IMMUNOGENETIC STUDIES OF THE PIGEON, *COLUMBA LIVIA*

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Abstract

Reagents made from rabbit anti-pigeon red blood cell sera detected differences in erythrocyte antigens among individual pigeons. The differences detected by several of the reagents are inherited in a regular Mendelian manner, the positive reaction always dominant to absence of a reaction.

A graded series of reactivities in pigeon red blood cells was observed in the use of rabbit Reagent A. All the cells tested were found capable of absorbing all agglutinins from this reagent. Positive cells differing in intensity of reaction all absorbed activity at the same rate, indicating that the differences between them are quantitative rather than qualitative in nature. These quantitative differences are inherited in a reasonably straightforward manner.

Rabbit Reagent E was shown to be composed of several qualitatively distinct fractions. Three subtypes were detected by the use of several E sub-reagents.

Six isoimmune sera were produced, two of which (RC and H) were analyzed in detail. Each serum was shown to be complex, but the different antigens detected by each were probably related. Matings of positives x negatives which had positive and negative offspring produced them in approximately equal numbers.

The E subtypes and the antigens recognized by the isoimmune sera were seen to be closely related in some as yet unexplained manner. The possibilities that these relationships consisted of linkage or allelism of the causative genes are discussed.

Positivity to Reagent A was found in 14-day pigeon embryos.

Antigen C was detected on the cells of some newly-hatched squabs, but the antigens recognized by Reagents E and H were first detected on the cells of 7-day-old squabs. Cells of positive squabs reached maximum intensities of reaction to all the reagents in one to three weeks after hatching.

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I. INTRODUCTION

The modern science of immunogenetics was established by the discovery of the blood groups in man in 1900 (1, 2). Landsteiner's discovery was based on isoagglutinins normally present in human sera, but during the past few decades the techniques of immunization have vastly extended the range of known blood groups in animals and man. Individual differences in erythrocyte antigens have been found in cattle, chickens, ducks, sheep, horses, dogs, goats, rabbits, rats and mice. Discussions of the findings with several of these species may be found in Wiener (3). Some of the latest publications in which references to other work performed since the publication of Wiener's book may be found, include: man (4), cattle (5, 6), chickens (7), dogs (8, 9), rats (10, 11), and sheep (12). In most of these species, genetic studies indicated that each antigen was inherited as if it were controlled by a simple Mendelian gene and that presence of the antigen was dominant to its absence. Two exceptions, inheritance by recessive genes, are known to the writer (12, 13).

Landsteiner and van der Scheer (14, 15) demonstrated that the inheritance of species-specific antigens could be investigated by studying cells of interspecific hybrids. These authors found that the cells of the mule contained antigenic elements which were characteristic of each of its parent species. It was also shown (16) that immunization of one species (domestig guinea pig) with the cells of another related species (Brazilian guinea pig) produced an antiserum that reacted more strongly with the cells of the parent used as donor in the immunization, than with the cells of an F_1 hybrid between the two species. It was early pointed out (14) that "...several possibilities seemed to exist with reference to the properties of the hybrid cells. They could correspond to those of one of the parents or they might contain elements of both parents, entirely or in part. Finally, the appearance of a new substance might have occurred."*

Three investigations of species hybrids, two in pigeons (17, 18) and one in ducks (19, 20) have yielded results consistent with the assumption

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* p. 214

that a "hybrid substance" not found in either parent species was present on the red cells of the hybrids. This phenomenon has been variously interpreted as an interaction of the genes from the parents (18), a steric effect (21), control of an agglutinogen by a recessive gene (7) and a physiological compensation exhibited by the hybrid (22).

The findings reported in the present study resulted from attempts to determine whether intra-specific differences in erythrocyte antigens could be found in pigeons, in contradistinction to the many species-specific antigens known in pigeons and doves. (See Irwin (23, 24) for descriptions of the species-specific antigens of various Columbidae.)

II. MATERIALS AND METHODS

A. Pigeons

1. Source

The pigeons used in this study were "wild-type" blue bars and blue checkers and some Carneaux and White Kings, all of Columba livia. Some twenty birds were on hand at the time the problem was begun and were used as a matter of convenience. These formed the group on which all the preliminary analyses were performed, and also constituted the breeding stock with which the genetic program was undertaken.

2. Bleedings

Bleeding of the pigeons was done by making a small incision with a razor blade in the brachial vein and bleeding directly into a tube of isotonic sodium citrate (formula: 0.5% NaCl + 2% $Na_3C_6H_5O_7 \cdot 2H_2O$). Approximately 4 ml. of blood was taken from one individual at each bleeding. Care was taken to be certain that all bleeding had stopped before releasing the bird. Repeated bleeding (e.g., certain birds were bled once a week every week for more than a year) had no evident ill effects on the pigeons.

Toward the end of the study, the technique of cardiac puncture was used with some success. A V-shaped platform was constructed to which, on opposite sides, were nailed two 2" wide leather straps. The bird to be bled was placed on its back in the V with its head and neck hanging over the edge of the platform. One strap was passed over the keel and fastened on the other side of the platform, effectively pinning the wings. The other strap passed in the other direction, restraining the feet. The area bordered by the clavicles and the crop was swabbed with alcohol. The bird's neck and head were held down with the left hand, and with the right hand a 21 gauge, 1 1/2" long needle on a 10 ml. syringe was inserted so that the needle passed between the clavicles and coracoids. The path of the needle was parallel to the long axis of the bird. As much as 8 ml. of blood was withdrawn at a single bleeding with no evident harmful effects. This technique has been used about 200 times with no fatalities.

3. Immunizations

At various times, pairs of pigeons were cross-immunized with each other's red blood cells, for several courses of injections. A

course of injections was as follows:

1) 0.5 ml. of a 50% red blood cell suspension intra-muscularly (into the breast muscle);
2) two days later, 1.0 ml. of a 50% red blood cell suspension intra-peritoneally, and
3) four days after the first injection, 0.5 ml. of a 50% red blood cell suspension intravenously (into the brachial vein).

The birds were immunized one week and trial-bled the next. If no antibodies were present, the following week another course was begun. This routine was continued for three courses of injections, after which time, if no agglutinins could be found, the immunization was discontinued.

Blood collected for isoimmune sera was put into dry tubes and stirred rapidly with a thin hardwood splint until clotting was complete. If the clot were broken into small pieces as soon as it formed, much better recovery of the serum was obtained than would have been possible by allowing the clot to stand and shrink as is customarily done with rabbit sera. The latter method would permit recovery of only about 50-70% of the serum. Bleeding in quantity was done on two successive days, a total of about 15 ml. of blood being taken, yielding about 6 to 6 1/2 ml. of serum. The birds survived this treatment in apparently good health.

Iso-antisera collected on two days were pooled and stored in 1 ml. aliquots in 1 dram shell vials without heating. When serum was needed, only the contents of one vial would be thawed. Some of the serum was removed, and the remainder was replaced in the freezer. The maximum number of times the contents of any one vial was frozen and thawed ranged from seven to ten. In contrast to the sera of other birds (25) pigeon sera remain clear during storage under these circumstances; at no time was any flocculation observed.

4. Sexing

Birds of unknown sex were sexed by laparotomy. An incision was made in the left ventrolateral body wall; the dorsal mesentery was pierced and the left gonad directly observed. Neither anesthesia nor asepsis were required for this operation.

Some pigeons were sexed by observation of their nesting behavior; a female would remain on the nest most of the day, giving up her place to the male only in the late afternoon and evening.

5. Matings

All matings were rigidly controlled. Each pair of birds to be mated was put in a separate cage and effectively isolated for as long as the mating was in progress. Eggs laid during the first eight days were discarded, since the paternity of squabs from such eggs might be questioned. Four days after the second egg of each clutch was laid, both eggs were candled for fertility. Eggs not showing signs of a developing embryo were discarded by the sixth day. All squabs, before being removed from the cage and placed in a large holding room, were banded and their descriptions recorded, so that in no case could parentage be in doubt.

All birds were kept in a below-ground-level building, under artificial light. Those not used in matings were kept encaged in a 9' x 19' room under a 10 hour day-length schedule. They were fed standard polished mixed-grain pigeon feed. Although the number of birds kept in this room increased constantly to a maximum of 250, over a two year period only 12 died. Fourteen breeding cages, in a different room, were kept in use at all times. Each cage was 30" in all dimensions, with a platform placed about 18" off the floor on which were two nests. Feeders and watering cans were suspended outside the cage at cage-floor level. The feeders were divided into three compartments; one held a mixture of treated (mineralized) pigeon grits, charcoal and steamed oyster shells; the second held recleaned wheat grain and in the third was a commercial all-purpose chicken feed in pellet form. Birds in matings were subjected to a 13 hour day of artificial lighting. Productivity was high under these conditions, almost all matings producing a clutch of two eggs every four to six weeks. Approximately 75% of the eggs produced living squabs (Table 1).

6. Numbering

The original birds (birds hatched before the beginning of this study) were numbered 1 to 17, 27, 28, and 30 to 35. These were the parents in most of the first set of matings. The numbers missing from this series were assigned to the first offspring to be produced; these were later renumbered according to the following procedure:

As the eggs were laid in each mating, they were lettered consecutively. If the egg hatched, the squab was designated by both the number of the mating and the letter of the egg (e.g., the fourth egg to hatch in mating 5 would be squab 5D).

Table 1

Productivity of the Pigeon Matings Under Conditions Described in the Text.

No. of Matings	Duration of Mating	Eggs Produced	Squabs Produced
1	12 mos.	20	12
4	11 "	73	61
4	10 "	66	53
2	9 "	28	22
2	8 "	26	21
2	7 "	25	17
5	6 "	56	42
9	5 "	80	63
6	4 "	50	31
Totals 24		424	322

Figure 4 (page 45) is a diagrammatic representation of all the productive matings made (the matings numbered 4, 13, 15 and 16 were not included because they were non-productive), and illustrates all the lines of descent. Female and male parents of each cross are represented by dotted and solid lines respectively, drawn from the parent in question to the mating number. This form of presentation of matings is derived from Hollander (26).

B. Preparation of Rabbit Antisera

This section covers only antisera prepared in rabbits. The preparation of pigeon iso-antisera was discussed above (Section IIA3).

1. Immunizations

Rabbits were injected intravenously three times weekly for three weeks with 0.5 ml. of a 20% suspension of thrice-washed pigeon red blood cells in 0.85% saline. Beginning on the third day after the last injection, 1 ml. samples of blood were taken from the marginal ear vein, allowed to clot and the serum poured off. Each sample was titered by the doubling dilution method (i.e., halving the concentration of serum in each succeeding tube) for agglutination tests of the cells of the donor pigeon. Trial bleedings were continued daily until such time that the dilution curve reached a plateau (i.e., the maximal dilution at which agglutination was visible remained the same for two days). This point was reached in all cases in six days. At this time the rabbits were bled by cardiac puncture. After the blood had clotted, the serum was removed and heated at 56° for 30 minutes to inactivate the complement.

2. Reimmunizations

Three months after the first series of injections, in the case of rabbit 13, and fourteen months in the cases of rabbits 7, 8, 9, 10 and 15, all except rabbit 7 were reimmunized with cells of the pigeon used in the original immunization. Pigeon 7, used to immunize rabbit 7, had since died. Pigeon 18, which seemed to have the same blood type, was therefore used as a source of cells for the reimmunization of rabbit 7.

The course of injections for the reimmunization was as follows:

1) 1.0 ml. of a 50% suspension of red blood cells intraperitoneally;
2) two days later, 0.5 ml. of a 20% suspension of red blood cells subcutaneously, and
3) again two days later, 0.5 ml. of a 20% suspension of cells intravenously.

Antibody titer was followed both in the serum and in the reagent (i.e., serum absorbed by appropriate cells and titered against the homologous cells) wherever possible. In some instances it was not possible to detect any antibodies after absorption, and these rabbits were bled at the peak of general antibody level. All rabbits were bled seven to nine days following the last injection, 50 ml. of blood being taken by cardiac puncture on each of two successive days.

The two lots of serum collected from each rabbit were pooled and heated. Aliquots were then put in 1 dram shell vials and stored in the deep freeze. Serum was taken from each vial as needed and the contents remaining were refrozen. Each lot of serum was thus thawed a minimum number of times. Even when certain sera were frozen and thawed repeatedly, no obvious denaturation (precipitation) or loss of activity was noticed.

C. Definitions

Terms to be used in this thesis will be assigned the following definitions:

1. Reagent - the supernatant fluid after a given serum has been fractionated by absorption.
2. Sub-reagent - the supernatant fluid after a reagent has been absorbed by certain cells positive to it, so that there are no longer agglutinins present for the absorbing cells, but activity is still shown toward other positives.
3. Type - the symbolic representation of the antigenic consti-

tution of the red blood cells of a given pigeon, as determined by the typing reagents.

4. Subtype - the antigenic character of certain cells as detected by the use of sub-reagents.

5. Positive - cells which give a definite reaction to the typing reagent and which can remove activity from the reagent by absorption.

6. Negative - cells giving zero reaction to the typing reagent; activity for positives is not removed by absorption with negatives. (This definition does not hold for Reagent A and will be discussed in Section IIIA4).

D. Absorptions

1. Analysis of the Serum

The cells of from five to ten pigeons were used in preliminary attempts to fractionate each serum. A measured volume of the packed, washed cells from an individual bird was placed in each of three tubes. The cells were washed three times with saline and packed hard after the last wash so that all the saline could be removed, to minimize dilution of the serum. To the first tube was added a volume of serum at a dilution of one to eight (1/8) in saline (i.e., one part serum to seven parts saline) equal to the volume of cells in each tube. Serum and cells were thoroughly mixed, allowed to stand five minutes at room temperature, centrifuged and the supernatant transferred to the second tube. This second absorption proceeded for fifteen minutes at room temperature and twenty minutes in the refrigerator, whereupon the second tube was centrifuged and the supernatant transferred to the third. This tube was given twenty minutes at room temperature and thirty minutes in the refrigerator. An aliquot of the supernatant from the final absorption was tested against the absorbing cells at a further dilution of 1/2, in order to test for completeness of absorption. (There should be no agglutination if absorption is complete.) If absorption was incomplete, fresh cells were added until all activity for the absorbing cells was removed. The reagents thus prepared were diluted 1/3 in saline and tested for agglutinins against the cells of each of the birds used in the fractionation.

2. Preparation of Reagents

a. Primary Reagents

The red blood cells of three apparently negative pigeons were

pooled, washed three times in fresh saline and used for absorbing the serum to make the reagent. Since only about 4 ml. of blood a week was taken from each bird, giving a total of about 6 ml. of cells available for each absorption, the following procedure was utilized to secure maximum efficiency. The total amount of cells was apportioned into three tubes. An aliquot of serum, diluted 1/8, equal in volume to the cells in one tube, was passed through the absorption procedure described above (D1). The cells in the first tube were always solidly agglutinated and were discarded. Fresh diluted serum was added to the second tube and then transferred to the third. The cells in the second tube were solidly agglutinated and so were discarded. Fresh diluted serum was then added to the third tube. All the tubes of reagent were stored in the deep freeze until the following week, when fresh cells were obtained. The reagents were then reabsorbed in their original sequence until absorption was complete as determined by agglutination tests of the reagent, diluted 1/3, against each of the absorbing cells.

After the individual lots of reagent of the previous week had been reabsorbed, fresh serum was added to the first tube of cells and passed on through the absorption chain. The second and third tubes also each were saturated with fresh serum in turn. The new lots of incompletely absorbed reagent were stored in the deep freeze until the following week. Completely absorbed reagents prepared each week were added to the previously made stock, and stored.

It must be noted here that the methods used in preparing reagents differed from those used in the analysis of the serum (Sec. D1). In the preparation of reagents, 2 ml. of cells were obtained from each bird and sub-divided into three tubes. For each series of three tubes, 2 ml. of diluted serum was absorbed, which means that each 2 ml. of reagent was initially absorbed by a total of only <u>one</u> volume of cells from each bird. Serum so absorbed required at least three more absorptions, for a total of two volumes of cells from each pigeon, in order for complete absorption to occur.

The procedure described above was used in preparing reagents A, B, D, F and I. Reagent E, after the above protocol had been followed, required further absorption to remove certain very weak or doubtful reactions.

The reagents made from rabbit antisera and the details of their

preparation are listed in Table 2.

