IMMUNOGENETIC STUDIES OF DROSOPHILA MELANOGASTER II. INTERACTION BETWEEN THE RB AND V LOCI IN PRODUCTION OF ANTIGENS[1]

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INTRODUCTION

THE discovery, at the turn of the century, of the human A-B blood groups, and the subsequent explanation of their heredity on the basis of triple alleles, opened a whole new field in genetics, one termed by IRWIN (1947 and previously) "Immunogenetics." Intensive search for such "genetic characters as yet only detectable by immunological reactions" has led to their discovery in such diverse animals as man and other primates, birds, rodents, cattle, and Protozoa. (The reader is referred to the following reviews: on immunogenetics, IRWIN 1947; on immunology, LANDSTEINER 1947; BOYD 1947.) With the exception of the antigens of Protozoa, where cytoplasmic inheritance introduces a novel situation (SONNEBORN 1947), most of these immunogenetic characters have been antigens of vertebrate erythrocytes.

From the genetic point of view, the most striking feature of this work has been the finding that, with but a few exceptions, there seems to be no interaction between genes involved in the production of antigens. This apparent one-to-one relationship between gene and antigen has led to the widespread suggestion that antigens stand in some sort of special relationship to genes, a relationship shared perhaps only with daughter genes and enzymes, in that they may be direct or primary products of their causative genes. If this be the case, immunogenetics should prove to be an important tool in the study of the nature of genic action and specificity (cf. WRIGHT 1945).

Whether or not this one-to-one relationship may be extended to all types of antigens is unknown. With only a few exceptions the relationship has been demonstrated for antigens of red blood cells. These substances, in the cases that have been analyzed, have proved to be haptens rather than complete antigens. Along with these immunochemical data, "the striking serological differences between the cells of individuals of the same species, the frequent occurrence of so-called heterogenetic antigens . . . , and the fact that blood cells of closely related species exhibit sometimes much greater differences than the respective serum proteins," led LANDSTEINER (1947, p. 76) to the conclusion that "there exist two systems of species specificity in the animal king-

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dom, the specificity of proteins and that of cell haptens. The proteins, it would seem, undergo gradual variation in the course of evolution, while haptens are subject to sudden changes not linked by intermediary stages."

In the few cases where serum antigens have been subjected to immunogenetic analysis the one-to-one relationship seems to hold (CUMLEY and IRWIN 1942; CUMLEY, IRWIN, and COLE 1941, 1943; IRWIN and CUMLEY 1942). Unfortunately, the antigens in these cases have not yet been chemically identified, and while they are probably proteins this is by no means certain.

Certain other exceptional cases seem to argue against an invariable one-to-one relationship between gene and antigen. These are the "hybrid substances" that have been found in crosses between closely related species and within species (GORDON 1938; IRWIN 1932; IRWIN and COLE 1936; MCGIBBON 1944; SOKALOWSKAJA 1936; THOMSEN 1936). The explanation for the occurrence of these hybrid substances which first offers itself is the interaction in the hybrid of genes derived from the parents (IRWIN and CUMLEY 1945).

The existence of hybrid substances raises a number of questions which are crucial for the one-to-one hypothesis. Are the hybrid substances really exceptions, or are all or most antigens produced by gene interaction which has remained undetected due to the constancy of genetic background resulting from the inability to produce mutation on a large scale in vertebrates? If the hybrid substances are exceptions, wherein do the genes producing them differ in effect from those producing antigens without interaction?

It seems abundantly clear that the resolution of these problems will come only after the association of specific antigens with particular loci in an organism better adapted for genetic analysis than are the vertebrates.[3] A previous article (FOX 1949) reported the development of immunological methods capable of detecting antigenic differences between strains of *Drosophila melanogaster*. This article reports the initial results of the application of these methods to the problem of accomplishing the association mentioned above.

GENETIC MATERIALS AND METHODS

The most direct method available for the association of gene with antigen is that of inducing single visible gene-mutations in an isogenic stock and testing for ensuing antigenic change.

Derivation of isogenic stock.—Twenty separate attempts were made to derive an isogenic stock by means of the following series of crosses:

$$sc\ lz/ClB \times Cy\text{-}RNS/Pm^2;\ Payne\ Dfd\ ca/Sb$$

to give, in the F_1, females of the following genotype:

$$+/ClB;\ +/Cy\text{-}RNS;\ +/Payne\ Dfd\ ca.$$

[3] BURNET and FENNER (1948), in a review which appeared as this paper reached final stages of preparation, arrive at a number of conclusions similar to those reached here. In particular they urge "a combined biochemical, genetic, and immunological study of some convenient bisexual . . . organism" which would "eliminate the complications inherent in the use of vertebrate cellular antigens."

Such females, taken singly, were then backcrossed to their own fathers;

$+/ClB$; $+/Cy\text{-}RNS$; $+/Payne\ Dfd\ ca \times Cy\text{-}RNS/Pm^2$; $Payne\ Dfd\ ca/Sb$

yielding, among others, the four following types of offspring:

1. $+/Y$; $Cy\text{-}RNS/Pm^2$; $Payne\ Dfd\ ca/Sb$
2. $+/+$; $Cy\text{-}RNS/Pm^2$; $Payne\ Dfd\ ca/Sb$
3. $+/Y$; $Cy\text{-}RNS/+$; $Payne\ Dfd\ ca/+$
4. $+/+$; $Cy\text{-}RNS/+$; $Payne\ Dfd\ ca/+$

Flies of types 1 and 2 were mated singly *inter se*, giving the "Marker Stock" below, and flies of types 3 and 4 were mated singly in hopes of obtaining isogenic wild type flies among their offspring. In none of the twenty attempts, however, were wild type flies produced; each of the attempts to produce isogenicity had rendered one or more lethal or semilethal genes homozygous. Instead, a balanced *Dfd* stock was obtained ("Isogenic Stock," below).

