protein (GPC-5*). The results of this inhibition assay are presented in Figure 3. GPC-5, GPC-7 and A/J whole serum completely inhibit this precipitation. Partial inhibition is also found with RPC-5 and 5563 myelomas (both γG_{2a}) and with BALB/c whole serum.

At least two specificities are detected in this assay. The first is found on both GPC-5 and GPC-7 but is not present on BALB/c γG_{2a} or γG_{2b} myelomas, or in fact in BALB/c immunoglobulins (since these latter three do not give complete inhibition). The second specificity, which is shared by GPC-7 (NZB γG_{2a}), RPC-5 (BALB/c, γG_{2a}) and 5563 (C3H, γG_{2a}) but not by MPC-11 or MPC-37 (both BALB/c, γG_{2b}), is then, by definition, a γG_{2a} specificity. GPC-5 also carries this specificity. GPC-5 myeloma does not, however, inhibit other specific γG_{2a} assays, and hence does not have the other

specificities of NZB γG_{2a}. The use of rabbit antisera has therefore extended the observations with mouse isoantisera. In summary, GPC-5 carries the following antigens:

γG_{2a}: Ig-1.5 and at least one of the γG_{2a} class defining specificities.

γG_{2b}: Ig-3.3 and all the known NZB γG_{2b} class defining specificities.

GPC-5 is therefore the first analyzed mouse myeloma protein *not* carrying the entire set of isospecificities normal to its class and allotype. GPC-5 is also the first myeloma protein in either man or mouse shown to carry the antigenic specificities, and inferentially part of the amino acid sequences of two classes of immunoglobulins, i.e., the gene products of two loci (see Fig. 4).

The participation of parts of two loci in the determination of GPC-5 structure may be similar to the genetic determination of Lepore type hemoglobins. These interesting variants of hemoglobin were shown by Baglioni (1962) to contain the N-terminal part of the δ-chain joined to the C-terminal part of the β-chain. The total number of amino acids in the new δ-β chain being the same as in the β-chain. Baglioni postulated that the formation of hemoglobin Lepore genes is the result of nonhomologous crossing-over between corresponding points of the β and δ genes. Such a crossover would result in the formation of unequal products, one of which is a new gene being partly δ and partly β (see Fig. 5).

Such a nonhomologous crossover could also be invoked for the formation of the hypothetical GPC-5 gene. But since the allotypic specificities controlled by the two loci involved are both of NZB type, this recombinational event must have been *intra*chromosomal, i.e., between sister chromatids rather than involving the two homologous chromosomes.

Precedence for such a genetic event is found in two studies by Laughnan. Examples are known in both Maize and Drosophila of adjacent serial duplications of portions of a chromosome (Laughnan, 1961; Peterson and Laughnan, 1963). These duplications retain synaptic homology, i.e., the capacity to pair in meiosis. The presence of these duplications results in phenotypes distinct from those resulting from chromosomes not carrying the duplications. Therefore, reversions to normal are easily recognized and are due to recombinational events. Using marker loci (Peterson and Laughnan, 1963) found a number of reversions which were not ascribable to conventional *inter*chromosomal crossing-over and which he explained as *intra*chromosomal crossover. A double loop configuration of the chromosome was suggested as a basis for this event (see Fig. 6). Alternative non-precedented recombinational processes as well as a mutational origin cannot be excluded.

These speculations as to genetic origin are offered to stimulate further investigation into the detailed chemical structure of this myeloma protein in order to decide among the alternatives.

Regardless of the genetic mechanism involved, the origin of the GPC-5 gene may have occurred during the development of the tumor or may have occurred during the evolution of the NZB genome or may be a regular somatic event occurring in plasma cell differentiation. If the latter two were correct, then this myeloma is the expression of a "normal" immunoglobulin, which should exist in normal NZB serum. Studies are in progress to determine whether this is so.