Table 2

Reagents Prepared from Rabbit Antisera

Reagent	Rabbit No.	Immunized with cells of pigeon no.	Serum absorbed by pooled cells of pigeons no.
A	13	13	2,4,5
B	9	9	13,17,1A
D	15	15	13,17,1A
E	7	7	1,3,6
F	8	8	1,3,6
I	13B	13	10,7C,6H

b. Sub-reagents

Each sub-reagent was prepared by absorbing the reagent with the cells of each of three birds separately. The cells of each bird were apportioned to four tubes and a volume of undiluted reagent, equal in volume to that of the cells in one tube, was absorbed in the first three tubes. A fresh lot of reagent was placed in the second tube and passed through to the fourth. Both lots were then tested, separately, for completeness of absorption against the cells of each of the three birds used to make that sub-reagent. The following week, fresh cells were obtained and the same procedure followed, adding the new preparations of each sub-reagent to a common stock. Each sub-reagent was actually, therefore, a pool of three sub-reagents each of which had been absorbed by the cells of a single bird.

The sub-reagents are listed in Table 3 together with the cells used in their preparation.

Table 3

Details of the Preparation of E Sub-Reagents

Sub-reagent	Reagent E absorbed separately by cells of pigeons no.
E_2E_3	11,14,8B
E_2	11B,14A,14D
E_3	28A,28B,28C

E. Readings

All typing was done at the reagent dilutions listed in Table 4. Since the rabbit sera had been diluted 1/8 in preparing the reagents, the total serum dilution in each rabbit reagent was 1/8th the dilution shown for the reagent. The iso-immune sera were used unabsorbed, at the indicated dilutions.

Table 4

Dilutions at which Reagents Were Used

Reagent	Dilution
A	1/5
B	1/4
C (isoimmune)	1/20
D	1/4
E	1/3
F	1/5
H (isoimmune)	1/15
I	1/5
all E sub-reagents	1/3

All tests were performed with a twice-washed 2% suspension of red blood cells and in standard agglutination tubes (10 x 75 mm). The tubes were boiled in detergent, washed several times with tap water and then at least three times with distilled water. In each tube one drop of the 2% cell suspension was added to two drops of the reagent at the indicated dilution. The tubes were allowed to stand two hours at room temperature, being shaken every half hour. They were then spun in an International Clinical Centrifuge in a ten-place angle head (specially constructed, not available commercially) at approximately 4,000 rpm for twenty seconds. The tubes were shaken and read as the pellet of cells was resuspended. All negative readings and readings below "3" in intensity were rechecked one hour later.

The symbols used in recording the test results, and their meanings, are as follows:

0 - the button of cells, formed by centrifuging, resuspends evenly and slowly on gentle shaking.

? - the button resuspends somewhat more quickly and irregularly.

t (trace) - the button forms definite jagged lines as it is

resuspended, but no clumps are visible.

1 - a few faint clumps are visible.

2 - several to many small clumps are evident.

3 - many clumps, somewhat larger than the "2" classification can be seen.

4 - a few large clumps are formed.

5 - the button breaks into two large clumps.

6 - the entire button resuspends as one clump.

Readings of the different reagents were checked microscopically. To a drop of the reagent on a slide was added one drop of the 2% cell suspension. The slide was gently agitated for 10-15 minutes at room temperature before examination. It was found that cells which, in the centrifugation test, had given reactions recorded as below the rank of "3" in intensity failed to demonstrate any agglutination on the slide. However, readings from "t" to "1" after being centrifuged and gently shaken contained definite clumps of cells when viewed microscopically. Zero readings by centrifugation were also confirmed in a similar manner.

Several lytic systems were also investigated, using the normal, unheated sera of pigeons, chickens, guinea pigs, rabbits, sheep, cattle and man as sources of complement. All were inactive in the pigeon cell-rabbit antibody system. All except pigeons, however, had normal agglutinins for the cells of the twenty five pigeons used.

Since macroscopic readings after centrifugation seemed to be more sensitive than microscopic readings without centrifugation, and since no lytic system could be found, it was decided to use the first method in this study. Some attempts were then made to increase the sensitivity of this method of reading. In one, a drop of a 1% purified egg albumin solution in saline was added to each tube and the tubes read in the usual manner. In another, the tubes were centrifuged, the supernatants removed and two drops of a diluted chicken anti-rabbit globulin serum added. The tubes were kept one hour at room temperature and read. Both attempts failed to improve the sensitivity of the simple centrifugation test.

III. REAGENT A

A. Serology

1. Analyses

This reagent was prepared from serum 13, obtained by immunizing rabbit 13 against the red blood cells of pigeon 13.

Before beginning a discussion of the detailed analysis of this antiserum, it is pertinent to present some general characteristics of anti-red blood cell sera. An antiserum produced against the red blood cells of one individual of a given species by a member of an unrelated species reacts with the cells of all members of the donor species. Thus we see (Table 5) that the cells of all the pigeons react with the unabsorbed antiserum; the serum is, in reality, "anti-pigeon". But an antiserum against so complex an antigen as the red blood cell contains a great variety of antibodies, some of which may be anti-pigeon (reacting with all pigeon red blood cells) and some of which may be directed against specific sites on the red blood cell which differentiate one pigeon from another. An analysis of such a serum is dependent on the complete removal of the anti-pigeon components by absorption with cells which lack the site or sites (i.e., are negative) against which antibodies detecting individual differences are directed, so that these antibodies remain in the supernatant fluid. This typing fluid may then be used to detect the sites on positive cells.

Preliminary investigation of serum 13 suggested that it might contain two antibody fractions which would serve to differentiate the cells used in the absorption (Table 5). However, several anomalies also appeared. The same types of anomaly have since been encountered several times in absorption analyses of different sera. Table 5 will be described in detail, both as an example of the reasoning used in the analysis of such tables and as an example of anomalous reactions. Discussions and explanations of tables to follow will be concerned mainly with the overall conclusions to be drawn from their analyses, rather than with step-by-step considerations.

In the table, the numbers in parentheses represent the reactions expected on the basis of the two antigens proposed. The numbers not in parentheses represent the reactions actually observed. As an aid in understanding such analyses, a theoretical case will be discussed first.

Table 5

Fractionation* of Serum 13 by Unselected Pigeon Red Blood Cells

Cells	Proposed Antigenic Constitution	Serum 13 absorbed by rbc of pigeons leaving in the agglutinins: 9 Anti-A + Anti-B	10 Anti-A + Anti-B	11 Anti-A + Anti-B	7 Anti-B	12 Anti-A	8 none	13 none	Unabsorbed Serum 13 at 1/1,000
9	O	0	0	0	0	0	0	(0)3	6
10	O	0	0	0	0	0	0	0	6
11	O	(0)4	0	0	0	0	0	0	6
7	A	3	4	4	0	4	0	0	6
12	B	3	3	3	3	(0)3	0	0	6
8	AB	6	6	6	6	6	0	(0)4	6
13	AB	6	6	6	6	6	0	0	6

* The serum, at a dilution of 1/8, was absorbed three times with fresh cells and tested at a further dilution of 1/3.

The numbers in parentheses represent the reactions expected on the basis of the two antigens proposed. The numbers not in parentheses represent the reactions actually observed.

Reagent A1 was prepared by pooling the supernates left over from the individual absorptions with cells 7,9,10,11 and 12.

The code to the numbers representing reactions may be found on page 7.

Thus, in those squares wherein two reactions are recorded, only the reactions enclosed by parentheses will be considered at this time. The others, the observed reactions, will be discussed immediately following the presentation of the theoretical case.

We may first observe that cells 9, 10 and 11 do not react with any of the reagents prepared by absorbing serum 13 with the red blood cells listed in the columns. Cells 9, 10 and 11 can be considered negative in this analysis. Cells of pigeon 7 exhibit the least number of reactions and must differ from the negatives by at least one antigen, which we may designate as A. Cells 12 differ in their reactions from both 7 and the negatives, and therefore, must have at least one other antigen, which will be designated as B. Cells 8 and 13 give test reactions consistent with the assumption that they are AB.

The reactions given by the reagents prepared by the absorptions provide a test of the system proposed. Cells 7, containing antigen A, should remove anti-A from the serum and leave in anti-B. The reagent prepared by absorbing with 7 should then agglutinate the cells which have antigen B; namely, 8, 12 and 13. Absorption by 12 should leave agglutinins for cells containing A; namely, 7, 8 and 13. 9, 10 and 11, being negatives and not having either antigen, leave both anti-A and anti-B in the serum. Reagents prepared by absorbing with these cells should, therefore, agglutinate all the positive cells; 7, 8, 12 and 13. Lastly cells 8 and 13, containing both antigens, should remove all activity (agglutinins) from the serum.

There are a few general rules applying to analyses of this sort, which aid in interpreting the results:

1) Two or more cells which remove activity for each other are identical in their antigenic constitution with respect to the serum under investigation.
2) If cells A remove activity for cells B, while B does not remove activity for A, then A has at least one antigen recognized by this antiserum, that is absent in B.
3) If cells A leave in activity for cells B and B leaves in activity for A, each has an antigen, recognized by this antiserum, that is absent in the other.

Cells 9 and 10, which remove activity for each other, are identical by rule 1. Similarly, 10 is identical to 11 and therefore, by

extension, 9 should be identical to 11. However, the reagent prepared by absorbing the serum with cells 9 actually leaves agglutinins in for cells 11 (reaction in parentheses). This is the first anomaly, that the criterion for identity of cells does not appear to apply in this system.

Rule 2 is illustrated by comparing the reactions of cells 7 and 10, for example. 7 absorbs activity for 10, but 10 leaves in activity for 7, indicating that 7 has at least one antigen (a) absent in 10.

The reactions of 7 and 12 illustrate rule 3. 7 leaves in activity for 12 and 12 leaves in activity for 7. These two cells differ by at least two antigens, 7 having one (A) and 12 having the other (B).

The absorptions in each case are complete as measured by the removal by the absorbing cell of agglutinins for itself, except in the case of the cells of pigeon 12 (reaction in parentheses). It is observed here that while the absorption was incomplete (i.e., 12 left in activity for itself) activity was removed for 9, 10 and 11.

In the last column, absorption by the homologous cells 13 is complete in that all activity for 13 is removed, but contrary to the usual expectation that the homologous cells should remove all activity from an antiserum, 13 leaves in activity for 8 and 9. One could invoke the presence of a normal antibody and suppose that 13 lacks the antigen which reacts with this normal agglutinin while 8 and 9 have it. But if 7, 10, 11 and 12 have the antigen, 13 should have left in activity for them also, and if they lack it, then they should have left in activity for 9. Whatever the origin of the antibodies concerned with these reactions, therefore, their behavior is anomalous.

Another explanation for the abberrant behavior of the reagent prepared by absorbing the serum with 13 is that although 13 is the homologous cell, it is not as effective as 8 in removing agglutinins. This concept, which suggests a quantitative difference rather than a qualitative one, will be discussed and elaborated later.

Reagents left over from absorbing with cells 7, 9, 10, 11 and 12 were pooled and some preliminary typings performed in an effort to detect any positives (such as 7, 12, 8 and 13). With this typing fluid (designated the A1 reagent) both positives and negatives were found in the remainder of the flock.

Since only small quantities of cells can be obtained from one bird at any one bleeding, the cells of nine pigeons negative to the A1 reagent were pooled and the pool then used to prepare a large quantity of reagent from serum 13. This lot of reagent was designated A2. When the A2 reagent was absorbed with a series of cells positive to it, the cells of one bird, 15, which had been positive by typing both with A1 and A2 and had been positive in an absorption analysis of A1, were found to leave in agglutinins for all the other positives (Table 6). Absorption by 2, which did not react to the A2 reagent, removed activity for 15. In the absorption of A1, 15 had removed activity for, among others, cells 1, 16 and 17 but it failed to do so in the absorption of A2. It therefore appeared that the A1 and A2 reagents were distinctly different.

Table 6

Absorption* of Reagent A2**

Cells	Unabsorbed reagent at 1/10	Reagent A2 absorbed by rbc of pigeons: 1	8	13	15	16	17	1C	1D	2
1	5	0	0	0	3	0	0	0	0	4
8	4	0	0	0	3	0	0	0	0	3
13	3	0	0	0	3	0	0	0	0	3
15	3	0	0	0	0	0	0	0	0	0
16	4	0	0	0	3	0	0	0	0	3
17	4	0	0	0	4	0	0	3	3	4
1C	3	0	0	0	3	0	0	0	0	3
1D	4	0	0	0	4	0	0	0	0	4
2	0	0	0	0	0	0	0	0	0	0

* Reagent A2 absorbed by cells of pigeons previously classified as positive to the reagent. Cell 2 is the negative control. Test done at 1/10 dilution of the reagent after absorptions.

** Reagent A2 was prepared by absorbing serum 13 with the pooled red blood cells of nine pigeons negative to Reagent A1. See Table 5 for the source of Reagent A1.

During the course of these early investigations, the quantity of cells used in the absorptions was not very strictly controlled. It would seem (but is by no means certain) that an excess of cells 15 was used in absorbing A1 and a smaller quantity used in absorbing A2. If this were the true cause of the variable results observed, it would

suggest that 15 is actually positive but does not remove activity as efficiently as do the other positives tested in the absorption of A2.

Table 6 also illustrates an exception to the rule that cells which remove activity for each other from a given serum are identical in regard to that serum. Cells 1, 8, 13, 16 and 17 remove activity for each other, and yet 1C and 1D leave in activity only for 17.

Because of the anomalous reactions, the suspicion that the volume of cells used in an absorption may be critical, and the doubt as to whether the fractionations had revealed a single or double antigen-antibody system, a large absorption analysis of the crude serum was set up, using the cells of twenty-one pigeons (Table 7).

As might have been expected from the previous analyses of the serum and reagents prepared from it, Table 7 indicates that a simple analysis of the serum is impossible. Rule 1 is violated over a dozen times. For example, cells 1, 3, 6, 8, 16, 1C and 1E all remove activity for each other, but only 1 leaves in agglutinins for 14; cells 2, 4, 5 and 12 remove activity for each other, but the reagent prepared by absorption with 12 reacts with 11 and 1A, while those prepared by absorbing with 2,4, and 5 do not. The presence of the anomalous reactions makes it impossible to analyze the serum in terms of qualitatively discrete antibody fractions.

Because a specific explanation for the reactions of the reagents prepared from the serum was impossible, a more general solution was sought. It can be seen that the cells can be divided into two broad classes, which may be designated "positive" and "negative". An examination of the rows reveals that certain cells reacted with very few or none of the reagents tested. These cells, 2,4, 5, 9, 10, 11, 12, 14, 15, 1B and perhaps 1A, can be called "negatives". The others, 1, 3, 6, 8, 13, 16, 17, 1C, 1D, 1E and perhaps 1A would then be the "positives". Only a brief examination of the columns is necessary to indicate that this division is justified by the reactions obtained by use of the individually prepared reagents. Thus, the positives remove all, or almost all, of the activity from the serum while the negatives leave in activity for most of the positives.

If there are two general classes of cells, positive and negative, what then can be the cause of the many anomalies? Granted that the same volume of cells was used from each bird, we must return to the statement

Table 7

Absorption* of Serum 13, Indicating Degrees of Positivity and Negativity

	Serum 13 absorbed by cells of pigeons																					
Cells	1	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17	1A	1B	1C	1D	1E	Totals
1	0	3	0	2	3	0	0	4	3	5	4	0	3	3	0	0	0	2	0	0	0	36
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	2	0	3	3	0	0	4	3	5	3	0	3	3	0	0	0	2	0	0	0	31
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3
6	0	3	0	3	3	0	0	4	3	5	3	0	4	3	0	0	2	3	0	0	0	36
8	0	3	0	2	3	0	0	4	3	4	4	0	3	3	0	0	2	2	0	2	0	35
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	3	0	2	3	0	0	4	3	4	4	0	4	4	0	0	0	2	0	0	0	33
14	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
15	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	4
16	0	3	0	2	3	0	0	4	3	4	3	0	3	3	0	0	0	2	0	0	0	30
17	2	4	2	3	4	2	2	5	4	5	4	0	4	4	2	0	2	2	.5	.5	2	54
1A	0	0	0	0	0	0	0	3	0	4	2	0	2	2	0	0	0	.5	0	0	0	13.5
1B	0	0	0	0	0	0	0	2	0	3	0	0	0	0	0	0	0	0	0	0	0	5
1C	0	4	0	3	4	0	0	4	4	5	4	0	3	4	0	0	.5	3	0	0	0	38.5
1D	0	3	0	3	4	0	0	4	4	5	4	0	3	4	0	0	2	3	0	0	0	39
1E	0	2	0	2	3	0	0	4	3	5	3	0	3	3	0	0	0	2	0	0	0	30
Totals	5	30	2	25	33	2	2	48	33	59	40	0	35	36	2	0	8.5	23.5	.5	2.5	2	

* Serum 13 absorbed, at 1/8, by the red blood cells of the individual pigeons listed, and tested at a further dilution of 1/6.