"Marker Stock"
$+/Y$; $Cy\text{-}RNS/Pm^2$; $Payne\ Dfd\ ca/Sb$
$+/+$; $Cy\text{-}RNS/Pm^2$; $Payne\ Dfd\ ca/Sb$

"Isogenic Stock"
$+/Y$; $+/+$; $Payne\ Dfd\ ca/l(3)F$
$+/+$; $+/+$; $Payne\ Dfd\ ca/l(3)F$

By means of this series of crosses all chromosomes in the isogenic stock except the 4th trace to a single source. Barring crossing over in the F_1 female (the likelihood of which is sharply reduced by the inhibitors present), and barring spontaneous mutations, the isogenic stock is actually isogenic in chromosomes 1 and 2, and the marker stock carries the same first chromosome as the former. Since the isogenic stock remains balanced, its genotype is as listed above.

The usefulness of the isogenic stock is not impaired by the fact that it is not wild type. Because of the balanced nature of the third chromosomes, and disregarding chromosomes 4 for the moment, all flies in the stock are genetically identical (again barring crossing over and spontaneous mutation). The presence of the dominant marker *Dfd*, as a matter of fact, has value as a check for contamination of the stock.

Lack of control of the fourth chromosomes would seem to be the most serious source of heterogeneity in this stock. In the absence of suitable dominant markers and crossover inhibitors no attempt was made to render chromosome 4 isogenic. The results of this investigation seem not to have been influenced by this factor.

Derivation of mutant stocks.—Males of the marker stock were treated with 3000 r units of X-ray and mated to females from an attached-X yellow stock. Male progeny exhibiting "good" mutations were selected for further crosses designed to replace all rayed chromosomes except the single X-chromosome

bearing the mutant gene with unrayed chromosomes from the isogenic stock. Here again, no attempt was made to control chromosome 4.

The mutant males were mated to females from the marker stock (X′ indicates the X-chromosome bearing the mutant gene):

$$\left.\begin{array}{l} \text{X}'/\text{Y};\ \textit{Cy-RNS}/+;\ \textit{Sb}/+ \\ \qquad\text{or} \\ \text{X}'/\text{Y};\ \textit{Cy-RNS}/+;\ \textit{Payne Dfd ca}/+ \\ \qquad\text{or} \\ \text{X}'/\text{Y};\ \textit{Pm}^2/+;\ \textit{Sb}/+ \\ \qquad\text{or} \\ \text{X}'/\text{Y};\ \textit{Pm}^2/+;\ \textit{Payne Dfd ca}/+ \end{array}\right\} \times +/+;\ \textit{Cy-RNS}/\textit{Pm}^2\ \textit{Payne Dfd ca}/\textit{Sb}$$

to yield females of the following genotype among their offspring:

$$\text{X}'/+;\ \textit{Cy-RNS}/\textit{Pm}^2;\ \textit{Payne Dfd ca}/\textit{Sb}.$$

Each such female was then mated individually to one male from the isogenic stock, giving among their offspring males and females as indicated:

$$\text{X}'/+;\ \textit{Cy-RNS}/\textit{Pm}^2;\ \textit{Payne Dfd ca}/\textit{Sb} \times +/\text{Y};\ +/+;\ \textit{Payne Dfd ca}/\textit{l}(3)F$$

$$\text{X}'/\text{Y};\ \textit{Cy-RNS}/+;\ \textit{Sb}/\textit{l}(3)F$$

$$\text{X}'/\text{Y};\ \textit{Pm}^2/+;\ \textit{Sb}/\textit{l}(3)F$$

$$\text{X}'/+;\ \textit{Cy-RNS}/+;\ \textit{Payne Dfd ca}/\textit{Sb}.$$

These males and females were then mated singly *inter se*, yielding among their offspring

$$\text{X}'/\text{Y};\ +/+;\ \textit{Payne Dfd ca}/\textit{l}(3)F$$

and

$$\text{X}'/\text{X}';\ +/+;\ \textit{Payne Dfd ca}/\textit{l}(3)F.$$

Such males and females were mated individually to establish the mutant balanced stocks. The end result of this series of matings was, then, several stocks each of which may be considered to differ from the isogenic stock by a mutation at a single locus.

The mutations recovered by these methods were identified by means of tests for allelism. The two mutant stocks utilized in this investigation proved to be ruby and vermilion. Aceto-orcein smears of larval salivary glands disclosed no detectable X chromosomal aberrations in homozygotes, heterozygotes (with isogenic), and hemizygotes of each of the two mutant stocks.

A ruby-vermilion stock was derived from the two mutant stocks by mating ruby females with vermilion males; backcrossing the F_1 females to ruby males; mating the double mutant, crossover males to vermilion females; and extracting the double mutant stock by backcrossing females from the previous genera-

tion to double mutant males from the F_1 backcross. The resulting stock had the following genotype:

rb v/rb v; Payne Dfd ca/l(3)F

rb v/ Y; *Payne Dfd ca/l(3)F.*

This stock may be considered to differ from the isogenic stock by the mutations present individually in the two mutant stocks.