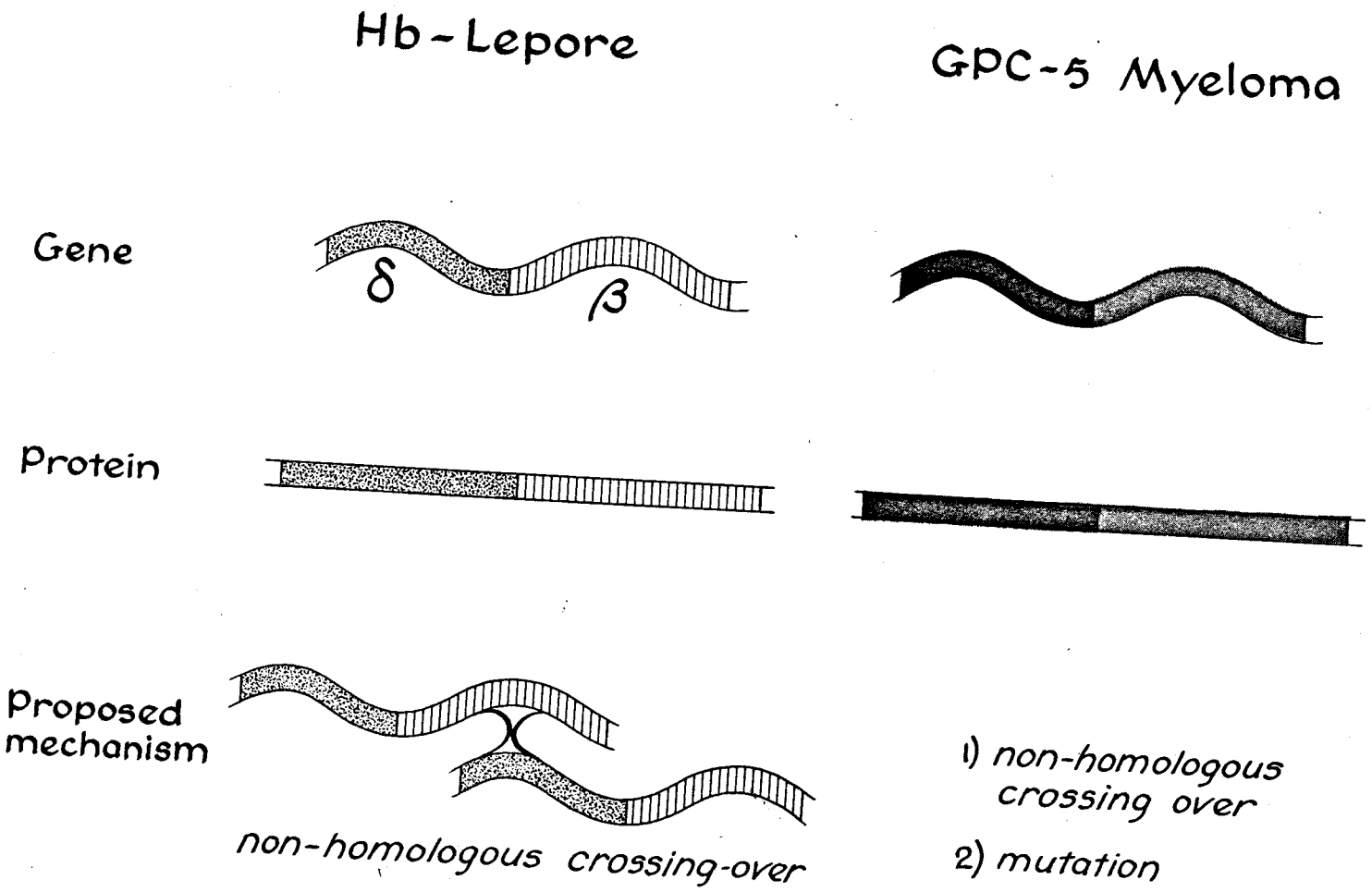


FIGURE 5. Parallels between Hb Lepore (Baglioni, 1962) and GPC-5 myeloma protein. Possible origins of the genes are depicted.

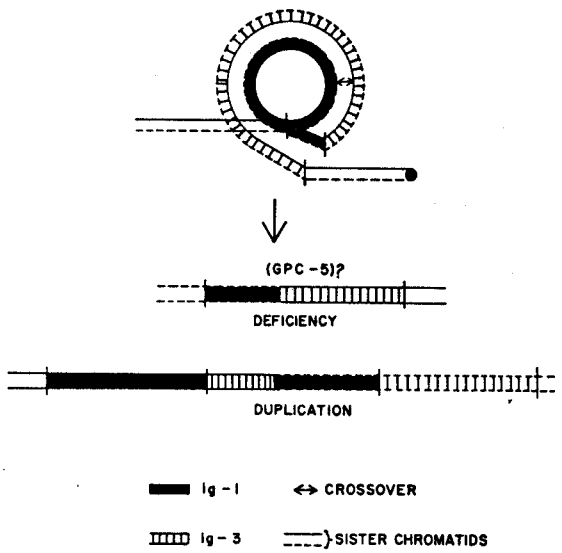


FIGURE 6. Diagrammatic representation of possible intrachromosomal exchange (after Peterson and Laughnan, 1963) resulting in GPC-5. A single chromosome region, posed at the chromatid stage, is drawn in a double loop configuration to allow maximum pairing homology. A crossover is indicated between the sister chromatids. For simplicity of repretation the Ig-1 and Ig-3 genes have been depicted as contiguous.

ISOANTIGENIC ANALYSIS OF γG_2 MYELOMA PROTEINS PRODUCED IN BALB/c OR C3H MICE

In the light of the preceding findings we felt that some of the previously described myeloma proteins arising in homozygous mice should be analyzed for their allotypic specificities since this had not been done in any detailed fashion.

This work is still in progress, but preliminary results show that specificities Ig-1.1, 2, 6, 7 and 8 are found only in two γG_{2a} myeloma studies (RPC-5 and 5563) and not in three γG_{2b} myelomas (MPC-11, -31, -37), whereas Ig-3.1 and 2 are only in the three γG_{2b}. Specificity Ig-1.6 is apparently lacking from 5563 and its absence may be in some way related to this myeloma being one of the longest maintained in passage.

These preliminary findings are consistent with the interpretation that a specificity is determined by alleles at only one locus. However, there is no basic reason for eliminating the possibility that an identical *allotypic* antigenic specificity may be found on the gene products (immunoglobulin molecules) of more than one locus.

DETECTION OF ALLOTYPES WITH HETEROLOGOUS ANTISERA

In the course of the antigenic analysis of GPC-5 with various rabbit anti-mouse immunoglobulin sera, it was found that in some inhibition assays, whole serum of some mouse strains would not completely inhibit the precipitation of the labelled antigen whereas other mouse sera would do so. This demonstrates that the particular rabbit antiserum used contains antibodies which react with the immunoglobulins of one strain and not of another. The corresponding antigens are hence allotypes. These are to be distinguished from other antibodies in the same rabbit antiserum which react with the immunoglobulins of all mouse strains. These latter antibodies react with

the class defining specificities which are present on all immunoglobulin molecules of a particular class, i.e., controlled by a particular *locus,* in all mouse strains, regardless of the allotypic allele at this locus. Thus, the gene at the particular locus has a certain region of DNA which is common to all mice (controls class defining specificity), and has another region which is variable between strains and determines the allotypic specificities.