Totals for the columns indicate degrees of negativity; the higher the total, the better the negative in absorptions. Totals for the rows indicate degrees of positivity; the higher the total, the better the positive in tests.

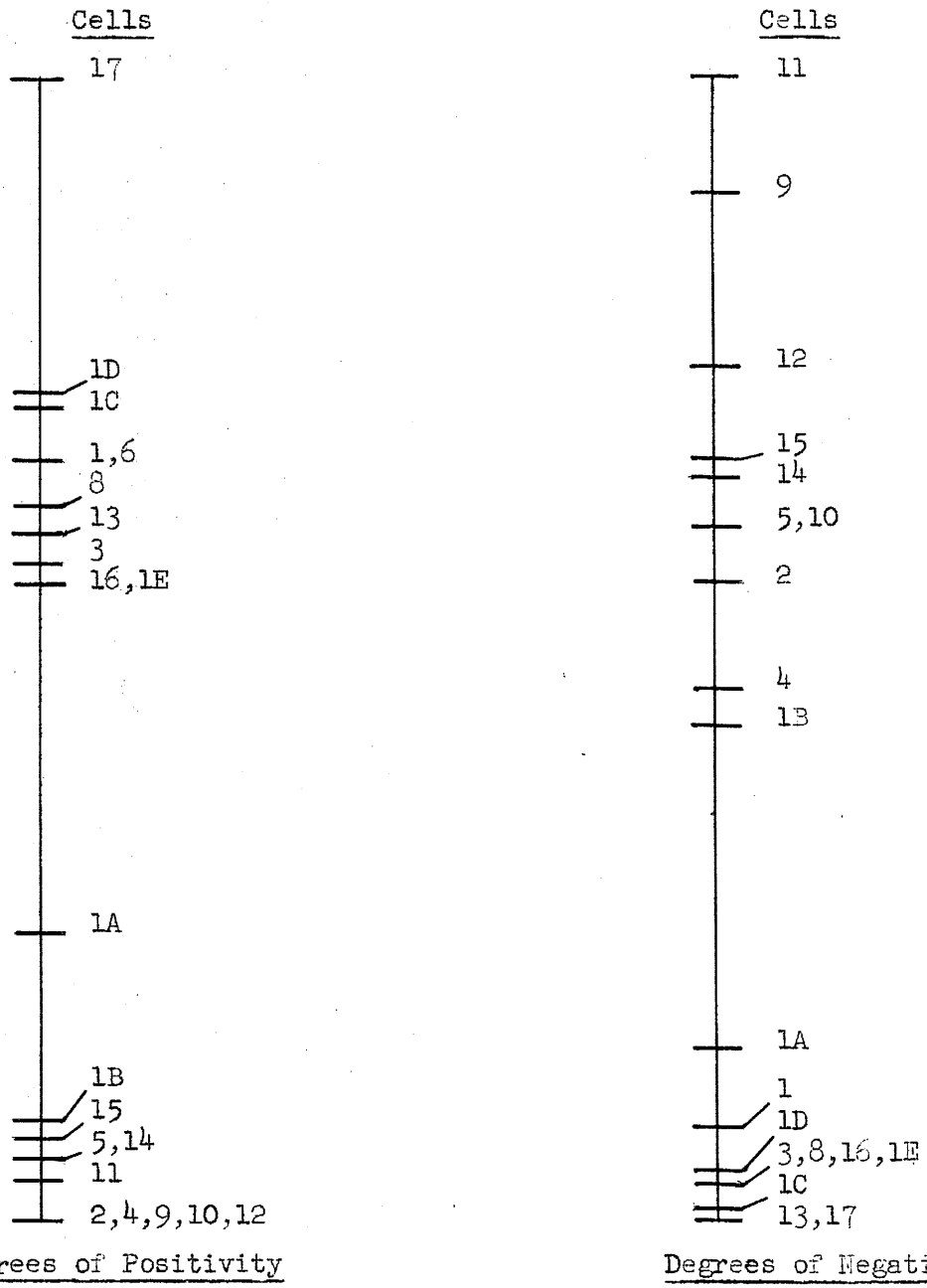
previously made, that the cells differ in the ease or efficiency with which they can absorb agglutinins from the serum. An indication of either the reactive ability or of the efficiency in absorption of the cells of a given bird is given by adding the numerical values of the reactions with which these cells are concerned. Adding across the table for any cell provides an index of the ability of the given cell to react with the reagents, in terms of "strength" of test reactions. Adding down the column for the reagent prepared by absorption with a given cell, an index is obtained of the ability of the cell to absorb activity from the serum.

The last column of the table, listing the row totals, suggests that the cells used in this analysis are divisible into four classes with regard to their reactions with the reagents (i.e., their "positivity"). The most positive are cells 17 with a total of 54, followed by the class with totals ranging from 30 to 39. Cells 1A have a total of 13.5 and the least positive cells (the "negatives") have totals from 0 to 5.

The column totals, listed in the last row, are not so easily divisible into classes. The most positive cells in tests give column totals in absorptions of from 0 to 5, while the least positive cells give totals of from 23.5 to 59. The conclusion to be drawn from these observations is that there is a range or gradation of activities in the ability of cells to act either as positives or as negatives. Cells giving the more positive test reaction remove more antibody from the serum when these cells are used in absorptions. We can visualize two scales, one ascending in order of positivity of cells in the test reaction, the other descending in order of negativity of reagents prepared by absorption with the cells (Figure 1). The positivity scale suggests a division into four classes, but any such division in the negativity scale is not apparent. The class of so-called negative cells (least positive), lying clumped in a very short part of the scale based on test performance (degrees of positivity), is seen to be spread out on the scale based on performance in absorptions (degrees of negativity), indicating that the "negatives" also differ from each other. In typing, the cells of a given bird cannot react more negatively than zero at the lowest dilution (i.e., the dilution of serum at which the absorption is performed), but in absorptions of the serum we see that even those cells which do not react at all in typing (and should be of one class) in reality

Figure 1

Illustrating the Quantitative Relations of the Reactions of the Cells Examined in Table 7



Degrees of positivity are based on test performances (agglutination reactions) of the cells. Degrees of negativity are based on the performances of the cells in absorptions.

The indices of cells 17 (highest positive) and cells 11 (highest negative) were taken as 100 and the figure drawn to scale.

differ in the amount of antibody they leave in the serum after absorption. A logical extension of this line of thought indicates that at least some of the negatives, if they do absorb some agglutinins, are in reality positive. Data on this point will be given later.

To sum up, because of the anomalous reactions observed in absorption analyses and because of the differences existing both among positive cells and among negative cells, we have postulated that there exist degrees of positivity and negativity in relation to the system detected with serum 13. The cells listed in descending order on the scale of positivity in tests are seen to be listed in ascending order on the scale of negativity in absorptions. Cells 1A, listed at the beginning of the discussion of Table 7 (p. 18) as perhaps negative, perhaps positive, are seen to occupy a special position on both scales. They react weakly in direct tests, and absorb agglutinins not as well as a good positive, but not as poorly as a good negative. Such cells, which are both positive to a degree and negative to a degree, might reasonably be expected to occur in the double scale of gradations.

Cells 2, 4 and 5, which absorbed activity for each other and whose reagents gave the same reactions, were chosen as the cells with which all absorptions would be done to make reagent A. Cells with higher ratings as negatives (e.g., 9, 11, and 12) might have been preferable in some respects, but the reagents prepared with them had not given identical reactions toward the other cells.

The supposition that negatives may actually be so weakly positive that they would react only below the limiting dilution of the reagent, and thus escape detection as positive in tests, can be verified by absorption tests. A preliminary indication that negatives might remove agglutinins for positives was obtained in a test originally designed to ascertain whether the cells of some newly obtained pigeons differed from each other (Table 8). Two of the absorptions, those for cells 33 and 34, were not complete. Cells 1F, 27 and 5A, as a result of the first set of absorptions, were shown to be negative in relation to the others, but on reabsorbing the reagents two more times several reactions disappeared; the negatives had absorbed more activity from the serum. Likewise, some of the positives which had been differentiated from each other in the first test removed additional activity in the second. Thus again we see that not only do negatives differ in their degrees of negativity

but positives also differ in their ability to remove activity for each other, activity for some being removed more easily than for others.

Table 8

An Illustration of the Effects of Reabsorption* on a Given Set of Reagents

	Serum 13 absorbed by rbc of pigeons									
Cells	1F	27	28	5A	30	31	32	33	34	35
1F	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0
28	2	2	0	2	0	0	0	0	0	0
5A	0	0	0	0	0	0	0	0	0	0
30	4	4	0	4	0	0	0	0	0	0
31	3,0	3,0	0,0	3,0	0,0	0	0,0	0,0	0,0	0,0
32	4,2	3,2	0,0	3,2	0,0	0	0,0	0,0	0,0	0,0
33	5,4	4,4	3,2	5,4	4,3	4	4,3	3,2	3,2	3,3
34	3,2	3,0	2,0	4,3	2,2	3	3,2	0,0	2,0	2,0
35	5,0	3,2	2,0	5,3	3,2	-	2,2	3,3	2,0	0,0

Serum 13, diluted 1/8, absorbed by cells of pigeons indicated and tested at a dilution of 1/3.

* Single figures and the first figure of the pairs of figures are results of absorbing the serum three times. The second member of each pair represents the results of five absorptions (the remainder of the reagent prepared by three absorptions reabsorbed two more times).

The dash indicates a combination not tested.

Table 9 illustrates the similar results that were obtained in the absorption of Reagent A by selected positives and negatives. Reagent A was prepared by absorbing the serum with the pooled red blood cells of pigeons 2, 4 and 5 until it no longer reacted with the cells of any of these three birds, tested separately. Further absorption by cells 5 decreased the intensity of the reaction for some positives and removed the reaction for others. All the other negatives except 25E also removed agglutinins from the reagent, and all the positives removed all or most of the activity, again indicating the similarity between positives and negatives in their reactions with this reagent.

Several negatives, including 5, were selected from the cells in Table 9 and used to absorb the reagent five times. The individual reagents so prepared were then tested against four of the strongest positives of Table 9, including 13, the homologous cells. Three of the four negatives removed all activity for all the positives. The fourth,

Table 9

Absorption* of Reagent A by Selected Pigeon Red Blood Cells

Cells	Reagent A at 1/3	Reagent A at 1/5	Reagent A absorbed by rbc of pigeons: 5	12	13	15	1F	34	2B	8A	9D	3L	3P	23B	27G	25E
5	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	3	3	2	t	0	1	0	0	0	0	0	0	?	0	0	3
15	t	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	5	5	2	2	0	1	t	0	0	0	0	0	0	0	0	3
2B	5	4	2	t	0	0	0	0	0	0	0	0	0	0	0	3
8A	6	4	2	1	0	0	0	0	0	0	0	t	0	0	0	3
9D	5	5	3	2	0	t	0	0	0	0	0	2	0	0	0	3
3L	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	?
3P	1	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23B	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3
27G	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Reagent A (serum 13 absorbed by pooled cells of pigeons 2, 4 and 5) absorbed three times, undiluted, with the cells of positives and negatives and tested at 1/3.

again cells 25E, left in activity for all positives, activity which incidentally was weakest for cells 13. It has already been noted that 13 was not as effective in removing agglutinins as was another positive (p. 16), and wherever absorption analyses have been performed using cells 13 it can be seen that there are positives which give stronger reactions with the reagents so prepared than does 13.

The action of the cells of pigeon 25E deserves special reference. These cells were the only negatives tested in Table 9 which did not remove all activity from the reagent. This was, of course, what was to be expected from a true negative in the classical sense. Did we, then, have two major types of negatives; the "false negatives" which were negative to the typing reagent but positive in absorption studies, and the "true negatives" which were negative in both tests and absorptions? The absorption study described in Table 10 was performed in an attempt to answer that question.

Table 10

Reagent A Absorbed* Undiluted by the Red Blood Cells of Selected Negative Pigeons

	Reagent A absorbed by rbc of pigeons													
Cells	2C	2R	3A	5D	6E	17H	22C	23F	24A	24C	25B	25F	30B	30D
13	t	0	0	t	0	0	0	0	0	0	0	0	0	0
34	2	0	0	0	0	0	0	0	0	0	0	0	0	0
2B	2	0	0	0	0	0	0	0	0	0	0	0	0	0
8A	2	0	0	0	0	0	0	0	0	0	0	0	0	0
9D	3	t	0	t	0	0	0	?	0	0	t	0	t	0
23B	2	0	0	0	0	0	0	0	0	0	0	0	0	0

* Each lot of reagent was absorbed three times with twice its volume of cells, and tested at 1/3.

Matings of pigeons had been in progress all during the course of this study. Birds positive to the A reagent had been mated to negatives in an attempt to learn something of the inheritance of the A character. Many of these positive parents had proven to be "heterozygous", in that some of their offspring had been positive, some negative. The negative offspring used in the absorption (Table 10) were of two classes: negative offspring of the cross of a negative with a weakly reacting heterozygote, and negative offspring of the cross of a negative with a strongly reacting heterozygote. The negative offspring within each cross were chosen at

random. The table does not list the type of cross from which each bird is derived, because the results were almost uniform.

All the reagents tested in Table 10 were deliberately over-absorbed, each lot of Reagent A being absorbed with twice its volume of cells. The results are easily analyzed: all the negatives except 2C, of the ten crosses investigated, can remove most, if not all, of the activity from Reagent A for six different positives, including the homologous cells. 2C, the female parent of 25E, was also shown to be an extreme negative in that it left activity in the supernatant after absorption whereas the other negatives did not. Table 10 also illustrates the fact that not all the negatives of mating 25 are of the 25E type; 25B and 25F behaved as negatives in the direct test, but removed the agglutinins when they were used in absorption.

Up to this point, a discussion of the causes of the differences among positive cells and the differences among negative cells has been avoided, because none of the data could resolve the problem. The problem is: are the differences we have observed among positives and among negatives qualitative or quantitative? Do these differences indicate the presence of different cross-reactive antigens on the cells or do they indicate the presence of a greater (on the stronger) or lesser (on the weaker) amount of the same antigen?

An answer to this problem was suggested by the status of the homologous red blood cells of pigeon 13. If a series or group of cross-reactive antigens existed, one might reasonably expect that the antiserum and any reagent prepared from it would react best (to the highest titer) with the homologous cells since most of the antibody reactive sites would be more closely complementary to the particular antigen of the homologous cell. But it has already been noted that 13 consistently reacts more weakly than do many other positives, with any given reagent prepared from serum 13. There are, in fact, cells which give positive reactions when Reagent A is diluted two to four times the maximal dilution at which it is possible to observe a reaction with cell 13. This is taken as suggestive evidence that the antibody specificity is directed against some antigen (or antigens) on the cells of pigeon 13 which exists in variable amounts on pigeons' cells.

An approach to the distinction of a quantitative from a qualitative basis for individual variation in this system was made by way of a

modification of comparative absorption curves as described by Race et al. (27). This method compares the change in titer of a given serum toward given positive cells when the serum is absorbed with different volumes of other cells.

Figures 2A and 2B illustrate the results obtained by absorbing Reagent A with the cells of four different positive pigeons and titering the resulting reagents against each of the same four. The abscissa represents the volume of cells used, while the ordinate represents the titers on a logarithmic scale. Using the logarithmic rather than an arithmetic scale condenses the vertical dimension of the figure. In a titration of any serum, using the method of reading previously described (Sec. IIE), the last two tubes in some cases may read "trace" and "doubtful" in the order of increasing dilution, while in other cases, the last two tubes may read "1" and "0" in the same order. Since the trace and doubtful reactions are reproducible, they represent real reactions and must be taken into account. For that reason, the titer of each reagent was scored as that dilution at which the first unambiguous zero was noted. The reagents were titered by the doubling dilution method (i.e., halving the concentration of reagent in each succeeding tube).

It is seen that the curves are all similar. The relationship between quantity of cells used in absorption and titer reduction is approximately the same for all the positive cells used. The differences at the end point of the test (absorption by one volume of cells) in the titers of the reagents so prepared were due only to the fact that the titers of the unabsorbed Reagent A differed for each of the cells tested. This observation is taken to mean that the four positives tested (and from this, we assume all positives) did not differ at all in kind, but only in degree. Had at least one of the positives differed from the others, the curves depicting the changes in titer of reagents prepared from it toward the other cells would have quickly reached a plateau, at which point further absorption would have not decreased the titer. (Such a phenomenon is illustrated in Figure 6A, p. 81; activity for cells 13 remained at a plateau when titered against successive reagents prepared by absorbing the serum with cells 28B, even though 28B removed activity for itself.)