IMMUNOLOGICAL TECHNICS

The development of immunological technics suitable for an investigation such as this has been described in a previous paper (Fox 1949).

Preparation of antigens.—Flies of each stock, collected from mass cultures and starved as previously described, were lightly etherized, rapidly frozen while still alive, and lyophilized. For these experiments a special lyophile apparatus was available, and its use was substituted for the improvised apparatus used in the previous work. When desiccation was complete, the flies were suspended in 0.85 percent NaCl buffered at pH 7.4 by 0.005 M phosphate in the ratio of 1 gm of dried material to 50 ml of saline. Reduction in a Waring blender and grinding in a Potter-Elvejhem homogenizer followed, after which the suspensions were rapidly frozen and stored in a deep-freeze.

Production of antisera.—The complete antigenic preparations were used as injection antigens. Healthy rabbits of approximately 2,000 gm in weight were given four intraperitoneal injections of doubling dose on alternate days, the first injection being 1 ml of the appropriate antigenic suspension. Each rabbit therefore received 15 ml of antigen. Ten to twelve days after the last injection the rabbits were aseptically exsanguinated from the heart, following a 24 hour fast to avoid opalescent sera. The sera were separated, preserved with merthiolate 1:20,000, rapidly frozen, and stored in a deep-freeze.

Reaction technics.—Measurement of the titer of each antiserum with its homologous and heterologous antigens, and absorption of each antiserum by the various antigenic preparations, were accomplished simultaneously by use of the optimal-proportions method of DEAN and WEBB (1926).

The antigenic preparations were centrifuged; the clear supernates, separated from the packed particles, served as the "antigens" in all of the precipitin tests. Optimal-proportion determinations of each serum with its homologous antigen and heterologous antigens were performed. The tubes from the exact optimal-proportion determinations were incubated for two hours at 37.5°C and stored in the refrigerator for at least 24 hours. At the end of this period absorption was complete, and the precipitate was thrown down by centrifugation. The supernates were then tested for antibody content.

The method used in these latter tests was the modified precipitin-ring technic of HANKS (1935). The antigens, dissolved at optimal-proportion concentration in 5 percent gelatin, were layered into the bottoms of microprecipitin tubes and allowed to harden. The supernate from each of the optimal-proportion tubes was then carefully layered over the homologous and heterologous

antigens. The tubes were incubated in a water bath at 37.5°C for one hour, at end of which time they were examined for the formation of a precipitin-ring at the interface between antigen and antiserum. Storage at 6°C for 24 hours followed, at the end of which period the tubes were returned to the water bath for five minutes to remove non-specific clouding of the gelatin, and final readings were taken. The following controls were performed: antigenic controls, consisting of the antigens dissolved in 5 percent gelatin overlayered by buffered saline; antiserum controls, consisting of the supernates layered over 5 percent gelatin; normal controls, consisting of normal serum layered over the antigens in 5 percent gelatin. The precipitin-ring tests were read as follows: −, negative; t, trace; +, weak ring; ++, strong ring; +++, very strong ring. In both the optimal-proportions tests and precipitin-ring tests, 0.85 percent NaCl buffered at pH 7.4 by 0.005 M phosphate was used for all dilutions.

Early in this work it was found that the results were obscured by heavy, non-specific precipitation of the antigenic preparations. The precipitate so obtained was found to be ether-soluble, and was therefore probably lipidal in nature. This difficulty had not been encountered in the previous work, and probably occurred here because of better preservation of organic material by the improved lyophile apparatus. The non-specific material was removed from the antigens by means of three successive extractions, in a separatory funnel, with cold ethyl ether. As a check against alteration of antigenic structure during this extraction, each precipitin-ring test was repeated using antigens extracted at different times. No evidence of alteration was encountered.

It should be noted that the supernates from a particular optimal-proportion determination were tested with all antigens simultaneously. This procedure reduced the possibility of subjective error, since the strength of reaction of the supernates with the homologous antigen could be directly compared with the strength of their reactions with the heterologous antigens.

RESULTS

Detailed results are given only for the optimal-proportions tests and for the analysis of isogenic. The data upon which the analysis of the other stocks are based have been deposited in the editorial office of GENETICS.

Optimal-proportions tests.—Table 1 gives the protocol used and the results obtained in a typical exact optimal-proportions test. The same protocol was employed in all such tests, except that in each case the initial antigen dilution was adjusted according to the results of the rough determination.

Table 2 gives the results of the optimal-proportions tests. For each antiserum the result with the homologous antigen is given first. The results for the anti-isogenic, anti-vermilion, and anti-ruby-vermilion sera indicate significantly higher titers for the tests with the homologous antigens. The anti-ruby serum gave no such results—the titers for all three antigens being substantially the same. There is a remarkable agreement between these differences and the results of the precipitin-ring tests reported below. In those tests, the same antisera demonstrate an antigenic fraction possessed alone by isogenic and a

TABLE 1

Protocol for exact optimal-proportion tests.