Several rabbit anti-mouse immunoglobulin sera have been examined to determine both the strain distribution (using the Ig-1 type strains), and the immunoglobulin class of these allotypes.

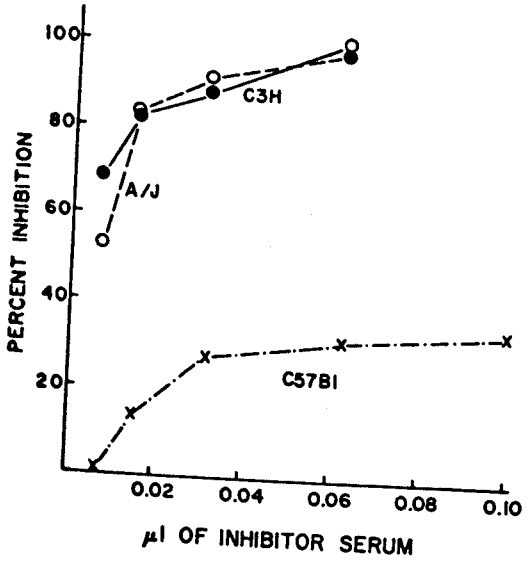
Three allotypic specificities have at present been recognized with rabbit antisera:

1. A rabbit antiserum was prepared to a slow cathodal starch black fraction of pooled C3H and C57BL/6 serum. This antiserum, after absorption with MPC-25, a γG_1 myeloma, was used in an inhibition assay with I^{125} labelled RPC-5 (a BALB/c γG_{2a} myeloma protein). All type strain sera (e.g., C3H, A/J shown on graph) completely inhibit except C57BL/6 (Fig. 7a).

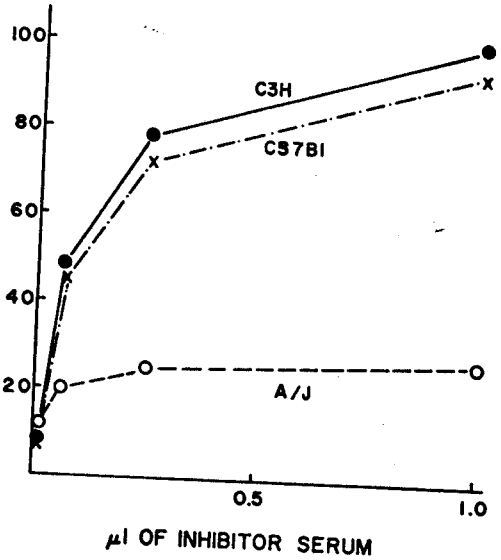
2. When this same antiserum is used with I^{125} labelled MPC-31 (a BALB/c γG_{2b} myeloma) C57BL/6 completely inhibits (Fig. 7b) as do all type strain sera except AKR and A/J.

3. As mentioned in the section on GPC-5, rabbit anti GPC-7 with I^{125} labelled GPC-5 is completely inhibited by NZB serum but not by BALB/c (Fig. 3). The results with other type strains show complete inhibition with AKR but only partial inhibition with all other sera.

In reaction 1 the H-chains (since the antiserum was absorbed to remove anti L-chain antibody) of C57BL/6 immunoglobulins lack an antigen which is present in the immunoglobulins of all other type strains. Since γG_{2a} myeloma was used in this assay as labelled antigen, and complete inhibition *was* found with C57BL/6 when a γG_{2b} myeloma was used as labelled antigen (in



7 (a)



7 (b)

FIGURE 7A. Inhibition of precipitation of I^{125} labelled RPC-5 myeloma protein by whole sera of various inbred strains. The antiserum was a rabbit anti-normal mouse γ-globulin, absorbed with PAPS-conjugated MPC-25 myeloma protein to remove anti L-chain activity.

FIGURE 7B. As for Figure 7a but using I^{125} labelled MPC-31 myeloma protein.

reaction 2) the allotype detected by this antiserum, absent from C57BL/6 but present in all other type strains is a γG_{2a} antigen. This corresponds to the distribution of specificity Ig-1.2 (Table IIa).

By exactly analogous reasoning, reaction 2 defines an allotypic antigen absent from AKR and A/J but present in the γG_{2b} immunoglobulins of all other type strains. This distribution is identical with Ig-3.4 (Table IIb).

The third allotypic specificity, detected by rabbit anti GPC-7, is present only on GPC-5, GPC-7 proteins and in AKR and A/J serum. This distribution is identical with Ig-1.5.

It is therefore apparent that the rabbit can recognize mouse allotypic specificities in addition to class defining specificities. The titer of rabbit antibodies directed to the allotypic antigens is rather low compared to the class specific antibodies, and sensitive techniques are needed to detect the anti allotype antibodies in the presence of the others. Although this is the first demonstration of heterologous antisera recognizing mouse allotypes, heterologous antisera have detected allotypic differences in both rabbit and human immunologlobulins (Bornstein and Oudin, 1964; Hess and Butler, 1962).

The finding that the particular allotypic antigen recognized by the rabbit has a similar strain distribution to one of the existing mouse isoantisera defined specificities does not however prove that these two antibodies are directed to exactly the same site. They may instead be antibodies directed to two entirely different antigens, or to different components in the one antigenic determinant. Experiments are in progress to decide between these alternatives.