Figures 3A and 3B illustrate the results of the application of the technique of comparative absorptions to the negative cells. The reagents prepared by absorbing Reagent A with each of the negatives were

Figure 2

Reagent A Absorbed by Positive Cells

Figure 3

Reagent A Absorbed by Negative Cells

The points represent the titer on a logarithmic scale after absorption by the indicated volumes of cells.

Code - cells used in tests

● - cells 9D

○ - cells 34

◒ - cells 9P

◓ - cells 13

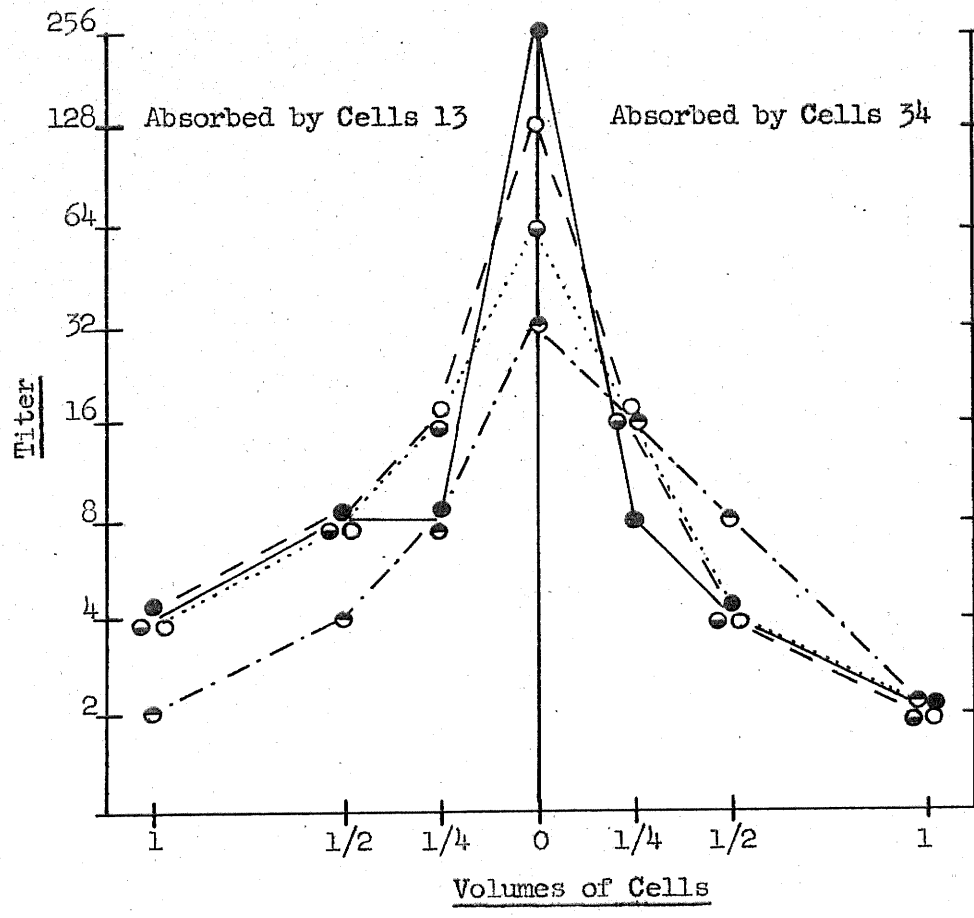


Figure 2A

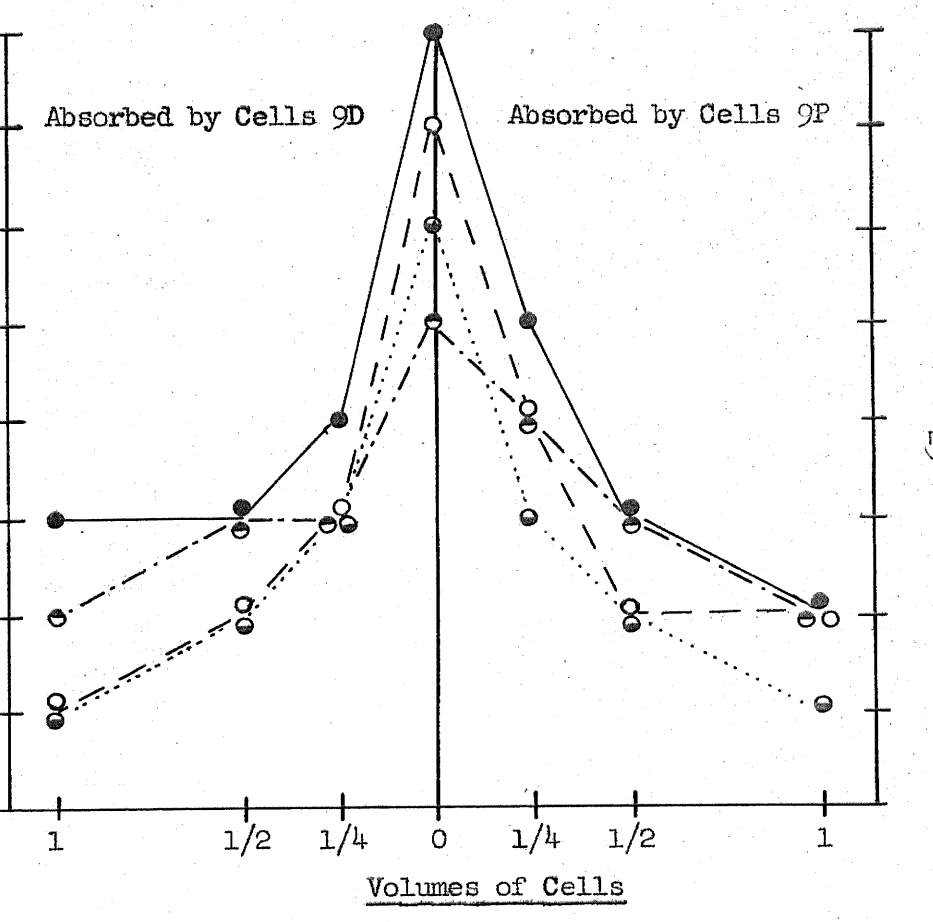


Figure 2B

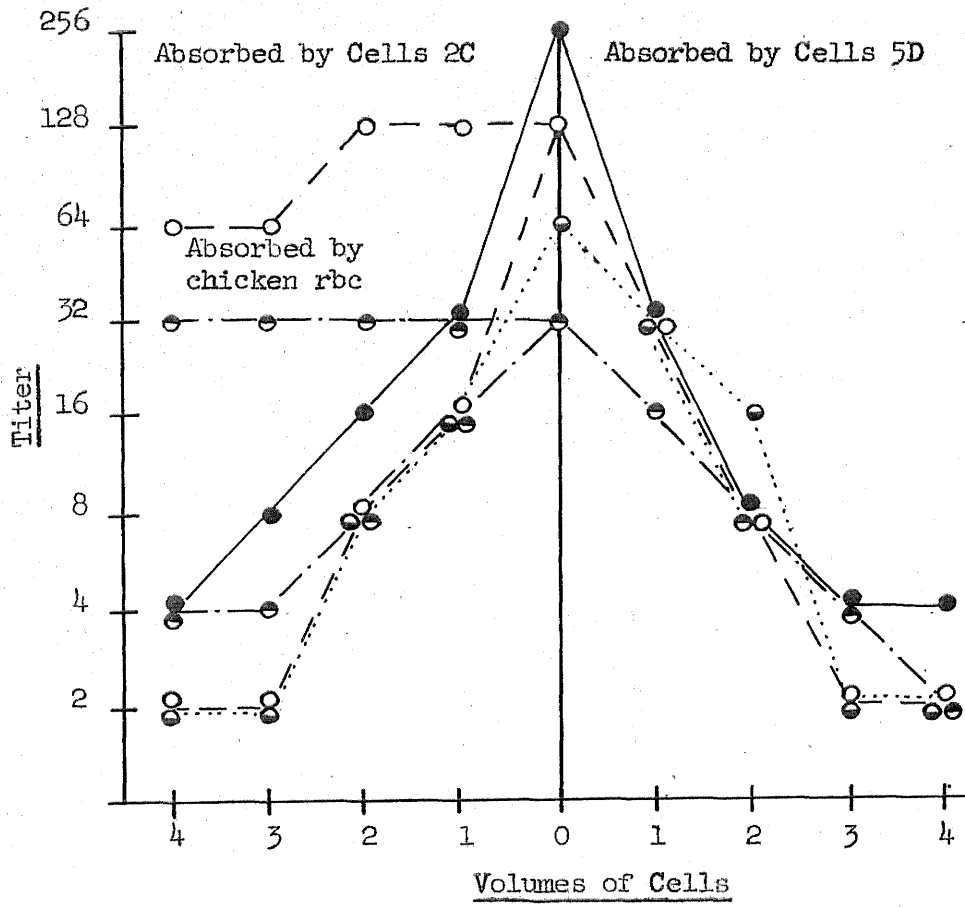


Figure 3A

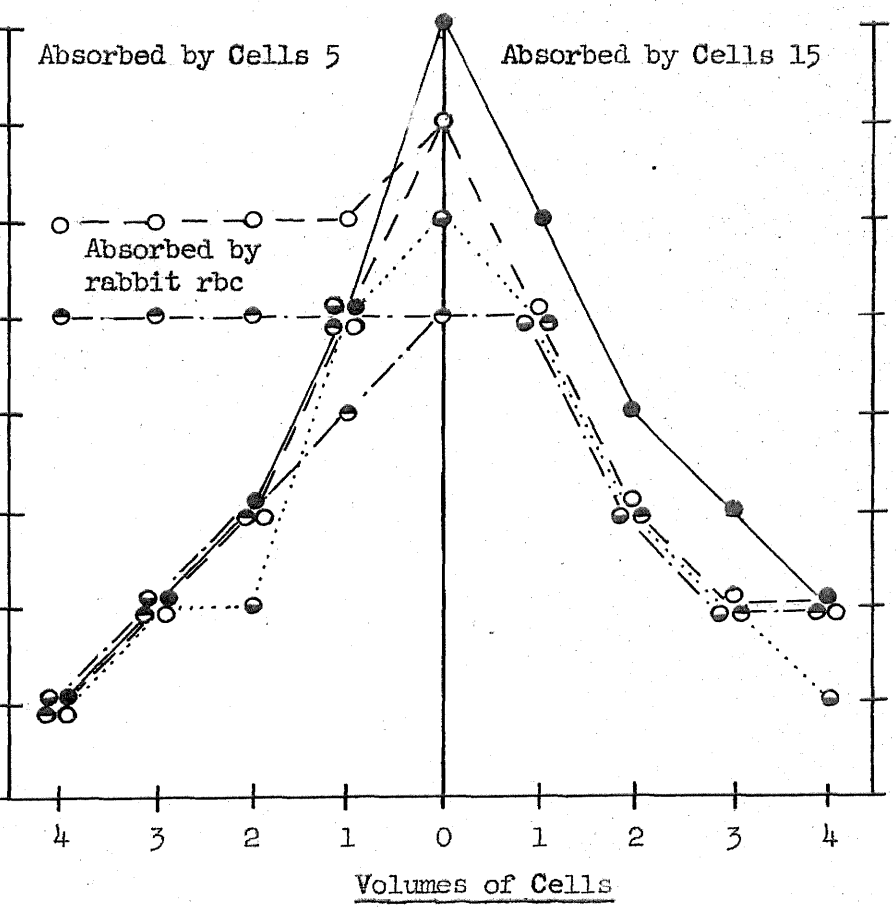


Figure 3B

titered against the same four positives as in the previous tests, after absorption by one, two, three and four volumes of cells. The extreme negative, cells 2C, is seen to give much the same fall in titer as the other negatives, showing that 2C is not intrinsically different, but is only lower on the scale of negativity.

As controls on both the specificity of the reaction and on the possibility of the activity being simply diluted in the absorption, the same procedure was followed using chicken (Fig. 3A) and rabbit (Fig. 3B) red blood cells. The cells of each animal were negative to the reagent. With each successive absorption there was a small decrease in the intensity of each reaction until finally the end point shifted one tube (cells 34 Fig. 3A and cells 34 Fig. 3B). After five absorptions, however, the titer for the specific activity still remained much the same as that for the unabsorbed reagent. Chicken and rabbit red blood cells, then, illustrate the behavior expected of true negatives to Reagent A.

2. The Reimmunized Serum

Rabbit 13 was reimmunized with the red blood cells of pigeon 13. After the rabbit had been bled in quantity an attempt was made to reduplicate Reagent A by absorbing the serum with cells 2 and 4, and testing the resulting reagent with a few selected positives. Because this attempt failed, an absorption analysis was performed.

Two different analyses, one using the cells of ten pigeons and the other using the cells of eleven other pigeons, indicated that cells which were positive to Reagent A as well as cells which were negative to Reagent A could remove all activity from the serum. Three cells were found, all negative to A, with which reagents could be made detecting weak activity in the A positive cells and either no or still weaker activity in the A negative cells.

Curves of comparative absorption analyses, performed with two of the negatives and one of the better positives, gave the same results as were obtained in the case of Reagent A. The negatives removed activity for both the positive and 13, the homologous cell, at almost the same rate as they had removed activity from the crude serum for each other. The difference between positive and negative was again in degree, not in kind.

Since the reactions observed were weak, no attempt was made to prepare a reagent from this serum.

3. Reagent I

The cells of pigeon 13 were injected into another rabbit (13B)

in an effort to obtain more Reagent A. Two absorption analyses of the 13B antiserum, each including the cells of ten pigeons, indicated that the antiserum was complex. The many anomalies in both analyses prevented any direct conclusion, but the cells of three pigeons, 10, 7C and 6H (all negative to Reagent A), seemingly more negative to the 13B serum than any of the other cells analyzed, were chosen to make a reagent. No further analyses were attempted; the reagent produced by absorbing the serum with the cells of the three pigeons listed was used only in typing. It was designated as Reagent I.

All offspring of matings in the study were typed with Reagents A and I at the same time. The similarities of the reactions indicated that Reagent A had been duplicated. The first stock preparation of Reagent I seemed to give slightly stronger reactions than those obtained with Reagent A, and many cells negative to the latter were weakly positive to the former. The second stock preparation, however, gave weaker reactions than those obtained with Reagent A. This may have been because more absorptions were used in the second preparation than in the first. Evidently, the same quantitative effect discussed earlier for A also prevailed for I. Overabsorption with negatives could remove additional activity.

In view of the fact that Reagent I duplicated Reagent A, no separate genetic analysis is necessary.

4. Discussion

Typing reactions obtained with and absorptions performed on Reagent A have indicated that the red blood cells of different pigeons have graded quantities of apparently the same antigen. At the typing dilution used, cells reacted negatively, very weakly, weakly, moderately or strongly, in a continuous spectrum of reaction intensities; no discontinuous divisions were discernable. Positive cells reacted to different titers of the reagent, but all were capable of absorbing all activity from the reagent. Differences among negative cells were also shown, in that absorption of the serum by various negatives created reagents of different strengths. Deliberate overabsorption of the reagent, using large excesses of negative cells, showed that they too were capable of specifically absorbing all agglutinins.

Quantitative differences (or at least differences in reactive abilities of different bloods to the same serum) related to the presence

of a single antigen have been known since Landsteiner and Levine (28, 29) first reported that two sera, anti-N and anti-P, detected individual differences among human bloods. The reactions observed with these sera varied from very weak or negative to very strong. For general typing with respect to N, however, sera were deliberately chosen which gave the least number of positives and which exhibited a distinct break between positive and negative reactions (30). These workers avoided a study of the phenomenon of graded reactions and chose instead to investigate the antigen on a qualitative basis, i.e., positive vs. negative cells.

Henningsen (31), using several sera from different sources, found a gradual transition from cells which were negative to anti-P through those which were weak to those which were strong. Race and Sanger (4) state that

> "The problem of grades of strength of blood group antigens... (also) arises in the work on the O...antigen of the ABO system, and on the D^u antigen of the Rh system."*

(The writer has not been able to find a specific reference in the literature to any report on a graded series of reactions observed with antigen O. The case of the D^u antigen will be discussed elsewhere in this thesis [Sec. VE].)

It was found (31) that all the bloods positive to anti-P could remove specific activity by absorption, as could some of those which were negative to anti-P. The general phenomenon, of bloods negative by typing but behaving as positives in absorptions, has occurred many times, but does not appear to have been fully investigated. Henningsen, in the study just mentioned, did not attempt to ascertain if the bloods negative by typing with anti-P but positive by absorption differed among themselves as to the efficiency with which they removed agglutinins, nor did he attempt to determine whether or not the "true" negatives (negative by typing, negative by absorption) could also remove activity by repeated overabsorption. Landsteiner and Levine (32) found that cells negative for M could remove anti-M activity only from certain rabbit sera (there was no evidence of a graded series of reactions in these cases), and that cells negative for N could frequently absorb activity from anti-N. Stormont (33) has also found that some K-negative bloods in cattle (anti-K is derived from an isoimmune serum) can absorb

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* p. 74.