Serum: S-13, Anti-isogenic — Incubation temperature: 37.5°C
Dilution—1:2
Antigen: D-3-1, vermilion — Optimal proportion:* 40
Initial dilution—1:50

TUBE	1	2	3	4	5	6	7	8
Saline (ml)	—	0.1	0.15	0.2	0.23	0.25	0.27	0.29
Antigen (ml)	0.4	0.3	0.25	0.2	0.17	0.15	0.13	0.11
Antigen dilution	1:50	1:67	1:80	1:100	1:118	1:133	1:154	1:182
Antiserum (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Time to pp't. (Min)	20	19.75	18.25	19.5	21	23	24.5	25.75

$$* \text{ Optimal-proportion} = \frac{\text{Dilution of antigen}}{\text{Dilution of antiserum}} = \frac{80}{2}$$

fraction possessed in common by ruby-vermilion and vermilion, but fail to demonstrate a fraction specific to ruby. The differences between optimal-proportions may, then, be due to these antigenic differences.

TABLE 2

Optimal-proportions.

ANTISERUM	ANTIGEN	OPTIMAL-PROPORTION
S-12 (Anti-isogenic)	D-1-1 (isogenic)	12.3
	D-2-1 (ruby)	50
	D-3-1 (vermilion)	40
S-14 (Anti-ruby)	D-2-2 (ruby)	29.4
	D-1-2 (isogenic)	21.4
	D-3-2 (vermilion)	29.4
S-15 (Anti-vermilion)	D-3-3 (vermilion)	4.2
	D-1-3 (isogenic)	12.3
	D-2-3 (ruby)	8
S-20 (Anti-ruby-vermilion)	D-4a-1 (ruby-vermilion)	6.2
	D-1a-1 (isogenic)	37.6
	D-2a-2 (ruby)	42.72
	D-3a-1 (vermilion)	2.7–3.1

Antigenic analysis of isogenic.—Tables 3, 4, and 5 give the results which allow analysis of the antigenic structure of isogenic. Antigenic controls, antiserum controls, and normal controls were all negative. Absorption of the anti-isogenic serum by the homologous antigen removed all antibodies, indicating specificity of the serum.

The results summarized in tables 3 and 4 indicate that upon absorption of the anti-isogenic serum with increasing amounts of either ruby or vermilion antigens a point is reached where all antibodies to antigens possessed in common by isogenic and the two mutants are removed, leaving antibodies to antigens specific for isogenic. This point is reached almost simultaneously for both mutants, regardless of which is used for absorption. This finding can only mean that neither mutant possessed, to the first order, more in common with isogenic than does the other, and that isogenic possesses an antigenic fraction lacking in both of the mutants.

TABLE 3

Analysis of isogenic with respect to ruby.

Serum: S-13, Anti-isogenic Absorbing Antigen: D-2-1, ruby

Precipitin-Ring Tests—Hanks Technic. 24 hours readings.

ANTIGEN	TUBE							
	1	2	3	4	5	6	7	8
D-1-1, isogenic (1:12 in gel.)	++	++	++	++	+++	+++	+++	+++
D-2-1, ruby (1:50 in gel.)	−	−	−	t	+	+	+	+
D-3-1, vermilion (1:40 in gel.)	−	t	t	+	+	+	++	++
D-1-1, isogenic (1:12 in gel.)	++	++	++	++	++	+++	+++	+++
D-2-1, ruby (1:50 in gel.)	−	−	−	−	t	+	+	+
D-3-1, vermilion (1:40 in gel.)	−	−	t	t	+	+	+	++
D-1-2, isogenic (1:12 in gel.)	++	++	++	++	++	+++	+++	+++
D-2-2, ruby (1:50 in gel.)	−	−	−	t	t	+	+	+
D-3-2, vermilion (1:40 in gel.)	−	t	t	+	+	+	+	++
D-1-2, isogenic (1:12 in gel.)	++	++	++	++	++	+++	+++	+++
D-2-2, ruby (1:50 in gel.)	−	−	−	t	+	+	+	++
D-3-2, vermilion (1:40 in gel.)	−	t	t	+	+	+	++	++

Closer examination of these tables reveals a second order effect. The slight, but consistently stronger reaction of vermilion with the absorbed serum would seem to indicate that isogenic may share minor antigenic similarities with vermilion.

TABLE 4

Analysis of isogenic with respect to vermilion.

Serum: S-13, Anti-siogenic Absorbing Antigen: D-3-1, vermilion
Precipitin-Ring Tests—Hanks Technic. 24 hours readings.

ANTIGEN	TUBE							
	1	2	3	4	5	6	7	8
D-1-1, isogenic (1:12 in gel.)	++	++	++	+++	+++	+++	+++	+++
D-2-1, ruby (1:50 in gel.)	−	−	+	++	++	++	++	++
D-3-1, vermilion (1:40 in gel.)	−	−	+	++	++	++	++	++
D-1-1, isogenic (1:12 in gel.)	++	++	++	++	++	++	+++	+++
D-2-1, ruby (1:50 in gel.)	−	−	−	t	+	+	+	+
D-3-1, vermilion (1:40 in gel.)	−	−	−	t	+	+	+	+
D-1-2, isogenic (1:12 in gel.)	++	++	++	++	++	+++	+++	+++
D-2-2, ruby (1:50 in gel.)	−	−	t	t	+	+	++	++
D-3-2, vermilion (1:40 in gel.)	−	t	t	t	+	+	++	++
D-1-2, isogenic (1:12 in gel.)	++	++	++	++	++	+++	+++	+++
D-2-2, ruby (1:50 in gel.)	−	−	t	t	+	+	++	++
D-3-2, vermilion (1:40 in gel.)	−	t	t	t	+	+	++	++

The results of the double absorption of anti-isogenic serum (table 5) are, at first sight, at variance with the above results. If both mutants lack the same antigens, as is indicated above, then absorption with both simultaneously should give the same results as absorption with either singly. Yet the precipitin-ring tests, after 24 hours' incubation, gave negative results for all antigens. Only after 48 hours at 6°C are results consistent with those of the single absorptions obtained. These latter results make certain the deductions based on the single absorptions, but the delay in the reactions remains unexplained.