FINAL COMMENT

We have presented in detail the concept of a chromosome region containing several distinct genetic loci, each coding for the structure of the H-polypeptide chains of one of the immunoglobulin classes, and of a separate region for the L-polypeptide chains. The allotypes of individual immunoglobulin classes were interpreted as controlled by alleles or alternate forms of the DNA base sequence at one or another of these loci. This representation is fully consistent with all the available data but we would like to stress the following information which should cause us to keep an open mind on this concept.

No crossovers between immunoglobulin loci have been observed despite efforts to find them. Approximately 1,000 progeny of test crosses which would have revealed crossovers have by now been examined without a single recombinant having been found. This may simply indicate that the chromosome region is a small one and that with continued looking crossovers will be found.

Secondly, there are eight alleles of Ig-1 and four and five alleles of Ig-2 and Ig-3 respectively. If the alleles of these three loci associated independently, one would have $8 \times 4 \times 5 = 160$ possible chromosome types. However, within the eighty or so inbred strains studied for allotypes by others and ourselves, at most eight such chromosome types have been found, indicating a high or absolute degree of association between alleles at loci on a particular chromosome. This association is consistent with a low crossover rate and the origin of the presently available laboratory mice from a few ancestors within the past several hundred years.

ACKNOWLEDGMENT

The authors are grateful for extensive help from Leonore A. Herzenberg in preparation of this manuscript.

APPENDIX

Special Methods Developed for Studies of Genetic Control of Mouse Immunoglobins

Materials

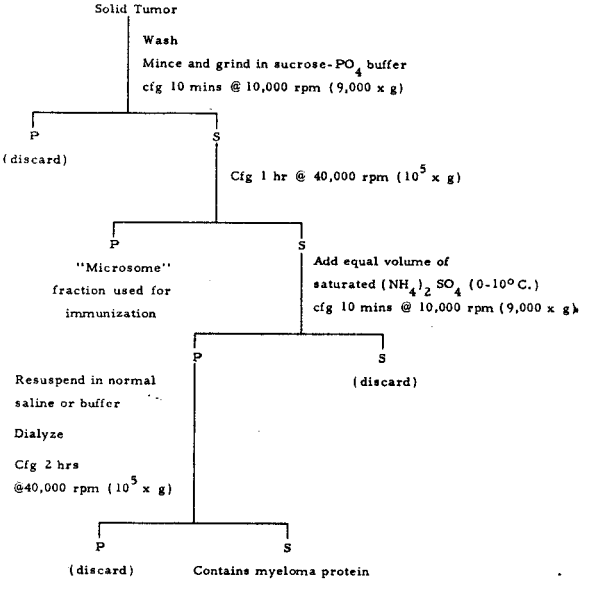
Mice and mouse sera used in these studies were obtained from many laboratories. For a summary of sources see Herzenberg *et al.*, (1965).

Three per cent bovine serum albumin (BSA) (Armour Pharmaceutical Co., Kankakee, Illinois) in 0.05 M tris buffer (final pH 7.6) is used as diluent for antigens and 0.05 M tris, containing 3 per cent BSA and 10 per cent normal rabbit serum (with final pH 7.6) is used as antiserum diluent (S-dil).

Isolation of Myeloma Proteins

For tumors: Solid subcutaneous tumors (weighing 3 to 10 g) are excised and blotted to remove excess blood and then ground with a solution of sucrose (0.25 M) and $CaCl_2$ (.004 M) in distilled water (1 g tumor/10ml solution), in a loose fitting conical glass homogenizer. From this point, all operations are carried out below 10C. The homogenate is centrifuged for 10 mins at 3,000 x g and the pellet (containing nuclei, cell membranes and cellular debris) discarded. The supernatant is spun again for one hour at (10^5 x g), yielding a pellet containing microsomes which have myeloma protein associated with them, and clear supernatant containing soluble myeloma protein. To this supernatant, an equal volume of saturated ammonium sulfate solution at 10C. is added to precipitate the myeloma protein. The precipitate is collected by centrifugation (10 minutes at 9,000 x g) dissolved in normal saline and dialyzed against normal saline (or buffer). Insoluble material after dialysis is removed by centrifugation (2 hours at 10^5 x g).

Myeloma protein prepared in this way is usually contaminated with less than 0.5 per cent of gamma globulins present normally in serum, and is free of other serum proteins detectable in immunoelectrophoresis (Scheidigger, 1955) with a polyvalent rabbit antimouse normal serum. Contamination with cellular constituents is detectable by polyacrylamide gel electrophoresis (Davis, 1964), however, these contaminents represent less than 10 per cent of the total protein and do not interfere with the immunoglobulin studies. Myeloma protein purified of contaminants may be obtained by DEAE chromatography (Fahey, 1962b), starch block (Kunkel, 1954) or preparative polyacrylamide gel electrophoresis (Jovin, 1964).



Iodination With I^{125}

Iodination of approximately 100 μg. portions of gamma globulin is performed by the method of Greenwood *et al.*, (1963), with an average labelling of less 1 atom I^{125} per molecule. Passage through anion exchange resin columns followed by dialysis against saline is used to remove unbound iodide. Approximately 5 to 30 per cent of the added iodine becomes convalently bound to the protein, and is therefore TCA precipitable. With some preparations 10 to 15 per cent of the radioactivity was not easily removed by

dialysis but was nevertheless not precipitable by TCA. In these cases, precipitation of the protein by 50 per cent saturated ammonium sulfate (0-10°C.) was an effective means of separating the protein from the nonprecipitable radioactivity.