K antibodies, and that bloods negative to some of the weaker B system reagents can often exhaust all the B antibodies. In the latter system, there is a range of cell types with regard to affinity for the antibodies.

Landsteiner and Levine (32) suggested that the species-specific antibodies and those recognizing individual differences within a species might be "in some sort of combination." There is some uncertainty involved in interpreting such a statement. The modern interpretation of the phenomenon might be that the sera under study contained antibodies which were capable of cross-reacting with all human bloods, but that they reacted more readily with the cells of some individuals than with others.

An explanation for the ability of putative negative cells to absorb agglutinins from a heterospecific antiserum could be that the cells merely differ in the amounts of many species-specific antigens they contain. Conceivably, this might explain both the observations described in this study, where some negatives are positive by absorption tests (and all are believed to be), and the effects observed with anti-P in which only certain negatives are positive by absorption, but in which the other negatives have not been proven to be negative by overabsorption studies. This suggestion would also serve to explain why the homologous cells of pigeon 13 react to a lower titer of Reagent A than do the cells of other birds. But it does not explain the fact that these differences are subject to simple genetic segregation, and that they do, in fact, simulate a one gene-one antigen form of inheritance. Certainly numerous species-specific antigens can be expected to require the control of numerous genes.

On the other hand, the "antigen" concerned might be a <u>single</u> species-specific antigen, present in various amounts on the red blood cells of all pigeons. On this hypothesis, one should never be able to detect any true negatives. We cannot say whether or not true negatives to Reagent A exist; many negatives were tested in this study but our sample of the genetic material of pigeons in general was very small. The original flock consisted of only twenty-six birds of which only ten were negative by the typing reaction. Henningsen claims that bloods negative to anti-P by typing can be either positive or negative in absorption studies, but he did not perform the definitive overabsorption studies. Final decision on this suggestion, that one is studying the quantitative expression of a single antigen present to some degree on

the cells of all members of the species, must be held in abeyance until true negatives can be shown to exist, or until sufficiently extensive studies offer a high probability that they do not exist.

Quantitative effects without a graded series of reactions have several times been shown to occur. These observations have indicated a dosage effect in which the heterozygote (only one allele producing the antigen in question) has been shown to give weaker reactions than the homozygote. This situation has been found in studies of the Rh system; anti-e (34), anti-c (35), anti-C^W (36) and anti-f (37) give marked dosage effects with particular sera, reacting to a higher titer with bloods from homozygous persons than with bloods from heterozygotes. The first case of a dosage effect (not recognized as such at the time) was reported by Landsteiner and Levine (29) who remarked that reactions for N seemed to be stronger where M was absent. That M and N behaved as alleles was demonstrated later (30). Race et al. (38) also reported that a particular anti-Fy^a serum seemed to distinguish the bloods of most, but not all, heterozygotes from those of most homozygotes, with variations in activity appearing in both classes. Dosage effects have also been found in organisms other than man, such as cattle (39), chickens (40) and rats (11). Different quantities of the same antigen can be produced and individuals are sometimes observed to fall into discrete quantitative classes.

It has been tacitly assumed in most of the reports cited that the presence of one allele causes the production of a certain amount of antigen and the presence of two alleles simply causes production of more of the same antigen. Owen (11) however, advanced the hypothesis that two different alleles may compete for a common substrate which is limited in quantity. In the heterozygote, this competition may result in decreased production of either antigen, not because the allelic dosage is limiting but because the quantity of substrate is insufficient for maximum expression of either allele. No definitive evidence for this hypothesis has yet been obtained, but both in rats (22) and in the human blood groups (41), supporting data are known. It has also been claimed (42) that since not all antisera to a specific antigen give a dosage effect, the antigens produced by the homozygote and the heterozygote may be qualitatively different.

Genetic effects on quantitative differences at the molecular level are known in other fields. Studies on abberrant hemoglobins, for

example, have indicated a straightforward relation between the amounts of aberrant and normal hemoglobins and homozygosity or heterozygosity (43, 44). It appears that certain of the quantitative differences observed reflect differences in the rates of formation of the qualitatively different adult and abnormal hemoglobins (45). Landman and Bonner (46) described mutant strains of Neurospora which utilized lactose poorly as a carbon source, and found that these strains formed smaller quantities of an enzyme which seemed to have the same properties as the enzyme in the normal wild type.

Certain theoretical implications of these considerations in the field of serology depend upon the absolute distinction of qualitative from quantitative differences. Irwin and Cole (18) used as proof of species differences the fact that cells from one member of one species reacted to a different titer than the cells of a member of another species, with the same antiserum. But it has been shown both in the present study and in Henningsen's work with anti-P (31) that differences in titer do not necessarily reflect qualitative differences. Buchbinder (47) also observed that titration values need not be correlated with qualitative differences, even when two different taxonomic families are involved. He found that while the red blood cells of members of the Treronidae (fruit pigeons) never reacted to dilutions higher than 1/320, and cells of the Columbidae (true pigeons) reacted to dilutions as high as 1/2560, of the same antiserum, erythrocytes of members of either family could absorb all agglutinins from the serum. We may only conclude that differences in titer of reaction may indicate qualitative differences, but that additional evidence is necessary before the unquestionable distinction of qualitative from quantitative differences is established.

Additional evidence is, of course, provided by absorption data. But here too, there may be some doubt; absorption analyses may not always be definitive in deciding between qualitative and quantitative differences. Either a small quantity of A_1 cells or an excess of A_2 cells can absorb all alpha agglutinins from a human B serum (48). This appears to be the same quantitative effect as has been encountered in the present study. Landsteiner and Witt (49) and Landsteiner and Levine (50) however, have shown by other more sensitive tests that A_1 and A_2 agglutinogens actually differ qualitatively, but that A_2 cells have a weak affinity for the agglutinins which react strongly with A_1 cells. Other confirmatory evidence

for the qualitative difference has come from Olbrich and Walther (51) and Wiener (3). Three other instances of qualitatively different cells absorbing all activity from the same antiserum are known, all of which are in the Rh system (27, 52). These will not be discussed at this time (see Sec. VE).

How then can we interpret the results of the studies with Reagent A? We have seen that titer differences do not necessarily represent qualitative differences, nor does absorption of all activity by all cells necessarily indicate a single, uniform antigen. The critical evidence in the present study is taken to be the absorption data presented in Figures 2 and 3. The fact that various positives absorbed activity from the reagent at the same rate as did the homologous cells is interpreted to mean that all positives possessed the same antigen. Had some of the positives possessed different but similar antigens, absorption by these would be expected to have depleted the reagent at different rates, even though these might have eventually exhausted the reagent of all activity. Strong supporting evidence for the quantitative nature of the phenomenon is the fact that the homologous cells react to a lower titer than do other cells, for in the words of Landsteiner (53) "...it is certain that antibodies react most strongly upon the homologous antigen..."* Stormont (33), on the other hand, has evidence that cells other than the homologous ones may have a greater affinity for the antibodies. However, for the two reasons cited, and especially the former, the author believes that individual differences with regard to Reagent A have an essentially quantitative basis.

A practical implication in this work lies in the techniques generally used to absorb and prepare typing fluids. If a quantitative effect is suspected, especially if a graded series of reactions is observed, then the choice of negatives to absorb the crude antiserum can be critical. Unless "good" negatives (that is, extremely weak positives) are chosen, much of the specific activity of the serum may be removed. This same conclusion has also been reached by Stormont (33).

B. Genetics

1. Experimental Results

Because of the great variation in the reactions to Reagent A

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* p. 266.

at the typing dilution of 1/5, the cells were rather difficult to classify. It was arbitrarily decided that reactions of "0", "?" or "t" were to be classed as negatives in the typing reaction, and all reactions of "1" or above were to be considered positive. The reagent was titered by the doubling dilution method (end point-first unambiguous zero) with the cells of every pigeon. Such titrations were performed only once on the red blood cells of each bird, and although these results cannot be considered absolutely reproducible, the resulting data were indicative of a consistent trend. In Tables 11 and 12 are listed, by family, the number of offspring and their classifications according to the "zero" end point. The dilutions of serum tested ranged from 1/8 (Reagent A undiluted) to 1/1024; the red blood cells of one pigeon (not in this table) gave an end point at a reagent dilution of 1/512 (serum 1/4096). The cells of pigeon 13, the homologous cells, gave an end point of only 1/32 for the reagent.

In all the families listed, the offspring are observed to fall into reactivity classes clustering around the values characteristic of the parents. For example, in mating 6 (Table 11) the parents are of reactivity classes 1/2 and 1/32 and the offspring are of classes 1, 1/2 and 1/32, 1/64. None of the crosses of negative by negative give obviously positive (dilution class 1/16 or above) offspring. In the grouping of the offspring there is always a small spread of one or two dilutions on either side of the parental classes. This small but definite spread is the cause of the anomaly to be discussed shortly.

As a general rule, it may be stated that immunogenetic studies have indicated that the presence of a particular antigen is usually dominant to its absence, in the sense that the antigen is regularly expressed in the heterozygote, and that segregation patterns suggest that one pair of alleles is responsible for the presence vs. the absence of the antigen. Tables 12 and 13 indicate that the A system does not constitute an exception. When the typing reaction is used merely to differentiate between positives and negatives at a particular dilution of the reagent (1/5), families in which one parent is positive and the other negative, and which have both positive and negative offspring, have them in approximately equal numbers (Table 13). Classification of the families starred in the table may be considered doubtful. Mating 17 was a mating of a weak positive with a negative; it gave a preponder-

Table 11

Crosses Involving Antigen A in Which at Least One Parent Is Negative

Mating Number	Dilution Class of Parents F.	Dilution Class of Parents M.	Parents F.	Parents M.	Dilution* of Reagent A: Number of offspring whose rbc gave the first zero reaction at the indicated dilution 1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
27	1	1	1F	5C		4	2					
24	1	1/2	14	3C	4	2	2					
26	1/2	1	4	7C	6	2	1					
5	1	1/4	10	12	3	1						
31	1/4	1	15	5E	3	3		1				
3	1/4	1/2	15	5	2	4		2				
17	1	1/8	10	1A		3	6	3				
30	1/32	1	8F	1B	2	2		1		1	3	
37	1	1/32	2T	3					1		2	
2	1	1/64	14	1	6					2	1	3
8	1/64	1	32	1B						9	3	2
14	1/64	1	17	5A		2	4	1			4	1
25	1	1/64	2C	1	5	1				1		
38	1/64	1	32	5C					1	2		
28	1	1/128	2E	34			2	2	7	7		
41	1	1/128	10	14D	1	1	1			1		
35	1/512	1	33	1B						2	1	1
6	1/2	1/32	2	3	2	3				4	2	
19	1/2	1/32	11	2B	1	2				2	1	
22	1/2	1/32	2	6A	2		2		1	3	2	
23	1/2	1/32	6D	3	6					2	2	1
7	1/2	1/64	4	6	4				1	4	2	

A tabulation of + x - and - x - matings, illustrating the variability in reactivity obtained in the offspring.

* The dotted line represents the dilution at which Reagent A was used for routine typing, i.e., 1/5. All offspring to the left of the dotted line are considered negative, all to the right positive.

Note: Reagent A is already at a serum dilution of 1/8.

Table 12

Additional Crosses Involving Antigen A

Mating Number	Parents F.	Parents M.	Dilution Class of Parents F.	Dilution Class of Parents M.	Dilution* of Reagent A: 1	1/2	1/4 : 1/8	1/16	1/32	1/64	1/128	1/256	Total +	Total 0	p** for 3:1
					Number of offspring whose rbc gave the first zero reaction at the indicated dilution										
1	13	16	1/32	1/32	2		1	1		2			4	2	0.46
9	33	1E	1/512	1/16					2	4	1	1	8	0	0.10
10	30	35	1/128	1/16				1	2	6	2		11	0	0.06
18	39	1C	1/32	1/64	1			2	5		4		11	1	0.16
29	10C	35	1/64	1/16					4	2	2		8	0	0.10

* The dotted line represents the dilution (1/5) at which Reagent A was used for routine typing. All offspring to the left of the dotted line are considered negative, all to the right positive.

** The last column lists the probabilities, as computed from the binomial expansion, that the observed deviation or greater may have been obtained with the given number of offspring.

Table 13

Segregation in the Crosses of A-Positive x A-Negative Pigeons

	Mating Number	Parents F.	Parents M.	Offspring +	Offspring 0	p** for 1:1
1. Positive offspring mated to their negative parents	23	6A	2	5	6	
	30	8F	1B	5	4	
				10	10	
2. Negative offspring mated to their positive parents	25*	2C	1	1	6	0.062
	22	6D	3	6	4	
				7	10	
3. Positives of a + x 0 cross, mated to any negative, other than the negative parent	19	2B	11	3	3	
	41	14D	10	1	3	
	40	14E	14F	1	1	
				5	7	
4. + x 0 crosses in original population, in which progeny segregated	2	14	1	6	6	
	6	2	3	6	5	
	7	4	6	7	4	
	14*	17	5A	6	6	
	17*	10	1A	3	9	0.08
	28*	2E	34	16	2	0.001
				44	32	
Grand total				66	59	
excluding family 28				50	57	0.5

* Families which may not actually be segregating and are discussed in the text.

** In those families in which the expected classes would contain five or more members, p was computed by Chi-square, without correction. p for family 25 was computed from the binomial expansion.

ance of negative progeny and the three offspring classified as positive were weakly reactive. By another criterion (e.g.,typing at reagent dilution of 1/8), such a mating might be classed as a cross of two negatives with only negative progeny. However, the total number of offspring in this family is small, and the numbers observed in each class are still consistent with the classification given in the table. The one positive offspring in family 25 is a strong positive; the mating gave an excess of negatives but the distribution does not deviate significantly from the expected 1:1. The distribution observed in family 28, however, is very unlikely to be due to a sampling error; this mating probably represents a cross of a homozygous positive by a negative. The two negative offspring (Table 11) are borderline cases, in that they are distinguished from positives only at one dilution of the reagent. Omitting family 28, the corrected totals of observed positives and negatives fall well within the limits of probability for the 1:1 ratio that would be expected if the positive vs. negative alternative were determined by a single pair of alleles.

One family in Table 11 (mating 8), a cross of a positive by a negative, yielded 14 offspring, all positive, indicating that the positive reaction is dominant to the negative. Family 28 may be taken as additional evidence in favor of this point; the presence of the two negative offspring need not prejudice that conclusion. Other crosses, in which both parents were positive (matings 1 and 18, Table 12), produced offspring in numbers suggestive of a 3:1 ratio. The determination of the true number of offspring in each class (positive or negative) is again complicated by the borderline cases, i.e., weak positives and weak negatives which are just to the right and to the left, respectively, of the arbitrary margin between the two classes represented by the dotted lines in Tables 11 and 12.

Although the segregation pattern illustrated in Table 13 strongly suggests control by a single pair of alleles, certain exceptions were noted in matings of two negative parents. According to a single-gene hypothesis such matings should give rise to only negative progeny, but some positive offspring were obtained (Table 14). The anomalous positive offspring were only weakly positive; all gave a reaction of grade "1" to the typing fluid. They were thus just barely classifiable as positives, but the reactions were nonetheless real and were repeated many times.

To determine whether the presence of these positives was simply another manifestation of the variability previously encountered (Tables 11

Table 14

Matings of Negative by Negative Parents

Mating Number	Parents		No. of Offspring		Anomalous Offspring
	F.	M.	+	0	
3	15	5	2	6	3L,3P
5	10	12	0	4	
24	14	3C	1	7	24M
26	4	7C	0	9	
27	1F	5C	1	5	27G
31	15	5E	0	8	
Total			4	39	

Reactions listed in this table were classified only by typing, not by titers.

and 12) or represented a true case of a substance which the parents lacked, appearing in the cells of the offspring, some of the positive progeny and their negative parents were included in an absorption analysis. Data on this analysis have already been presented (Table 9). It is clear that the "negative" parents 15 and 5 can each absorb activity from the reagent for their positive offspring 3L and 3P, as can the negative parent 1F for its positive offspring 27G. We have seen that the classes into which offspring fall display some variation about the parental classes. These anomalous offspring, then, are merely examples of this kind of variation.