Antigenic analysis of vermilion.—The results obtained in the analysis of anti-vermilion serum were essentially similar to those described for anti-isogenic serum. The precipitin-ring tests indicated that upon absorption of anti-vermilion serum with increasing amounts of either isogenic or ruby antigens a point was reached where all antibodies to antigens possessed in com-

TABLE 5

Specific antigens of isogenic.

1. *Double Absorption*

Serum: S-13, Anti-isogenic
Dilution—1:2
Antigens:
D-2-1, ruby
Initial dilution—1:25
D-3-1, vermilion
Initial dilution—1:25

Incubation: 2 hrs. at 37.5°C, followed by 36 hrs. at 6°C

TUBE	1	2	3	4	5
Saline (ml)	—	0.2	0.3	0.4	0.46
D-2-1 (ml)	0.4	0.3	0.25	0.2	0.17
D-3-1 (ml)	0.4	0.3	0.25	0.2	0.17
Final dilution of each antigen	1:50	1:66.7	1:80	1:100	1:117.5
Antiserum (ml)	0.8	0.8	0.8	0.8	0.8

2. *Precipitin-Ring Tests*—Hanks Technic. 24 and 48 hours readings.

ANTIGEN	TUBE									
	24 hours					48 hours				
	1	2	3	4	5	1	2	3	4	5
D-1-1, isogenic (1:12 in gel.)	−	−	−	−	−	+	+	+	+	+
D-2-1, ruby (1:50 in gel.)	−	−	−	−	−	−	−	−	−	−
D-3-1, vermilion (1:40 in gel.)	−	−	−	−	−	−	−	−	−	t
D-1-1, isogenic (1:12 in gel.)	−	−	−	−	−	+	+	+	+	+
D-2-1, ruby (1:50 in gel.)	−	−	−	−	−	−	−	−	−	t
D-3-1, vermilion (1:40 in gel.)	−	−	−	−	−	−	−	−	−	t
D-1-2, isogenic (1:12 in gel.)	−	−	−	−	−	+	+	+	++	++
D-2-2, ruby (1:50 in gel.)	−	−	−	−	−	−	−	−	t	+
D-3-2, vermilion (1:40 in gel.)	−	−	−	−	−	−	−	−	t	+
D-1-2, isogenic (1:12 in gel.)	−	−	−	−	−	+	+	+	++	++
D-2-2, ruby (1:50 in gel.)	−	−	−	−	−	−	−	−	−	−
D-3-2, vermilion (1:40 in gel.)	−	−	−	−	−	−	−	−	−	t

mon by the three stocks were removed, leaving behind antibodies capable of precipitation with antigens specific to vermilion. There was no indication that either isogenic or ruby possessed more in common with vermilion than the other.

Double absorption of the anti-vermilion serum gave results which were in complete agreement with the results of the single absorptions. Since both heterologous antigens lacked the same fractions, the supernates, after double absorption, contained the same antibodies as they did after single absorption.

Antigenic analysis of ruby.—The results obtained with anti-ruby sera were not similar to those obtained with anti-isogenic and anti-vermilion sera. In absorbing with either of the heterologous antigens a point was reached where reaction in the precipitin-ring tests ceased simultaneously with all three antigens. These results indicated, therefore, failure to demonstrate antigenic fractions possessed by ruby which are not possessed by both of the other stocks.

Antigenic analysis of ruby vermilion.—Analysis of anti-ruby-vermilion serum yielded evidence indicating that the double mutant, ruby vermilion, shares an antigenic fraction with vermilion to the exclusion of isogenic and ruby. Identical results in the precipitin-ring tests indicated that ruby-vermilion and vermilion are antigenically identical. Despite the absence of reciprocal tests, it is reasonable to conclude that the fraction demonstrated here is identical with that already shown to be possessed by vermilion to the exclusion of ruby and isogenic.

DISCUSSION

Combination of the optimal-proportions and precipitin-ring technics has yielded results which indicate antigenic differences between the stocks employed in these experiments. For purposes of convenience, these results have been summarized, in a qualitative manner, in table 6. From these results we may draw the following conclusions. 1) Isogenic possesses an antigenic fraction specific to itself. This we shall designate as Antigen 1. 2) Vermilion possesses an antigenic fraction specific to itself. This we shall call Antigen 2. 3) No specific antigen has been demonstrated for ruby. 4) Ruby-vermilion is identical with vermilion, and therefore possesses Antigen 2. In addition to these "stock-specific" antigens, the strong cross reactions of all of the unabsorbed antisera indicate that all four stocks possess the great bulk of their antigens in common, presumably because of the identity of their residual genotypes. These antigens may be designated as "species-specific."

Can these differences be attributed to mutations at the *rb* and *v* loci, or are they associated with other genetic differences between the stocks? The answer to this question depends upon the genetic methods employed, and may be analyzed in terms of sources of genetic variability between the stocks.

Genetic variability between the stocks will depend upon the methods used in the derivation of the mutant stocks. The most important considerations here are 1) lack of control of chromosome 4, and 2) effects of the X-ray treatment.