Production of Rabbit Antisera

Rabbit antisera are made against mouse whole sera, partially purified normal immunoglobulins, purified myeloma proteins and fragments (papain digestion products, i.e., F and S pieces) of immunoglobulins. Two injections, each of approximately 100 μg of purified protein (50 μg in the case of the papain fragments) or 100 λ of whole serum, evoke antisera which give strong precipitin arcs in immuno-electrophoresis.

The first injection of the antigen emulsified in Freund's adjuvant is given in the foot pads and in several subcutaneous sites. After approximately four weeks, a second injection of the antigen, without adjuvant, is given intramuscularly. Starting at one week after the second injection and continuing weekly thereafter for several weeks, 30 to 60 ml of blood are drawn from the heart.

The same protocol, used with the "microsome" fraction prepared from a myeloma tumor (see above) as antigen usually leads to minimal contamination of the antiserum with antibodies specific for immunoglobulin or serum proteins other than the immunizing myeloma protein. These sera do, however, usually contain anti-L-chain antibody which must be absorbed to obtain specific antisera.

Production of Mouse Anti Allotype Sera

A basic schedule of immunization is followed for preparing the majority of antisera produced. Minor changes in antigen dosage and timing are occasionally made with little apparent affect on the response. A primary subcutaneous injection of 10 μl of whole serum per mouse in complete Freund's adjuvant is followed three weeks later by two weekly intraperitonal injections, each of 10 μl. The mice are bled one week later and individual sera tested for the presence of anti allotype antibodies. Positive mice are bled weekly and "boosted" every third week. Mice failing to produce detectable antibodies are alternately injected and bled at weekly intervals for about one 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. For example, whereas BALB/c mice immunized with normal (C57BL/6) serum failed to produce any detectable isoantibodies to C57BL/6 gamma globulin, immunization of the same strain with a C57BL/c 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^1) (Erickson, 1964) was also effective as an antigenic serum.

Effective immunization has also been obtained by challenging the animal with gamma globulin in the form of an antigen antibody complex. Recently we have used the following method developed by Dresser, Taylor and Wortis (personal communication).

Mouse antisera to *Bordetella pertussis* are prepared by two injections of 2×10^9 bacteria intraperitoneally, with a four week interval between injections. One week after the second injection mice are bled and sera from each mouse strain pooled (*pertussis* organisms received courtesy of Glaxo, Ltd England via NIMR, Mill Hill, London England).

Equal volumes of a suspension of *Bordetella pertussis* (20×10^9/ml) and 1.5%

dilutions of the above antiserum pools are incubated for 30 minutes at 37C. to allow attachment of the antibody to the bacteria. Mice are then injected with a 0.1 ml aliquot of this mixture. At four and eight weeks this procedure is repeated. Recipient mice are bled seven to ten days after the last injection and weekly thereafter.

Precipitation of I^{125} Labelled Gamma Globulin

Antisera from immunized mice and rabbits were tested for ability to form a line in immunodiffusion and for abilty to precipitate I^{125} labelled gamma globulin of the appropriate type. Precipitation of labelled antigen was found to be consistently far more sensitive than immunodiffusion in agar. Many sera that did not show any precipitation in agar, precipitated the labelled antigen even with antiserum dilutions of 1:1000.

All precipitation reactions employing labelled gamma globulin are carried out in 6 x 50 mm culture tubes. For the reaction, 50 μl of labelled antigen in 3 per cent BSA is added, with rapid mixing to 50 μl of varied dilutions of the antiserum in S-dil. The tubes are left at 37C. for three hours, chilled to 4C. and then centrifugated in the cold at 10,000 x g for ten minutes; 50 μl of the supernatant is carefully removed and placed in a 10 x 75 mm diposable tube containing approximately 0.5 ml of normal saline. These are then counted in a well type crystal gamma scintillation counter.