Figure 4 summarizes all the matings involving antigen A, using the arbitrary classification determined by typing with Reagent A at a dilution of 1/5. The inheritance of the antigen (or at least the characteristic of being positive to some degree to Reagent A at 1/5) is seen to be reasonably straightforward, except for matings 3, 24 and 27 in which negative parents produced positive offspring. The distribution observed in the figure is explicable on the basis of a single locus determining the reactivity of the cells of any bird. In any particular family (Table 11), the offspring fall into discrete classes clustering around those of the parents. The extensive variation underlying this classification strongly suggests that the classes differ quantitatively rather than qualitatively, with respect to antigen A.

Figure 4

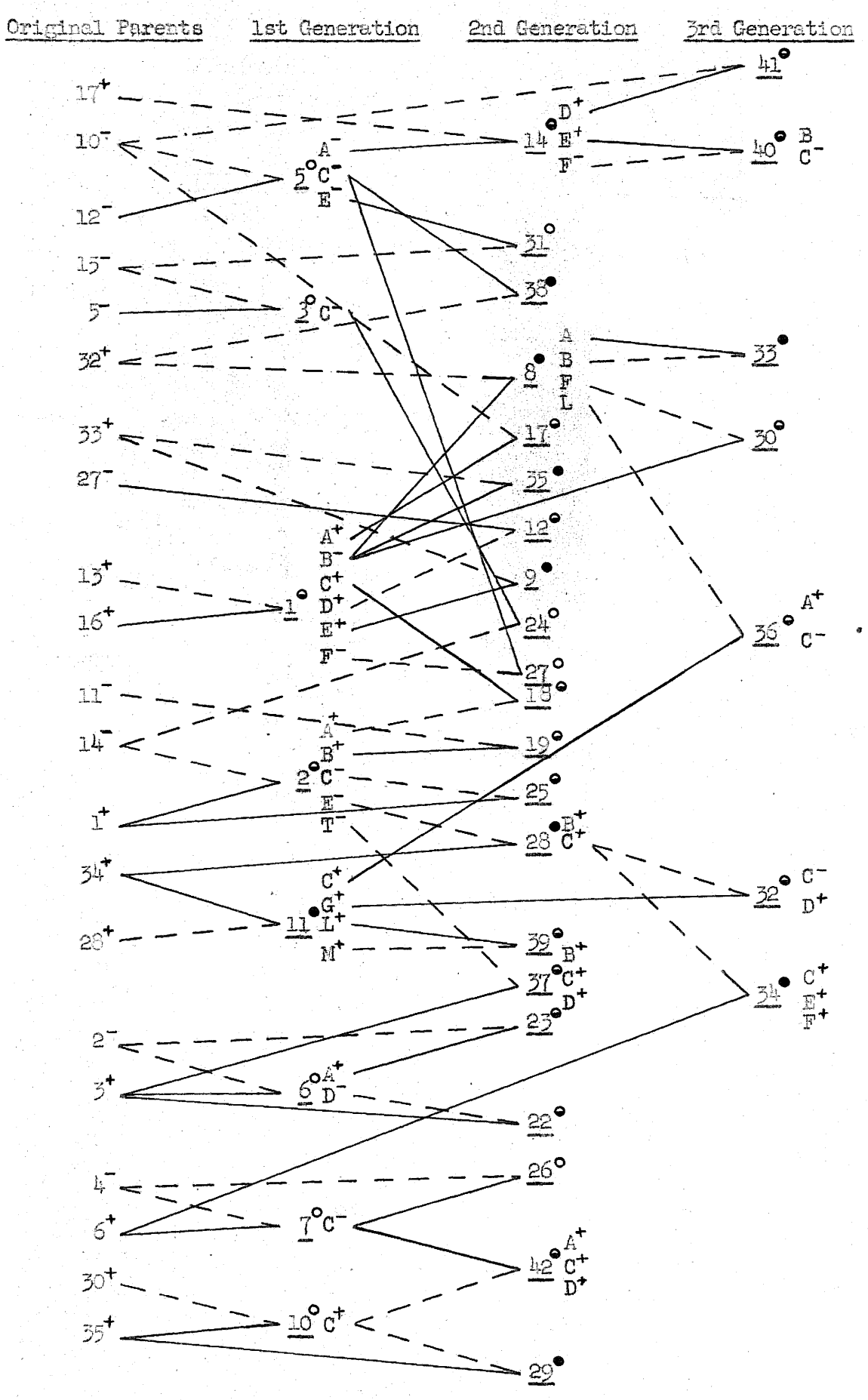
The Inheritance of Positivity to Reagent A at the Routine Typing Dilution

A dotted line drawn from a pigeon number to a mating number (underlined) indicates the bird to be the female parent. A solid line indicates the male parent.

Symbols and Code

○ - All members of the family were negative.
● - All offspring were positive.
⊖ - Some offspring were positive, some were negative.
\+ - The individual pigeon was positive.
\- - The individual pigeon was negative.

The exceptional positive offspring were classed as negative for the purpose of this illustration.


Original Parents
1st Generation
2nd Generation
3rd Generation
17+
10-
12-
15-
5-
32+
33+
27-
13+
16+
11-
14-
1+
34+
28+
2-
3+
4-
6+
30+
35+
5 A- C- E
3 C-
1 A+ B- C+ D+ E+ F-
2 A+ B+ C- E- T-
11 C+ G+ L+ M+
6 A+ D-
7 C-
10 C+
14 D+ E+ F-
31
38
8 A B F L
17
35
12
9
24
27
18
19
25
28 B+ C+
39 B+
37 C+ D+
23
22
26
42 A+ C+ D+
29
41
40 B C-
33
30
36 A+ C-
32 C- D+
34 C+ E+ F+

2. Discussion

a. Inheritance of a Quantitative Character

The reasons put forth in the preceding sections justify the working hypothesis that we are concerned with one antigen present in varying amounts, and that only one gene is concerned with the elaboration of this antigen. It is possible that the variation observed in the apparent amounts of the antigen possessed by the cells of different birds is controlled by a series of alleles at what can be called the "A" locus.

Segregations observed in the familial studies strongly suggest allelic determination of positivity vs. negativity for Reagent A (Tables 11 and 13). For example, in each of the matings 2, 6, 19 and 23 (Table 11), the offspring form two discrete classes. Since the positive progeny in these families are strong positives, their positive parents might be said to be heterozygous for a "strong" allele. Similarly, the distribution observed in families 31 and 3 might be due to the segregation of a "weak" allele. But the distributions obtained in families 30, 14 and 22, in which no sharp discontinuity is evident, complicates the hypothesis of allelic determination.

It has also been shown (Fig. 1) that negatives differ from one another. If we are to assign several alleles on the basis of strength of reaction to the positives, then it would appear equally probable that different negatives have different alleles. An additional complication is that there is no way to determine what contribution the different negative alleles make to the variation observed in individuals heterozygous for A. It may be that this variation is non-genetic in origin, but if it is genetic it is certainly too complicated to be resolved by the data at hand. Some clue to the inheritance of these variations might have been obtained if enough positive siblings of different classes, having different proposed alleles, had been mated and produced large enough families so that the variation could be accurately analyzed on a "progeny test" basis.

Perhaps an important aid would be random typing of many unrelated birds. With the proper statistical analysis, one could then much more easily detect the presence of discrete groups, if they existed. If a truly continuous variation were found, the number of alleles, if one assumes that multiple alleles are indeed the causative factors, could become very large. And in fact, the only analagous situation which has

been recorded (54), for which much more extensive data were available, led Fisher (55) to point out that a major cause of variation might be exogenic.

Henningsen (54) found that random typing of a human population suggested continuous variation in the P antigen. Familial studies indicated regular one-gene control of the presence of P. Classes were arbitrarily fixed at every two dilutions, i.e., cells reacting to dilutions 1 and 1/2 were designated as having weak receptors (Pw), 1/4 and 1/8 as having receptors of medium strength (Pm), and cells reacting at 1/16 or greater as having strong receptors (Ps). All negatives which could be shown to be positive by absorption were classified as Pw. It was unknown whether or not these also represented several "classes". In the familial data presented, no positive offspring was of a dilution class more than one dilution step from its positive parent; one dilution step was not considered a significant difference. Directly analagous to the results obtained in the present study (mating 3, Table 11, for example) are the results obtained in one of the families analyzed by Henningsen; a cross of a Pw x Pw produced a Pm (which was only one dilution away from its positive parent and therefore not "significant"). At a slightly higher serum dilution, this cross would have been classified as a cross of two negatives producing a positive offspring.

The variation in reactivities to anti-P within the population was such that known heterozygotes could not be distinguished from homozygotes. If any Pw x Pw cross produced a Pm, the Pm was assumed to be the homozygote. If a Pm x Pm produced a Ps, the Ps was the homozygote. But the fact that Ps x Ps produced negatives was interpreted to mean that the strongs were "strong" because of the action of genes at more than one locus. The strongs could not have been homozygous dominant and produced progeny of the recessive class. It seems that being forced to invoke additional genes which act to produce more of the same antigen (an unprecedented concept in the field of immunogenetics, one for which there is no experimental support) places the entire distinction drawn between homozygotes and heterozygotes on a tenuous foundation. The genotype of a person cannot be inferred from the reactive strength of his cells, because the different genotypes present a continuous variation.

Landsteiner and Levine (56) had suggested multiple gene inheritance to explain the variation in the P receptor. On the basis of

his study, Henningsen postulated at least three alleles for positivity and one for negativity, where negative cells were defined as cells which cannot remove activity from the serum. Fisher (55), however, in an analysis of Henningsen's genetic and random data, concluded that one important cause for variation lay within the heterozygotes as a class, which ranged from strong to weak, and to a lesser extent within the homozygotes as a class, which ranged from strong to medium. The remaining variation might be due to genes of small effect acting as cumulative factors, or to multiple alleles at the P locus, or it might even be exogenic. Fisher concluded by pointing out that an important fact antagonistic to the existence of multiple alleles was the failure to demonstrate qualitatively different antigens, distinguishing "strong" from "weak", as had been done in the studies on human A_1 and A_2. We need only observe here that the data and interpretations of human antigen P are analogous to our study of pigeon antigen A.

Of great theoretical importance in this study of pigeons would be the demonstration of true negatives, for until these are known it cannot be said that an antigen (i.e., quality) is segregating, but only that a quantity is being distributed in a more or less fixed manner within any one family.

b. Species Differentiation and Hybrid Substances

In one of the most extensive immunogenetic investigations that have been made, Irwin and his co-workers have studied the inheritance of erythrocyte antigens as a means of determining relationships among various species of pigeons and doves. [For reviews of a large number of papers, see Irwin and Cumley (57) and Irwin (23, 24, 58, 59).] By means of rabbit antisera, some antigens have been found which are apparently present on the red cells of several species. Other antigens are apparently specific for the species being investigated.

While there is no doubt of the essential reality of these findings, several results, and especially the interpretation of these results, are subject to reexamination in light of observations made in the present study. Irwin and Cole (60) assumed that any bird whose cells removed activity for itself but not for others lacked at least one antigen that was present on the cells of the others. In the same paper, it was suggested that a contrast in titer of two or three tubes (by the doubling dilution method)

would at least be indicative of the presence of unlike substances on the contrasting cells. There need not, however, be any correlation among the titers of various cells to a given antiserum and the number of antigens present on these cells. The presence or absence of agglutination cannot be accepted without additional evidence as a test for the presence or absence of an agglutinogen. And typing of the bloods of a few members of a species [e.g.,(24)]does not necessarily indicate whether or not the species has the antigen, for negatives by typing may be positive by exhaustive absorption. Furthermore, if a few members of a species are negative the antigen may nevertheless be segregating within the species. If positives which can absorb all activity from a reagent are found among representative members of a species, then of course the question of segregation is academic, for the species can at least be said to have the antigen.

The apparent regularity of the segregation of the antigens and the fact that the blood cells are produced early in embryonic life led to the formulation of the statement (60) that

> "...the production of biochemical components of the erythrocyte may reasonably be considered as a direct action, or very nearly so, of the genes, thereby avoiding the complications of the chain of many reactions presumably involved in the formation of other genetic characters."*

The comment that a "hybrid substance" had been recognized (17), apparently produced as a result of gene interaction, did not at first arouse widespread interest. As more became known about different hybrid substances, however, and as immunogenetic studies in different organisms suggested that the relationship between gene and antigen was commonly one-to-one, the hybrid substance took on greater theoretical importance. Irwin (17) showed that offspring of the cross Streptopelia risoria (Ring dove) x Spilopelia chinensis (Pearlneck) had antigens on their erythrocytes which could not be detected on the red cells of the parents. The same phenomenon was found in offspring of crosses of Columba livia (common pigeon) x Streptopelia risoria (17, 18) but not in a cross of Columba livia x Columba guinea (triangular spotted pigeon) (61). Both occurrences of a hybrid substance involved the Ring dove, but the substances were not

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* p. 106

identical (18).

Irwin and Cumley (62) showed that the hybrid substance in the Pearlneck x Ring dove crosses could be divided into several components, each of which could be shown to be associated with an antigenic character specific to Pearlneck. At the same time, the suggestion that the hybrid substance merely represented a greater concentration of antigens normally possessed by one or both parents (a quantitative effect) was denied by Irwin, who cited as evidence the fact that four absorptions of an anti-hybrid serum by the parental cells failed to remove activity for the hybrid cells. But where a quantitative effect does exist (e.g., this study), four absorptions may not be enough for a very weak positive to absorb all activity for a strong positive. It must be mentioned, however, that the hybrid substance has appeared many times and seems to be inherited very regularly. Irwin asserted (23) that only about half the species hybrids in pigeons and doves possess antigens different from those of the parents.

In the present study, positive progeny have appeared in the cross of negative x negative birds. In these anomalous instances, the parental cells can remove activity for the cells of the offspring without much difficulty. This evidence that a quantitative effect may simulate the occurrence of a hybrid substance suggests a reinvestigation of the claim that the hybrid substance is qualitatively different from any antigen possessed by the parents.

McGibbon (19) investigated the species hybrid of the cross Muscovy duck (Cairina moschata) x Mallard duck (Anos Platyrynchos platyrynchos). Using both rabbit anti-hybrid sera and isoimmune sera, a hybrid substance was detected. For the production of isoimmune sera the cells of two offspring were pooled and injected into each of the parents. Each antiserum was then absorbed by the cells of the other parent and tested with the cells of the hybrids. All hybrids reacted to the two types of absorbed antisera. Clearly, the offspring possessed antigens which were not only lacking in the cells of the parent but were actually antigenic to the parents. It is difficult to invoke a quantitative explanation for these results.

Fox (63, 64) using rabbit antisera, claims to have gathered evidence for an antigenic specificity produced by the interaction of two

genes controlling eye color, "ruby" and "vermillion", in Drosophila. His use of crude extracts of Drosophila, in view of the many complexities involved in the analysis of a precipitation system when several antigens are involved [see Kabat and Mayer (65)], makes his observations subject to some reservation. Analysis of such a system on a quantitative basis is not feasible, because overabsorption is not possible in a soluble antigen system, where the presence of excess antigen causes redissolution of an antigen-antibody precipitate (65, 66). The same situation exists in attempts to evaluate the report of Sokolowskaja (67) that she had found a hybrid substance in the serum of hybrids between Mallard and Muscovy ducks.

It is not necessary to postulate a distinctly new antigen for any case of the appearance of a hybrid substance, as was pointed out by Burnet and Fenner (21). The necessities of combining the parental antigens may induce a new structural specificity and not a chemical one, such that the new structure is antigenically unique. (See Landsteiner (53) for a discussion of the effects of stereochemical changes on antigenic relationships.) The suggestion is important because, if it were proven true, it would invalidate the necessity for assuming gene interaction to produce a new antigen. It is, however, difficult to see how the hypothesis might be tested.

Bryan and Miller (69) do not believe that a steric effect can explain their findings. Cells of heterozygous hybrids CC' [C is specific to Columba guinea, C' to Columba livia (70)] are capable of producing an antibody specific for heterozygotes, even when the reagent is made by absorbing the anti-CC' serum by the pooled cells of the respective parents of the birds tested. These workers believe that, since AA', BB', EE' and FF' heterozygotes [where A, B, E and F are specific to guinea and A', B', E', F' are specific to livia (70)] do not apparently produce a hybrid substance, a steric effect is not a likely cause of a new specificity. However, in the present author's opinion, the absence of evidence for steric interaction in any number of instances does not necessarily exclude the possibility that such interaction may occur in other instances.

IV. REAGENTS B, D AND G

Reagents B, D and G are discussed as a group because it is believed that they are very similar or identical reagents. B and D reagents gave identical reactions, and although only a preliminary study was performed on G it behaved enough like B and D to be classed with them.