Due to the method of their derivation, the mutant stocks would be expected

TABLE 6

Summary of results.

ANTISERUM	ABSORBED BY	TESTED WITH			
		ISOGENIC	VERMILION	RUBY	RUBY-VERMILION
Anti-isogenic	Isogenic	−	−	−	
	Vermilion	+	−	−	
	Ruby	+	−	−	
Anti-vermilion	Isogenic	−	+	−	
	Vermilion	−	−	−	
	Ruby	−	+	−	
Anti-ruby	Isogenic	−	−	−	
	Vermilion	−	−	−	
	Ruby	−	−	−	
Anti-ruby-vermilion	Isogenic	−	+	−	+
	Vermilion	−	−	−	−
	Ruby	−	+	−	+
	Ruby-vermilion	−	−	−	−

to possess, among others, a sample of fourth chromosomes derived from the isogenic stock. This being the case, isogenic antigens produced by loci on the fourth chromosome might be expected to occur in the mutant stocks as well. It will be recalled that isogenic was demonstrated to possess an antigenic fraction *not* possessed by the mutant stocks. This circumstance, along with the small size of the fourth chromosome, would seem to reduce, although not eliminate, the possibility that the antigenic differences between the stocks are due to genetic variability of the fourth chromosome.

The method of X-ray induction of visible mutations in an isogenic stock possesses the objection that invisible mutation may accompany the visible mutation and may be responsible for any observed antigenic change. The probability of such isoallelic mutation at 3,000 r is sizable, and cannot be disregarded.

Further consideration of the implications of this conclusion will be deferred until after an examination of the meaning of the antigenic changes on the hypothesis that they are due to mutation at the *rb* and *v* loci. This hypothesis is, after all, the most probable one, since mutation at these loci has definitely been demonstrated.

As a first step in the interpretation of these findings it would be well to examine the consequences of the hypothesis of a one-to-one relationship between gene and antigen in light of the genotypes of the four stocks used in this work. The genotypes of these stocks may be diagrammatically represented in the following manner:

Isogenic	*rb*$^+$	*v*$^+$	residual
Ruby	*rb*	*v*$^+$	residual
Vermilion	*rb*$^+$	*v*	residual
Ruby vermilion	*rb*	*v*	residual

If each locus produces antigens independently of the rest of the genotype, as is required by the one-to-one hypothesis, then a complete anti-isogenic serum would possess antibodies to antigens produced independently by *rb*$^+$, *v*$^+$, and each of the loci in the residual genotype. Absorption of such an antiserum by antigens of ruby would remove antibodies to the *v*$^+$ and residual antigens, leaving in the supernate antibodies to the *rb*$^+$ antigen. The supernate would, by virtue of these latter antibodies, react with isogenic and vermilion with equal strength. Similarly, absorption of the anti-isogenic serum by vermilion would leave antibodies to the *v*$^+$ antigen, and the supernate would react with isogenic and ruby with equal strength. The results of this investigation do not fit these predictions.

The best interpretation of these results is that, at least in isogenic, the *rb* and *v* loci interact to form a "compound" antigenic substance, that is, Antigen 1. Changes at either of these loci would, then, alter this substance, giving the results obtained in this investigation. It is entirely possible, of course, that other loci are involved in the production of this antigen, but since the effects of mutation of none of them have thus far been investigated, such interaction is entirely hypothetical.

A number of schemes intended to portray, in greater detail, the relationships between genes and antigens in each of the stocks are possible. Before proceeding to consideration of these schemes, implications of the possible occurrence of mutations at loci other than those of *rb* and *v* should be considered. It seems clear that regardless of this possibility the major conclusion of this paper, that is, the demonstration of genic interaction in the production of Antigen 1, remains justified. Even if the antigenic changes exhibited by the two original mutant stocks are not due to mutation at the *rb* and *v* loci, they must certainly be due to mutation at a *different* locus in each of these stocks.

Until such a time as critical work can be performed, the most economical hypothesis is that the antigenic changes observed were actually due to mutation at the *rb* and *v* loci. All further considerations will be based upon this hypothesis.

Figure 1 depicts the relationships of genes and antigens, without implication as to the mode of action of the genes or the chain of events leading to the production of the antigens. In isogenic, *rb*$^+$ and *v*$^+$ interact in an unknown fashion to produce Antigen 1, which is absent in the mutant stocks because of mutation at one or both loci. The possibility that loci of the residual genotype participate in the formation of Antigen 1 is indicated by a dotted line. Ruby possesses no specific antigens (so far as the present evidence goes)—the combination of *rb* and *v*$^+$ must, therefore, have no antigenic effect. Vermilion and the double mutant are identical in the possession of Antigen 2. This is evidently due to their common possession of *v*, which, with or without the partici-

pation of residual loci, produces Antigen 2 independently of the condition at the *rb* locus.