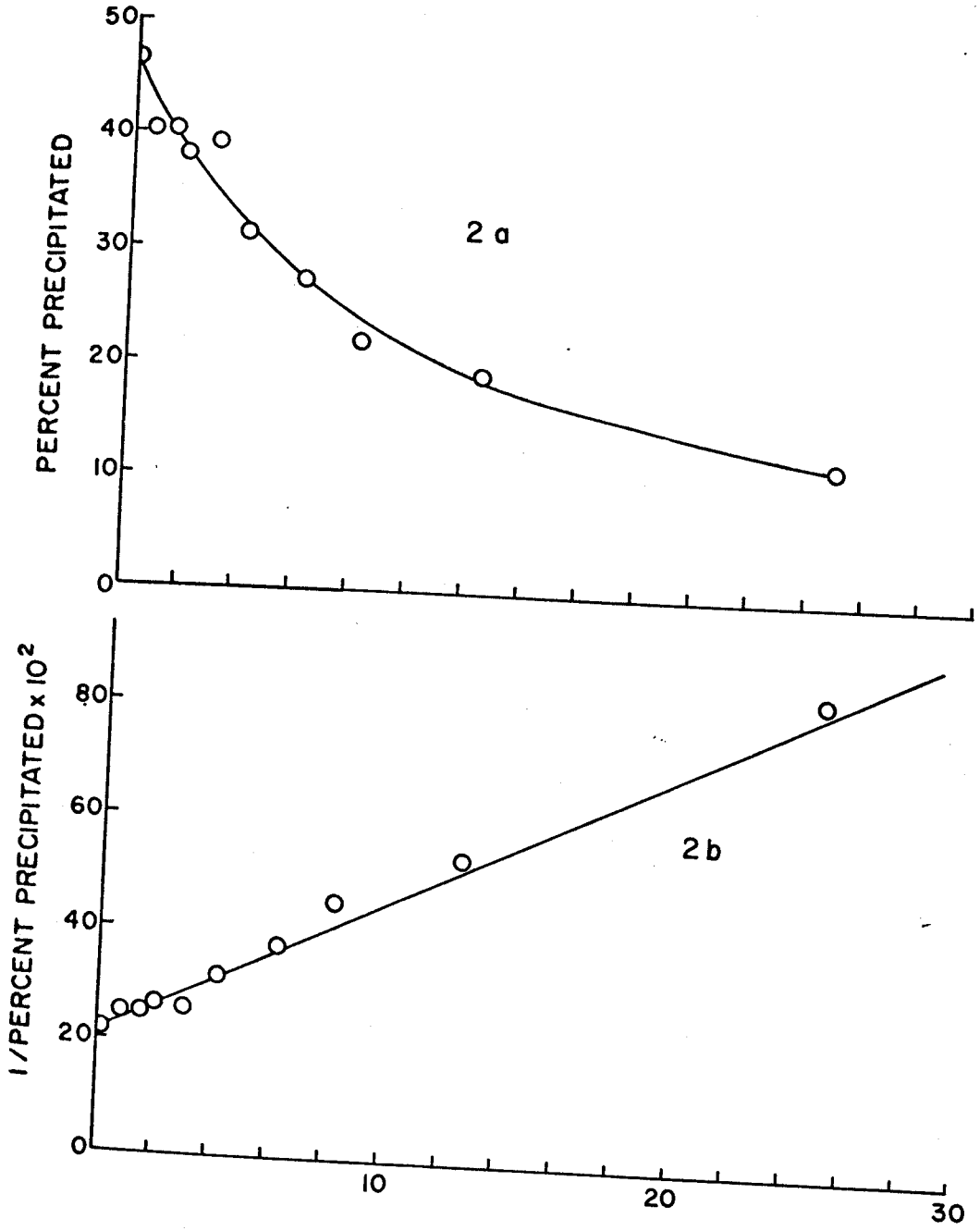
Inhibition of Precipitation of Isoantigens

An adaptation of a method of estimating gamma globulins based on the inhibition of precipitation of labelled reference antigens is employed. Concentrations of labelled antigen and antisera are chosen as follows: A standard concentration of labelled gamma globulin, giving about 10,000 counts per minute per 50 μl is chosen, and precipitated, as described above, with serial dilutions of isoantiserum. An amount of antiserum slightly below equivalence is then determined (i.e., an amount which precipitates 80 to 90 per cent of the precipitable counts).

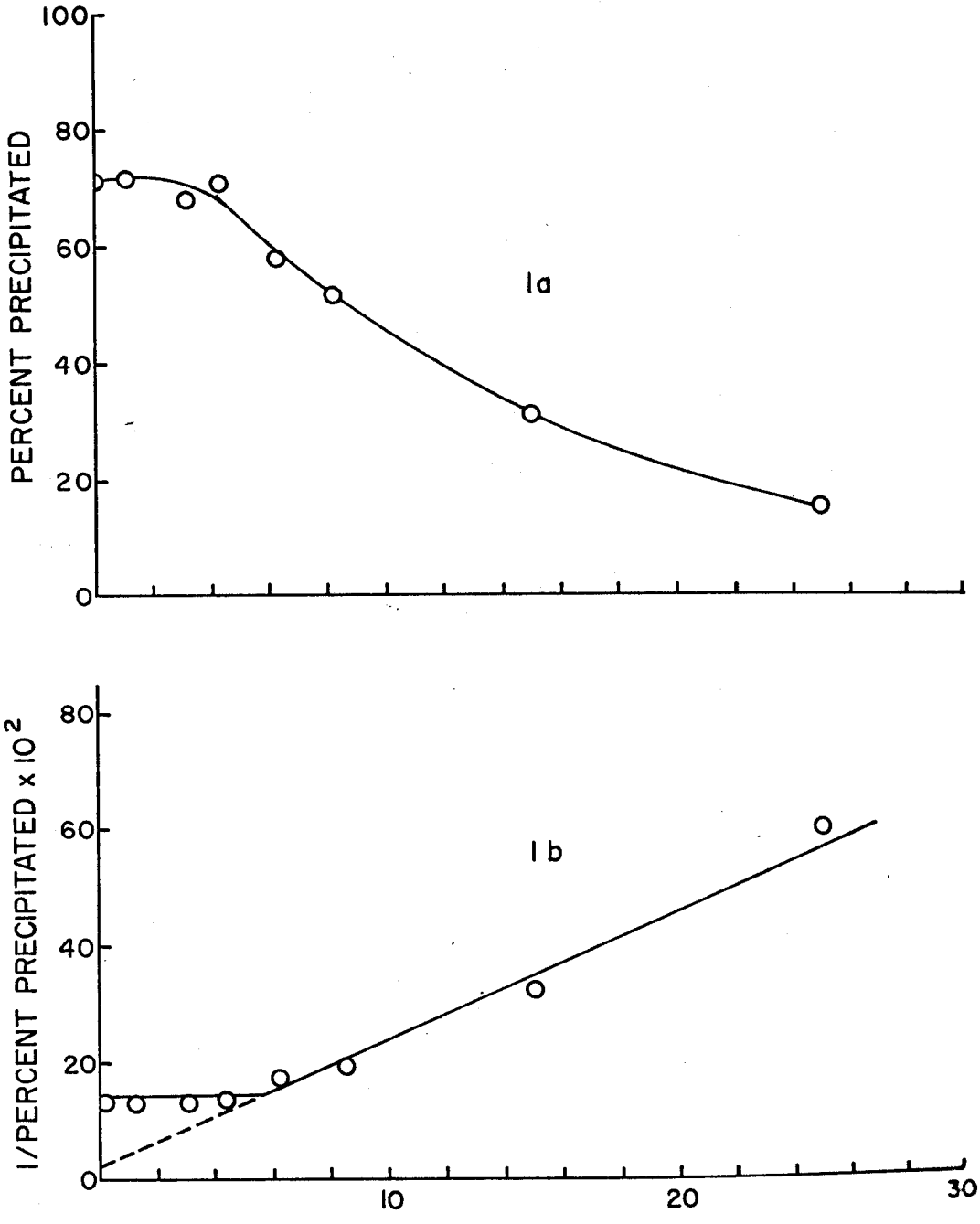
The inhibition assay is performed in a fashion similar to the precipitation of labelled antigens. The order of addition of the reagents is quite critical. Fifty μl of a dilution of labelled antigen is placed in all tubes, followed by the addition of 5 μl of serial dilutions (in S-dil) of test (inhibitor) whole sera. At the addition of 50 μl of diluted isoantiserum each tube is immediately mixed on a Vortex Jr. mixer (Scientific Industries, Springfield, Mass.). Two controls are included in each assay. One tube contained 55 μl of S-dil with 50 μl of labelled antigen and is the control of total radioactivity (i.e., no precipitation). The other control tube determines maximum precipitation (i.e., no inhibition) and contain 50 μl of antiserum, 5 μl of S-dil, with 50 μl of labelled antigen. Incubation, centrifugation and sampling are carried out as described above.