A. Reagent B

An initial fractionation of serum 9 (anti-pigeon 9) revealed the cells of one bird, pigeon 7, to be negative in relation to eleven others (Table 15). It can be seen that the type of anomaly discussed previously (Sec. IIIA1, i.e., cells removing activity for each other but differing in the reactions given by reagents prepared with them) is also evident in this table. In spite of this anomaly, the reagent prepared by absorbing the serum with cells of pigeon 7 was used to type the flock.

Table 15

Fractionation* of Serum 9

		Serum 9 absorbed by cells of pigeons							
Cells	Unabsorbed Serum	1	3	4	5	6	7	11	12
1	6	0	0	0	0	0	4	0	0
3	6	0	0	0	0	0	4	0	0
4	6	0	0	0	0	0	4	0	0
5	6	0	0	0	0	0	4	0	0
6	6	0	0	0	0	0	4	0	0
7	6	0	0	0	0	0	0	0	0
11	6	0	4	4	0	4	5	0	0
12	6	0	0	0	0	0	4	0	0

* Serum 9 absorbed at 1/8 and tested at a further dilution of 1/3.

Of eighteen birds typed, the cells of three were found to be negative. An absorption analysis indicated that one of the negatives in the previous test behaved as a positive in absorptions, and that one of the positives in the test was a negative by the absorption criterion. Reagent B was then made by using the pooled cells of pigeons 13, 17 (which had been thought to be positive by typing but proved to be negative

in the absorption study) and 1A.

It was found in making the reagent that absorbing with the pooled cells removed activity very readily for cells 13 and 1A, but repeated absorptions were necessary before the reaction for 17 was lost from the reagent.

B. Reagent D

Two absorption analyses performed with serum 15 anti-pigeon 15 yielded the same negatives, 7, 13 and 17, as were obtained for Reagent B. One of these analyses (Table 16) indicated a perfect single antigen-antibody system, insofar as the particular cells in the absorption were concerned. The other analysis, not presented because of its essential similarity to tables previously discussed, displayed the anomaly frequently encountered (Sec. IIIA1).

Table 16

Fractionation* of Serum 15

		Serum 15 absorbed by cells of pigeons							
Cells	Unabsorbed Serum	2	4	5	7	8	9	16	17
2	6	0	0	0	5	0	0	0	4
4	6	0	0	0	4	0	0	0	4
5	6	0	0	0	5	0	0	0	4
7	6	0	0	0	0	0	0	0	0
8	6	0	0	0	3	0	0	0	3
9	6	0	0	0	5	0	0	0	4
16	6	0	0	0	5	0	0	0	4
17	6	0	0	0	0	0	0	0	0

* Serum 15 absorbed at 1/8 and tested at a further dilution of 1/3.

This table is an illustration of a perfect single antigen-antibody analysis. Another absorption analysis of this serum using different cells gave several anomalous reactions.

Typing with a preliminary preparation of Reagent D indicated that 1A was also negative. Cells 1B were found to be negative on typing but proved to be positive in an absorption. Nine positives all removed activity for each other from the reagent.

Reagent D as used for general typing was prepared by absorbing serum 15 with the pooled cells of 13, 17 and 1A. Again, as in the prep-

aration of Reagent B, many absorptions were necessary to remove all activity for 17.

Reimmunization of rabbit 15 with the cells of pigeon 15 yielded a serum which apparently did not contain any antibodies recognizing intraspecific differences. The cells of nineteen pigeons (including 13, 17 and 1A) all removed activity for 15, the homologous cells.

C. Reagent G

The first fractionation of serum 10 anti-pigeon 10, comparing the cells of seven pigeons, revealed that 11 was a negative, and gave a straightforward analysis. Typing with the serum after absorption with the red blood cells of pigeon 11 indicated that 1E, 7, 13 and 1A were negative, while 17 was positive. Absorption of the serum showed, however, that 17 was negative and 1E positive. The other cells behaved consistently in absorptions and tests. Absorptions performed at various times with different volumes of cells yielded reagents of different strengths and different reactivities.

It was decided, in view of the weakness of the reactions observed and in view of the essential similarity with two other reagents (B and D) already on hand, to do no further analyses of this serum. Therefore, a stock of Reagent G was never made.

D. Typing Studies with Reagents B and D

Typing with Reagents B and D proved that these reagents were indeed identical, the only difference being that the reactions obtained with D were as a general rule weaker than those obtained with B.

A genetic study was impossible because almost all offspring of the matings at hand were positive, and all families had offspring whose cells reacted to the reagents with various intensities. A few negative offspring were noted, but their presence did not serve to define the genetic control of the characteristic. Unfortunately, even though 1A was a male and both 13 and 17 were females, no crosses were made among these, the only negatives noted in absorption studies.

V. REAGENT E

A. Serology

1. Analyses

The first fractionation of serum 7 anti-pigeon 7 gave a straightforward analysis for a single antigen-antibody system and yielded two positives and six negatives. The reagents remaining from the individual absorptions by the negative cells were pooled and used as a preliminary typing fluid. Tests with this fluid indicated that there were other positives and negatives in the flock; there was a preponderance of negatives.

The cells of eleven negative pigeons were pooled and used to make Reagent E_I. Typing with this reagent gave almost the same positives and negatives as had been previously observed, with the exception that two cells which had been negative in the original absorption were, with this reagent, classified as positive. The fractionation of Reagent E_I by positive cells indicated a complex and almost straightforward system (Table 17).

The results of the fractionation of E_I suggest a five-component system, with but two anomalies. The first is that the reagent prepared by absorbing E_I with the cells of pigeon 11 gives a trace of reactivity with cells 14. 11 and 14 are, by all other criteria, identical and have been assumed to be so in the analysis. The other anomaly is that 9, negative to the reagent, removes some activity for 11 which is positive to the reagent. This anomaly was reproducible but an attempt to liberate a specific antibody for cells 11 from cells 9, in which cells 9 were heated after being used to absorb the reagent, failed. This phenomonon, a negative removing activity for a positive, has been described and discussed in the section on Reagent A.

It was felt that making the reagent by absorbing with the pooled cells of 11 birds might have caused some of the complications observed, in that many kinds of cells in combination might remove agglutinins which each could not do separately. Therefore, as was done with the other reagents, the cells of three birds, in this case 1, 3 and 6, were used to absorb serum 7. Typing with the resulting fluid (E_{II}) gave some weak or doubtful reactions which were reduced by further absorption with other negatives.

Table 17

Fractionation of Reagent E_I [+] by Positives

Cells	Proposed Antigenic Constitution	Reagent E_I absorbed by rbc of pigeons leaving in the agglutinins, anti-									Unabsorbed Reagent E_I at 1/5
		11	14	16	1A	17	13	8	7	9	
		B,C,D,E	B,C,D,E	B,D,E	B,D,E	B,E	D,E	none	none	A,B,C,D,E	
11	A	0	0	0	0	0	0	0	0	t*	3
14	A	t*	0	0	0	0	0	0	0	4	4
16	AC	4	4	0	0	0	0	0	0	4	4
1A	AC	4	4	0	0	0	0	0	0	4	4
17	ACD	4	4	3	3	0	3	0	0	4	5
13	ABC	4	4	3	3	3	0	0	0	4	5
8	ABCDE	5	5	3	3	3	4	0	0	6	6
7	ABCDE	5	5	4	4	4	3	0	0	5	5
9	0	0	0	0	0	0	0	0	0	0	0

+ Reagent E_I prepared by absorbing serum 7 at 1/8 with the pooled red blood cells of eleven negative pigeons. Tests were done at a 1/5 dilution of the reagent after absorption.

Cell 9 is the negative control.

* Anomalous reactions.

Table 18 lists the reactions observed with various positives when Reagent E_{II} was absorbed by the cells of ten different negative pigeons. It can be seen that there was no significant diminution in activity of any of the individually absorbed reagents toward any of the positives.

Reagent E, as finally prepared and used for typing, had then undergone two sets of absorptions. Serum 7 had been absorbed with the pooled cells of pigeons 1, 3 and 6. This reagent had been divided into nine aliquots, each of which was separately absorbed three times with an equal volume of cells from each of the nine negative birds (excluding cells 1) listed in Table 18. The resulting individually absorbed fluids were pooled and used, at a dilution of 1/3, as Reagent E.

This reagent was fractionated by absorbing it five times with each of several different positives. The same fractionation had been attempted by absorbing only twice but too much uncertainty was involved in the analysis. Therefore, in an attempt to remove the uncertainties and to be sure that the reactions remaining were due to specific antibodies (and not like the system detected by Reagent A in which all positives could remove all activity) the reagent was deliberately overabsorbed (Table 19).

The reactions remaining, even after overabsorption, were not easy to analyze. Cells 11, 14 and 8B were obviously negative in relation to the others. 10A and 16 removed activity for each other and yet Reagent 10A gave entirely different reactions than did Reagent 16. Other anomalies (starred in the table) also served to confuse the analysis.

The cells most like each other, in reactions of the reagents prepared by absorbing with them and in reactions of the cells themselves, were grouped and considered alike. By this procedure, four general types were postulated. Since all the cells were positive to Reagent E, all shared a common antigen, E_1, which was the only antigen present on cells 11, 14, and 8B. A large group of cells was postulated as having antigens E_1 and E_3, while only one (28B) had E_1 and E_2. Although the reagent produced by absorbing with 10A is unique, 10A reacted with the other reagents as if it were E_1E_3 and so was included in that group.

From the data in Table 19, it is apparent that three sub-reagents could be made. Reagent E (which actually contains anti-E_1, anti-E_2 and

Table 18

Absorption* of Reagent E_{II}** by Negatives

Cells	Reagent E_{II} absorbed by rbc of pigeons										Reagent E_{II} at 1/5
	·1	10	30	1B	1C	1E	3C	5A	5C	10C	
14	1	1	t	1	1	t	t	t	t	t	t
32	3	3	3	3	3	3	3	3	3	3	3
34	5	5	5	5	4	3	4	5	4	4	5
8B	3	3	3	3	3	3	3	3	3	3	3
8S	3	3	3	3	3	3	3	3	3	3	3
10A	4	4	4	4	4	4	3	3	4	4	5
11B	4	4	4	4	4	3	3	4	3	3	5
14A	3	3	3	3	3	3	3	3	3	3	3
14D	3	3	3	3	3	3	3	3	3	3	4
28B	5	5	5	5	5	5	5	5	5	5	5

* The reagent was absorbed three times, each time by an equal volume of cells, by each of the negative cells listed. Tested at a 1/3 dilution of reagent against various positive cells.

** Reagent E_{II} prepared by absorbing serum 7 with the pooled cells of pigeons 1, 3 and 6.

Table 19

Fractionation[+] of Reagent E by Positives

Cells	Proposed Antigenic Constitution E-	Reagent E absorbed by rbc of pigeons leaving in the agglutinins, anti-E-														
		11	14	8B	16	10A	11B	14A	14D	8S	17	1A	28B	32	34	13
		2,3	2,3	2,3	2	2	2	2	2	2	2	2	3	none	none	none
11	1	0	0	0	0	1*	0	0	0	0	0	0	0	0	0	0
14	1	0	0	0	0	2*	0	0	0	0	0	0	0	0	0	0
8B	1	0	0	0	0	3*	0	0	0	0	0	0	0	0	0	0
16	1,3	3	3	4	0	0	0	0	0	0	0	0	3	0	0	0
10A	1,3	5	4	3	0	0	0	0	0	0	0	0	3	0	0	0
11B	1,3	4	4	3	0	2*	0	0	0	1*	0	0	4	0	0	0
14A	1,3	4	3	3	0	t*	0	0	0	0	0	0	4	0	0	0
14D	1,3	5	4	4	0	1*	0	0	0	0	0	0	5	0	0	0
8S	1,3	4	3	3	0	3*	0	0	0	0	0	0	4	0	0	0
17	1,3	5	6	6	0	3*	0	0	0	1*	0	0	6	0	0	0
28B	1,2	4	3	4	3	4	3	4	4	1	4	3	0	0	0	0
32	1,2,3	4	3	3	1	3	t	1	1	0*	1	t	3	0	0	0
34	1,2,3	5	5	4	1	4	3	4	4	1	2	2	4	1*	0	0
13	1,2,3	4	4	4	1	3	3	t	2	1	0*	1	4	?*	t*	0

+ Reagent E, undiluted, absorbed five times, each time with an equal volume of cells from the pigeons listed and tested at 1/3.

* Anomalous reactions; where positive, the reaction should have been negative and where negative, the reaction should have been positive, on the basis of the system proposed.

Cells 1A, whose test reactions were omitted from the table, reacted like 14A.

anti-E_3) absorbed by cells 11, 14 and 8B would give a reagent (E_2E_3) which should detect the types E_2 and E_3, either singly or in combination. Absorption by 11B, 14A and 14D (chosen because these cells react almost identically, as do the reagents made by absorbing with them) produces Reagent E_2, which should detect the type E_2. Reagent E, absorbed by 28B (later 28A and 28C, shown to be identical with 28B, in that each removed activity for the others, were also used), produces Reagent E_3, which should detect the type E_3. The table below illustrates the reactions expected for each of the cell types with sub-reagents of the postulated constitution.

Cell Types	Unabsorbed Reagent E	Reagent E absorbed by cells containing E_1 leaving in anti-$E_{2,3}$	$E_{1,2}$ leaving in anti-E_3	$E_{1,3}$ leaving in anti-E_2
*E_1	+	0	0	0
*$E_{1,2}$	+	+	0	+
?E_2	+	+	0	+
*$E_{1,3}$	+	+	+	0
E_3	+	+	+	0
*$E_{1,2,3}$	+	+	+	+
$E_{2,3}$	+	+	+	+
*e	0	0	0	0

* Types which have been found.

? The true classification of the one bird possibly of this type is uncertain.

The table shows that E_1 cannot be distinguished by typing when in combination with either or both antigens E_2 and E_3. Only an absorption performed with E_2E_3 cells would yield sub-reagent E_1. Unfortunately, only one E_2E_3 bird, other than the ones listed in Table 19 which apparently remove anti-E_1, was encountered in the flock. This bird, too, removed all activity from reagent E. The presence of E_1 on cells positive to the existing sub-reagents would only be detected by absorption studies. If E_1 were absent, these cells could not remove activity for E_1 cells.

General typing was undertaken with the reservation in mind that the system was by no means regular and straightforward. The reagents did not represent unit specificities, and anomalous results were to be expected. It was hoped that the appearance of such anomalies might serve to elucidate the composition of the reagents.

The cells of some fifty birds were typed with all the sub-reagents to check their consistency. All cells which reacted with Reagent E_2E_3 also reacted with one or both of the single sub-reagents. Cells which did not react with E_2E_3 were also negative to E_2 and to E_3. After the consistency of the reagents had thus been established, cells negative to Reagent E_2E_3 in later tests were not checked with the other single sub-reagents.

2. Reagent F

Serum 8, obtained by immunizing rabbit 8 with the red blood cells of pigeon 8, proved on absorption to be complex. The cells of birds 1, 3 and 6 were chosen as the negatives with which to absorb the serum to make Reagent F. (These were the same negative cells as had been chosen to make Reagent E.)

After extensive typing had been done, it began to appear likely that Reagent F was a weak duplicate of Reagent E. In order to test this supposition, some of the cells used in the analysis of Reagent E (Table 19) were used in a similar absorption analysis of Reagent F. The results verified the assumption; the same combinations of reagents and cells gave the same reactions as those recorded in Table 19. The reactions were, however, somewhat weaker. Indeed, some of the anomalies noted in Table 19 were reproduced in the fractionation of Reagent F.

Once the fact was ascertained that Reagents E and F were duplicates, typing with Reagent F was discontinued and no genetic analysis was attempted.

B. Genetics

As expected, the genetic results revealed anomalies in certain families. Other families, however, seemed to be characterized by a precise inheritance of the antigen or antigens responsible for the reactions. The anomalies will be discussed as they arise in the presentation of the genetic results.

1. Inheritance of Positivity to Reagent E

Reagent E gave a gradation in intensity of reaction of the cells similar to that noted in discussing Reagent A. Cells reacted within the range from "?" or "t" to "6". Typing a given cell as either positive or negative was arbitrary; all reactions from "1" to "6" in intensity were called positive and the others negative. In other words, when any clumps of cells, no matter how small, were visible, the reaction was noted as positive.

The inheritance of the quality of being positive to Reagent E, which contains at least anti-E_1, anti-E_2 and anti-E_3, is summarized in Table 20.

Table 20

Inheritance of Positivity to Reagent E

Number of Matings	Type of Mating	Offspring	
		+	0
11	0 x 0	0	89
1	0 x 0	1	6
3	+ x 0	49	0
12	+ x 0	42	59
5	+ x +	15	0
2	+ x +	7	7

All positive offspring have red blood cells which react "1" or greater in intensity to Reagent E.