Figure 1 should not be considered final. The failure of the anti-ruby sera to demonstrate specific antigens in ruby should not be taken to indicate that no such fraction exists. Variation in the production of antibodies by different

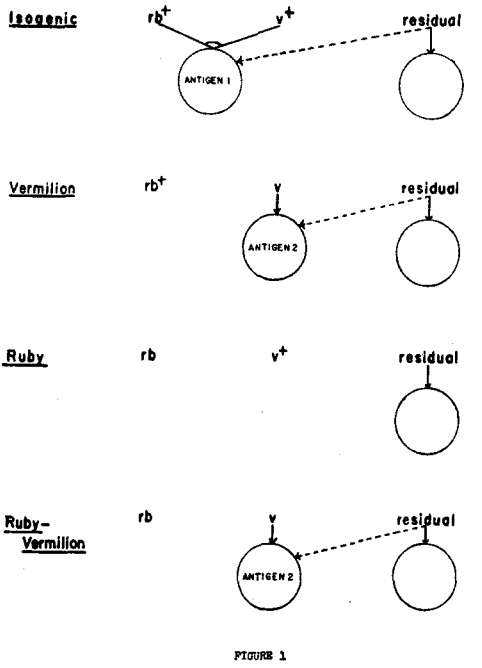

FIGURE 1

FIGURE 1.—Diagrammatic representation of relationships between genes and antigens.

rabbits is a well known phenomenon, and it may well be that the particular rabbits employed in the production of these antisera failed to form antibodies to such a specific fraction. If, upon testing additional anti-ruby sera, ruby is demonstrated to possess a specific antigenic fraction, then figure 1 will require revision.

In addition to this scheme (which implies nothing as to the physiological mechanisms of the genic control of antigen formation in this case) a number of alternative hypotheses concerned with the chain of events leading to Antigens 1 and 2, and consistent with figure 1, are possible. Figure 2 represents several such hypotheses.

The simplest hypothesis consistent with the results is that represented in figure 2a. Antigen 2 serves as substrate for the action of v^+ or its agent, being converted quantitatively to a non-antigenic substance which is in turn converted to Antigen 1 by rb^+ or its agent. Both *v* and *rb* fail to carry out the processes accomplished by their wild alleles.

If Antigen 2 be considered a product of these loci rather than a substrate for their action, then such a hypothesis as that in figure 2b becomes possible.

The same substrate is acted upon by v^+ and v, but with different results.

Figure 2c outlines a hypothesis which allows for the possibility that v^+ and v

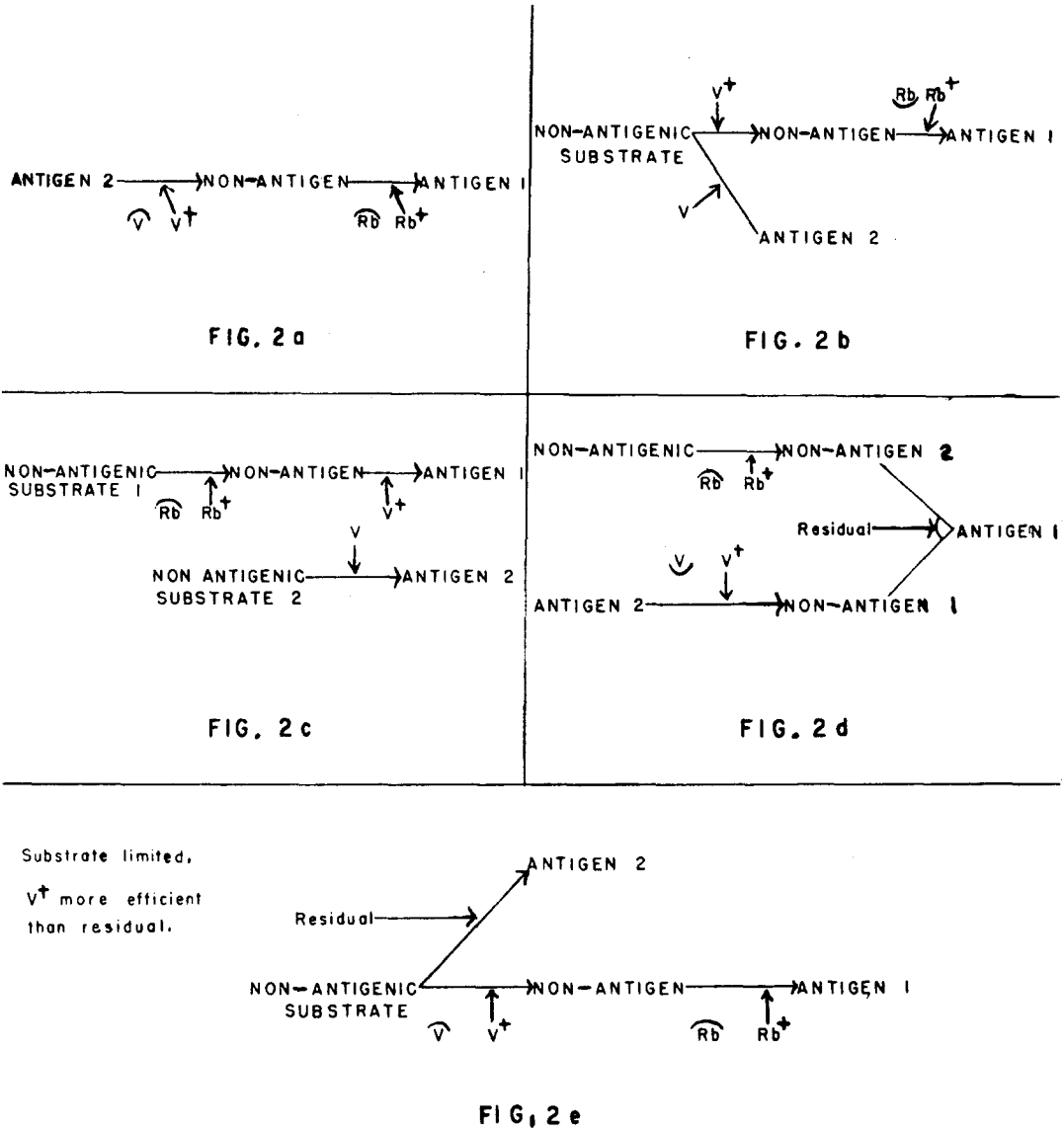

FIGURE 2.—Alternative hypotheses accounting for observed gene-antigen relationships.

act on different substrates. According to this scheme rb^+ acts on a prior link in the same chain of events as does v^+.