Quantitative Estimation of Immunoglobulins by Inhibition of Precipitation of I^{125} Labelled Antigens

To determine the amount of a particular immunoglobulin, either purified or in whole serum, a combination of antiserum and I^{125} labelled antigen is chosen in which (1) the inhibitor, when used at a high enough concentration, is able to completely inhibit precipitation (i.e., has all antigenic specificities of the labelled antigen which are detected by the antiserum chosen), and (2) other immunoglobulins present are unable to inhibit precipitation. The percentage of precipitation of the labelled antigen in the presence of the "unknown" is then compared to the percentage of precipitation in the presence of dilutions of a standard



FIGURES 2a, 2b. Typical inhibition of precipitation assay plotted directly and inversely. No antibody excess.



FIGURES 1a, 1b. Typical inhibition of precipitation assay plotted directly and inversely. Slight antibody excess.

preparation of inhibitor (e.g., purified immunoglobulin or pooled adult whole serum from a single strain). The standard is always tested at the same time as the "unknown."

In Appendix Figures 1a and 2a, the per cent of labelled antigen precipitated is plotted as a function of inhibitor concentration. In both cases, extrapolation between points is somewhat awkward. Therefore to facilitate the comparison between "unknown" and standard, a curve is obtained instead by plotting the reciprocal of the percentage of radioactivity precipitated in each tube against μl or μg of standard added to that tube. Plotted in this way, the standard curve for the standard is linear over a four-to-twenty-fold range (see Appendix Figs. 1b and 2b) as is the curve generated by dilutions of the "unknown."

The amount of antigen in a sample may be read from the standard curve or readily calculated from the equation of the standard line for samples within the linear range. For a large number of determinations the latter is done with the aid of a LINC computer.

The linear relationship between amount of inhibitor added and the reciprocal of percentage of radioactivity precipitated may be understood as follows:

When the amount of antigen precipitated by a given amount of antibody neither increases nor decreases with additional antigen, the fraction of a constant amount of radioactive antigen which is precipitated by a constant amount of antiserum varies according to the amount of unlabelled antigen present, that is, according to the specific radioactivity of the total antigen.

Thus if,

P = Fraction of the counts precipitated,

A_b = μg of antigen precipitated by the amount of antibody used in the assay,

A_g = μg of antigen in labelled preparation used in assay,

A_g^* = number of counts per minute in the amount of labelled antigen used in the assay,

C = concentration, in $\mu g/\mu l$ of the antigen in the inhibitor preparation and

v = volume, in μl of the inhibitor preparation added,

then,

$$P = \frac{\dfrac{A_g^*}{A_b \cdot \overline{A_g + vC}}}{A_g^*} = \frac{A_b}{A_g + vC}$$

$$\frac{1}{P} = \frac{A_g}{A_b} + \frac{C}{A_b} \cdot v$$

which is in the slope intercept form of the equation for a straight line when $1/P$ is plotted against v.

The inverse of the ordinate intercept of this line is equal to the fraction of antigen in the labelled preparation which is precipitable by the amount of antibody used.

In the experiment shown in curve b, Appendix Figure 1, where 70 per cent of the labelled antigen is precipitated there is an initial "lag" before the linearity is established. When the amount of antibody is decreased to precipitate only about 50 per cent of the labelled antigen the lag disappears. Although complete linearity extends the range of the assay somewhat, it is frequently more convenient to work with greater precipitation and ignore the early part of the curve. The observed lag is probably due to the necessity of reaching the antigen concentration above which the amount of antigen precipitated becomes constant, since the length of the lag increases with antibody concentration and decreases (not shown here) with higher concentrations of antigen. It is of interest to note that the lag portion of the curve in the reciprocal plot corresponds to the initial

shoulder of the S-shaped curve which results from the usual, direct plot of percentage of labelled antigen precipitated versus amount of inhibitor antigen added.