Should the two loci be involved in separate chains, then such a hypothesis as that in figure 2d may be formulated. Antigen 2 serves as substrate for v^+, being quantitatively converted to Non-antigen 1. A non-antigenic substrate

is converted to Non-antigen 2 by rb^+. Non-antigens 1 and 2 are combined by one or more loci in the residual genotype, or spontaneously, to form Antigen 1.

These four hypotheses all assume that the availability of substrate is not a limiting factor, and that the rate-constants of the various gene-controlled reactions (WRIGHT 1941) or the efficiency of the various genes (STERN 1943) do not differ. Other hypotheses may consider the possibility of limited substrate and different rate-constants or efficiencies. In figure 2e a non-antigenic substrate, possessing limited availability, may be utilized by both v^+ and one or more loci in the residual genotype. The rate-constant of the reaction controlled by v^+ is, however, greater than that of the reaction controlled by the residual genotype (that is, the efficiency of v^+ is greater than that of residual). Under these circumstances, Antigen 2 will not be formed in the presence of v^+; in the absence of v^+ Antigen 2 will be formed.

It is impossible, on the basis of the data at hand, to judge objectively the relative validity of these or equally likely hypotheses. It is nevertheless interesting to note the complexity of gene-product relationships encountered in a field which previously had been considered one of almost unique simplicity.

The relation between the results of this investigation and the known effects of the *rb* and *v* loci on eye-color is obscure. CASPARI (1946) has demonstrated that the gene *a* in *Ephestia kühniella*, the homologue of *v* in Drosophila, has the effect of causing the accumulation of tryptophane in the tissues, and the incorporation of that amino acid in proteins to a greater degree than in a^+/a^+. This difference in protein structure should have a reflection in antigenic specificity. Unfortunately, in Drosophila *v* does not cause a corresponding change in protein tryptophane, although it does increase the amount of free tryptophane (GREEN 1948 and personal communication).

The conversion of tryptophane to kynurenin under the influence of v^+ is well known (EPHRUSSI 1942). SEWALL WRIGHT (personal communication) has suggested that if kynurenin is a precursor of Antigen 1, then the production of Antigen 2 in the absence of v^+ but not in the absence of rb^+ means that v^+ and rb^+ act on different steps. His suggestion is in agreement with several of the schemes presented above.

The frequency of occurrence of genic interaction in the production of antigens can be determined only by investigation of a large number of other loci. Nevertheless, the possibility that this is a wide-spread phenomenon should be considered. It would be expected that any mutation affecting a biochemical synthesis, more particularly the synthesis of an amino acid, should have an effect on protein structure. Loci involved in a chain of reactions leading to the production of an amino acid would, then, be involved in genic interaction in the production of antigens. This is the case in bacteria, where biochemical and phage-resistance mutations are frequently accompanied by antigenic changes (LURIA 1947). It would be surprising if the same situation did not prevail in higher organisms.

In conclusion, a possible explanation for the difference in the evolutionary behavior of haptenic and protein antigens might be proposed. If it should be found that genic interaction is the rule in the production of proteins in contrast

to a one-to-one relation between gene and hapten, then the sudden, intraspecific changes in haptens would be attributable to mutations in the single genes involved in their production while the gradual variation of proteins would be easily understood on the basis of the many genes involved.

SUMMARY

1. An isogenic strain of *D. melanogaster*, two derived strains differing from the isogenic by X-ray induced mutations, one at the *v* locus and one at the *rb* locus, and a double mutant derived from the two latter strains have been subjected to antigenic analysis.

2. Anti-isogenic serum, when absorbed by vermilion antigens, still reacts strongly with isogenic antigens, but with neither vermilion nor ruby antigens. The same serum, when absorbed by antigens of the ruby strain, still reacts strongly with the isogenic antigens, reacts very weakly with vermilion antigens, and not at all with ruby antigens. These results indicate an antigenic component possessed by isogenic but not by either mutant.

3. Somewhat similarly, anti-vermilion serum, when absorbed by either isogenic or ruby antigens, still reacts strongly with vermilion antigens, but with neither isogenic nor ruby antigens. Vermilion, therefore, possesses an antigenic component specific to itself in relation to ruby and isogenic.

4. To date, anti-ruby sera have failed to demonstrate antigenic components specific to ruby, not possessed by isogenic and ruby.

5. Anti-ruby-vermilion serum gives results identical with those of anti-vermilion serum. The double mutant is concluded to be antigenically similar to vermilion.

6. Double absorptions of the various anti-sera give results consistent with those obtained by single absorptions.

7. These results may be taken to indicate a more complex situation than would be expected on the hypothesis of a one-to-one relationship between gene and antigen. At least the *rb* and *v* loci seem to be involved in the production of an antigen or antigenic complex by means of interaction. Several alternative hypotheses, consistent with the data, are discussed.

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