If the unknown is not identical to the I^{125} labelled antigen with respect to the antiserum used but only cross-reacts, i.e., if the inhibitor cannot *completely* inhibit precipitation, linearity is not obtained. This may be explained by the presence of two populations of antibody, only one of which is inhibitable by the inhibitor, the other being specific for the labelled antigen. It is of interest that linearity in such a case may be obtained by subtracting the percentage of radioactivity not inhibitable from the total percentage precipitated, before converting to the reciprocal. It is, however, much more satisfactory to choose appropriate combinations of antiserum and labelled antigen so that such manipulations are unnecessary.

Estimation of allotype (antigen) levels by this method may be carried out without interference even in the presence of a 20,000-fold excess of a non-cross reacting gamma globulin. Thus as little as 2 μg of allotype carrying immunoglobulin may be detected in a mouse with roughly 20 mg of total immunoglobulin.

Estimation of Proportion of Molecules Carrying a Particular Antigenic Specificity in an Immunoglobulin Preparation

In some experiments it is necessary to determine the proportion of molecules in an I^{125} labelled immunoglobulin preparation which carry a particular antigenic specificity. While precipitation of antigen, as previously described, is suitable for determining whether an antiserum has antibody activity to the particular antigen, this method may not give maximum precipitation of the labelled antigen since there may be soluble antigen-antibody complexes left in the supernatant. A method was accordingly devised which would detect *all* complexed antigen.

Labelled antigens migrate in polyacrylamide gel electrophoresis into a characteristic region of the gel. If the antigen is complexed with antibody molecules, its migration is greatly retarded. To determine the proportion of labelled antigen complexed with antibody, samples of an I^{125} labelled antigen are mixed with either normal serum or the particular antiserum, left to incubate at room temperature for one to two hours, mixed with an equal volume of 25 per cent sucrose in buffer and layered on spacer gel. Up to 100 μl of the reaction mixture is placed on the gel. The extruded gel is cut into 0.25 mm segments and each segment is counted in a well type scintillation counter. An example of this method is given in Appendix Table I with the calculation of amount of antigen bound.

Conjugation of Proteins to Polyaminopolystyrene

Fractionated serum proteins are conjugated with diazotized polyaminopolystyrene (PAPS) (Norsk Hydro-electrisk, Oslo, Norway) according to the method described by Webb and Le Presle (1961). From 20 to 150 mg of protein is conjugated with 1 to 3 g (moist weight) of PAPS from which the "fines" have been removed by repeated decantation. Conjugation is carried out at pH 8. Unreacted groups are blocked with glycine and/or β-naphtol: 50 to 90 per cent of protein is bound.

Absorption of Antisera With Immunoglobulins Conjugated to PAPS

Enough PAPS-conjugate suspension to give a packed volume of approximately 0.5 ml is allowed to pack by gravity in a column made in a 2.5 ml (Tomac) disposable syringe, the constricted end of which has been plugged with glass wool.

Up to 5 ml of the antiserum to be ab-

sorbed is layered on top of the column and allowed to flow through at approximately 2 ml per hour. After passage of the void volume, 1 ml samples are collected. When the antiserum meniscus reaches the volume bed, 2 ml of S-dil is layered on top of the column. Collection of samples is continued until all the antiserum has passed the column.

The completeness of absorption is tested for each sample by determining whether that sample is able to precipitate I^{125} labelled antigen of the absorbing type. Fully absorbed samples are then pooled and retested in the same assay. Incompletely absorbed samples are passed through a second column of the same PAPS-conjugated antigen, tested again, but are never pooled with the samples from the first absorbtion.

Usually the first two or three samples are fully absorbed and there is little dilution of the antibodies which do not react with the conjugated protein. Since the absorbed antibody remains behind on the column complexed with the conjugated antigen, no antigen antibody complexes or excess antigen are present in the absorbed antiserum. Using this method it is possible to avoid the interference of complexes or other contaminents usually introduced by absorbing material and therefore a single antiserum may be satisfactorily sequentially absorbed with several antigens.

Elution of Antibody Bound to PAPS-Conjugate

After the above absorption the column of PAPS-conjugated antigen, which now has antibody complexed with it, is washed with two volumes of S-dil. Then, to elute the antibody from the column, approximately 6 vols of 0.1 N HCl (pH 1.1) are passed through the column. The eluate is tested with pH paper until the pH drops (rather abruptly) to approximately pH 1. At this point, 0.5 ml samples are collected into tubes that have been prewashed with S-dil (to reduce adsorption to the glass of the small amount of eluted protein) and contain two to three drops of 0.5 M phosphate buffer, pH 7.5 which is sufficient to bring the sample to neutrality.

The eluted antibodies all react with the antigen conjugated to the PAPS, and are contaminated to a relatively small extent with other serum proteins. When the active samples are pooled and the protein iodinated with I^{125}, roughly 50 per cent of the counts bind to antigen. The bindable percentage may be increased by a second absorption and elution. Since the eluted antibodies presumably comprise less than 1 per cent of the serum protein of the original antiserum, this method gives at least a fifty fold purification of antibody.

ACKNOWLEDGMENT

The authors greatly appreciate the help of Michael Iverson, Richard Lanzerotti and Roy Riblet in development of the methods described in this Appendix and in preparation of the manuscript.

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