The one exceptional offspring in the mating of two negatives is probably not a real exception at all. One parent was classified as "t" and there is another "t" included in the offspring. The red blood cells of the one "positive" offspring only reacted weakly to the reagent (intensity of "1"). It is therefore felt that this case does not constitute a true exception. The fact that in eleven matings with a total of eighty-nine offspring, no additional positives appeared is sufficient evidence that a cross of two negatives will yield only negative progeny.

The three matings of positive x negative which gave all positive offspring had sufficiently large families (12, 14 and 18) to indicate that the positive parents were homozygous and that the presence of the positive character was dominant to its absence.

The total number of offspring in the positive x negative crosses which gave both positive and negative offspring is large enough to provide a significant analysis. The results, 42 positive and 59 negative offspring, represent a 1:1 ratio with a probability of about 0.1. Crosses of E_1 x negative involving only E_1 and no other E subtype (sections I and II, Table 21) yielded 20 positive and 31 negative offspring (p for 1:1 is about 0.1). The remainder, 22 positives and 28 negatives, representing the offspring of the crosses E_3 x negative (Table 23) which segregated for E_3 and negative progeny (no other E subtype involved), gives a better fit to the proposed 1:1 ratio (p is about 0.4).

The only back cross tested yielded four positive and five negative progeny. The other positive x negative crosses involved negative offspring mated to positive parents, negative and positive sibs, and birds which were unrelated to each other.

The numbers of offspring in the two classes obtained in the two crosses of positive x positive which produced some negative progeny are almost incompatible with a 3:1 ratio (p is 0.04), but the numbers are too small for a definite conclusion to be reached.

As a general observation, then, it would seem that the quality of being positive to Reagent E (which is known to contain antibodies of several different specificities) is inherited as if that quality were controlled by a single gene.

2. Inheritance of E_1

The identification of E_1 as a discrete antigen is doubtful, because of the limitations in the sub-reagents (see page 60). That E_1 is not merely a weaker form of either E_2 or E_3, for which activity is removed by the further absorption necessary to make the sub-reagents, is shown by the fact that although most E_1 cells are weakly reactive, there are several cells which react strongly to Reagent E and not at all to sub-reagent E_2E_3. This is taken to preclude the possibility that E_1 represents a quantitative variation of the other subtypes; it does apparently represent a qualitatively distinct specificity.

Table 21 tabulates the inheritance data on antigen E_1. Section I includes the matings of pigeon 14 and its offspring involved in positive x negative crosses and producing positive and negative progeny. The totals

Table 21

Inheritance of E_1^+

	Mating Number	F.	Parents Type	M.	Type	Offspring +	Offspring 0	p for 1:1
I.	2	14	+	1	0	6	6	
	24	14	+	3C	0	3	5	
	18	2A	+	1C	0	5	7	
	19	11	+	2B	0	1	5	
					Total	15	23	0.2
II.	9	33	+	1E	0	4	5	
	35	33	+	1B	0	1	3	
					Total	5	8	
			Total I + II			20	31	0.1
III.	8	32	+	1B	0	7	6	
	38	32	+	5C	0	1*	4	
					Total	8	10	
IV.	25	2C	0	1	0	1	6	
	Grand total, excluding IV.					28	41	0.1

+ E_1 cells were positive to Reagent E and negative to sub-reagent E_2E_3.

* This bird was typed as E- negative. For reasons described in Section VC however, the original typing was believed to be erroneous, and it was classed as E_1.

p was determined by the Chi-square method.

for the two classes in Sections I and II (which represent matings of E_1 x negative in which no other E subtype was involved) represents a 1:1 ratio with the indicated probability of about 0.1. Although the deviation is not significant, the consistent excess of negative progeny, together with the appearance of one weakly-reacting positive in a mating of a negative offspring of bird 14 with its negative parent (Section IV) suggest that the segregation of positives and negatives may not represent a strictly qualitative difference. It was observed that the positive offspring of E_1 x negative crosses were weakly reactive. The negatives included birds whose red blood cells gave reactions with Reagent E which were scored as "?" or "t". The variability in intensity of the reactions observed with Reagent E (almost always involving E_1 families) was of the same sort as has already been described in the case of Reagent A, suggesting that quantitative differences may have been the underlying causes for the excess of negatives. This does not mean that all negatives possessed the same antigen, for none of the negatives in Table 18 removed all activity for cells of pigeon 14. Unfortunately, none of the negative offspring of pigeon 14 were involved in the absorption analysis of Table 18, and none were ever used in a similar absorption.

Section III of the table lists the two matings of the one E_1E_3 bird which, when mated to negatives, produced E_1 and E_3 offspring in approximately equal numbers. The lack of an E_1 reagent made it impossible on the basis of typing alone to decide whether or not the E_3 progeny were also E_1. The problem of whether or not this family represents a true case of segregation for antigen E_1, although it has been included into the grand total of the table, must be left unsolved for the time being. The point will be further discussed in Section VC.

The only conclusion that can be drawn about the inheritance of E_1 is that it seems to behave as if its presence were controlled by a single gene. A more definitive statement is impossible because of complications very probably quantitative in nature.

3. Inheritance of E_2

The inheritance data on E_2 are meager (Table 22) but indicate a regularity in segregation suggestive of simple Mendelian control. Only one of the original pigeons, the male bird number 34, possessed E_2 detectable by typing. In two matings, one to an E-positive (subtype unknown

Table 22

Inheritance of E_2

Mating Number	F.	Parents Type	M.	Type	Offspring +	Offspring 0
11	28	?	34	+	3	4
28	2E	0	34	+	9	9
29	11M	+	11L	+	2	0
32	28C	+	11G	0	3	2

Positive offspring were all positive in reaction with sub-reagent E_2.

? This bird was typed as positive to Reagent E, but died before it could be typed with the sub-reagents.

but probably E_1) and the other to a negative, male 34 produced twenty-five offspring of which twelve were positive to sub-reagent E_2. Two of its E_2 progeny when mated together, produced two E_2 offspring.

Two birds, 32 and 13, which might have been classified as E_2 by absorption (Table 19) because they removed activity for the E_2 birds 28B and 34, could only be classed as E_3 by typing. Pigeon 13 did not produce a family large enough to indicate whether or not it would transmit the E_2 characteristic but 32 produced a total of 18 offspring in matings to negatives. None of these offspring were E_2 by typing.

4. Inheritance of E_3

The mating listed in Table 23 of a positive by negative which produced twelve offspring all of which were positive indicates that the presence of the antigen was dominant to its absence. The rest of the data are consistent with the assumption that the presence and absence of E_3 are controlled by a pair of alleles.

C. Genetic Relationships Among the E Subtypes

The symbols for the antigens will be represented by sub-scripts to the letter "E" and the symbols for the genes apparently controlling the antigens by super-scripts, (e.g., $\underline{E}^1$ is the gene controlling the antigen E_1). Absence of the antigen will be represented by the small letter, "e", with the appropriate sub- or super-script.

Table 23

Inheritance of E_3

Number of Matings	Type of Cross	Offspring	
		+	0
1	+ x 0	0	3
10	+ x 0	44	47
1	+ x 0	12	0
2	+ x +	3	5

Only reactions of grade "1" or greater to sub-reagent E_3 were scored as positive.

Note: Of the ten matings of + x 0 producing both types of progeny, six were of matings of E_3 x 0 in which no other E subtype was involved, and produced 22 positive and 28 negative offspring (p for 1:1 about 0.4).

1) The data presented in Table 24 suggest that E^1 and E^3 are alleles, if one accepts the changes indicated by the asterisks as valid. The changes were dictated by the observation that in all matings involving a parent heterozygous for both E_3 and C (C is an antigen detected by an iso-immune reagent which will be discussed later), E_3 and C seem to segregate together. In family 8, all E_3 offspring were C-positive and all E_1 offspring C-negative. The exceptional E-negative offspring in the total of eighteen progeny of pigeon 32 was found to be C-negative, suggesting it might really have been E_1. In family 30, with all E_3 offspring C-positive, the exceptional offspring (typed as E_1) was C-positive and its cells were weakly reactive with sub-reagent E_2E_3. That the reagents E and C are not the same is shown by the fact that there are cells which are C-positive, E-negative and C-negative, E-positive. In mating 33 (E_1 x E_3), in which both parents were heterozygous, only three classes of offspring would be obtained, since the E_1E_3 cells and the E_3 cells would give the same reactions. The numbers obtained wer not large, but the excess was in the proper class, i.e., E_3. In all considerations of E^1, it must be remembered that critical proof of allelism between E^1 and E^3 is lacking. Furthermore, since no reagent

Table 24

Data Bearing on the Relationship Between $\underline{E}^1$ and $\underline{E}^3$

Mating Number	F.	Parents Type	M.	Type	Offspring E_1	E_3	0
8	32	E_1E_3	1B	0	7	6	
38	32	E_1E_3	5C	0		4	1*
30	8F	( )E_3	1B	0	1*	3	5
33	8B	E_1	8A	( )E_3	1	5	2

Parentheses in the columns headed "Type" indicate that the E_1 character is unknown.

* The offspring indicated by an asterisk are believed to be erroneously classified, for reasons discussed in the text. One offspring of mating 38 was believed to be actually E_1 and one of mating 30 to be actually E_3.

exists which will distinguish E_1 when in combination with the other subtypes, such proof would be difficult to obtain. $\underline{E}^1$ and $\underline{E}^3$ seem to be alleles but the data are insufficient to be conclusive.

2) Pigeon 34, in two matings with negatives (families 11 and 28), produced a total of 25 offspring of which 12 were E_2 and the remainder E_3. The failure to produce E-negatives or E_2E_3 offspring indicates quite conclusively that $\underline{E}^2$ and $\underline{E}^3$ are not independent. That they could have been linked (entering the cross in repulsion) is not disproved, but since the concept of linkage demands a cross-over for its verification, until a cross-over appears $\underline{E}^2$ and $\underline{E}^3$ will be considered as alleles. Each can cause the detectable presence of its respective antigen either singly or in combination with the other. Postulating that $\underline{E}^2$ and $\underline{E}^3$ are alleles proposes the anomaly that pigeon 34 apparently has three alleles at the same locus; $\underline{E}^1$, $\underline{E}^2$ and $\underline{E}^3$. This inconsistency can be resolved by assuming we are dealing with complex antigens. Pigeon 34 could have either the genotype $\underline{E}^{1,3}/\underline{E}^2$ or $\underline{E}^{1,2}/\underline{E}^3$. Discussion on this point will be elaborated in Section VIIB.

3) Little is known about the relationship between $\underline{E}^1$ and $\underline{E}^2$. The data were meager and insufficient for even a preliminary analysis. However, if $\underline{E}^1$ and $\underline{E}^2$ can be considered as allelic to $\underline{E}^3$, then $\underline{E}^1$ should be

allelic to $\underline{E}^2$.

D. Some Complexities of the E System

1) It must be pointed out again that the reagents, although they are designated by symbols that give the impression that they represent unit specificities, do not actually represent single antibody fractions. It was stated in the description of these reagents (p. 57) that the sub-types were postulated as a "general sort of observation." If true unit specificities were involved, there should have been no anomalies observed in Table 19, from which the information leading to the production of the sub-reagents was derived.

2) The typing fluids, although they seemed to yield consistent genetic results, did not always type according to the true antigenic constitution of the cells. For example, cells 32 and 13 on typing were classed as E_3 but in absorptions (Table 19) they removed activity for E_2 cells as well as E_3.

3) Not only did the serum include a multiplicity of antibodies, but the cell-types detected by each of the single sub-reagents (E_2 and E_3) differed from each other, indicating a diversity of antigens.

a) Cells 10A, classed as E_3 by the absorption summarized in Table 19 and typed as E_3 by the reagents, when used in an absorption produced an obviously different reagent than did the other E_3 cells. Cells of pigeon 8S, also classed by typing as E_3, were seen to differ from the other E_3 cells in that they removed at least some of the activity for E_2 and E_2E_3 cells. 8S is almost identical with its parent, pigeon 32, in this respect. These facts suggest that there are at least three different types of E_3 antigens. All are detectable as E_3 by use of the sub-reagents, and the genetic data involving each are consistent with the hypothesis that a single antigen is being inherited. But one type (8S and 32) can remove activity for E_2 cells while the other two (10A and 11B, for example) cannot, and 10A is unique in that it does not remove agglutinins for E_1 cells.

b) A previously unmentioned fact is that an anomalous offspring appeared in family 2. The cross was E_1 x negative and although five offspring were E_1 and six were negative, the red blood cells of the twelfth reacted weakly to sub-reagents E_2E_3 and E_3. This fact was not discussed in Section VB2 on the inheritance of E_1 because it was the

only such exception and it was felt that it could be best discussed at this point. We cannot say whether the presence of one E_3 in a total of twelve offspring represents a mutation, a non-genetic change, or an indication that the inheritance of the antigen is complex (which is certainly not indicated in the other inheritance data on E_3). In an attempt to determine if 14, the E_1 parent, could remove activity for 2T, its E_3 offspring, and if 2T could remove activity for other E_3 cells, an appropriate absorption was performed (Table 25). Pigeon 14 removed agglutinins for its exceptional offspring, but a more remarkable observation to be made is that both 14 (an E_1) and 2T (an E_3) removed activity for <u>two</u> E_2 cells and not for a third. (It was seen in Table 19 that five absorptions of Reagent E by cells 14 did not remove activity for 28B, but in Table 25, it is shown that six absorptions did. One of these reactions may have been an artifact, and if so, it was probably that of Table 19, for 14 is seen to leave in activity for another E_2 cell, 28A.) 2T is seen to be like 8S and 32 in removing activity for E_2 cells, but 2T does not remove activity for E_3 cells, while 8S and 32 do. 2T is classified by typing as E_3, as are 8S and 32, but it is not known whether 8S and 32 could also subdivide the E_2 reagent. This one table indicates the complexities encountered and the relationships observed among the different "antigens", namely the facts that an E_1 parent gave rise to an E_3 offspring, E_1 and E_3 cells can remove activity (on overabsorption) for E_2 cells but not for E_3 cells, and E_2 cells are further divisible into two classes.

We see, then, that the cells reacting positively to sub-reagent E_3 are divisible into several kinds, each being inherited as if it were controlled by a single gene. We see also that more than one kind of E_2 cells can be distinguished within <u>one</u> family, indicating a segregation within a specific class of segregants. If an almost perfect segregation (i.e., positive <u>vs.</u> negative) is observed for any one antigen as detected by a given typing fluid, one is led to believe that a one gene-one antigen relationship obtains in the case under study. One therefore reasonably assumes that the positive offspring all have the same antigen (at least, within any one family). This has been shown to be false in the case of family 28, in which there was segregation for E_2 and its absence but in which the E_2 individuals were not all alike.

Table 25

Absorption* of Reagent E by the E_3 Exception in an E_1 Family

Cells	Reagent E absorbed by cells: 14	2T	Sub-reagent E_2E_3	E_2	E_3
14	0	0	0	0	0
2T	0	0	1	?	1
28A	2	2	3	3	0
28B	1	0	3	3	0
28C	0	0	2	3	0
11B	3	2	3	0	3
14A	2	2	2	0	2
14D	3	2	3	0	3

* Reagent E absorbed six times, undiluted and tested at 1/3. The E sub-reagents were also tested at 1/3, for comparison.

c) A further serological relationship between E_2 and E_3 is indicated by a developmental study, in which squabs were typed at various times during their growth. Two squabs of family 28 were found to be positive to both sub-reagents E_2 and E_3 at approximately fourteen days of age. By the time they had reached 20 days however, one had become E_2 and the other E_3, their cells reacting not at all to the opposite reagent. Whether each would absorb activity from the opposite reagent is unknown.

Figure 5 summarizes the matings involving all the E subtypes. The genetic results can be seen to be regular, despite the complexities listed above.

E. Discussion

In spite of the complexities and anomalies encountered in the various absorption studies, the typing studies yielded very regular results. Even though the reagent was known to be fractionable, inheritance of the positive-negative alternative appeared to be controlled by a pair

Figure 5

Summary of Matings Involving All the E Subtypes

A dotted line drawn from a pigeon number to a mating number (underlined) indicates the bird to be the female parent. A solid line indicates the male parent.

Symbols and Code

+ - Family segregating for E_1 and E_3.

‡ - E_1 bird.

‡ - E_1E_3 bird.

‡ - E_3 bird, E_1 constitution unknown. This symbol placed next to a mating number indicates that all the offspring were E_3.

X - Family segregating for E_2 and E_3.

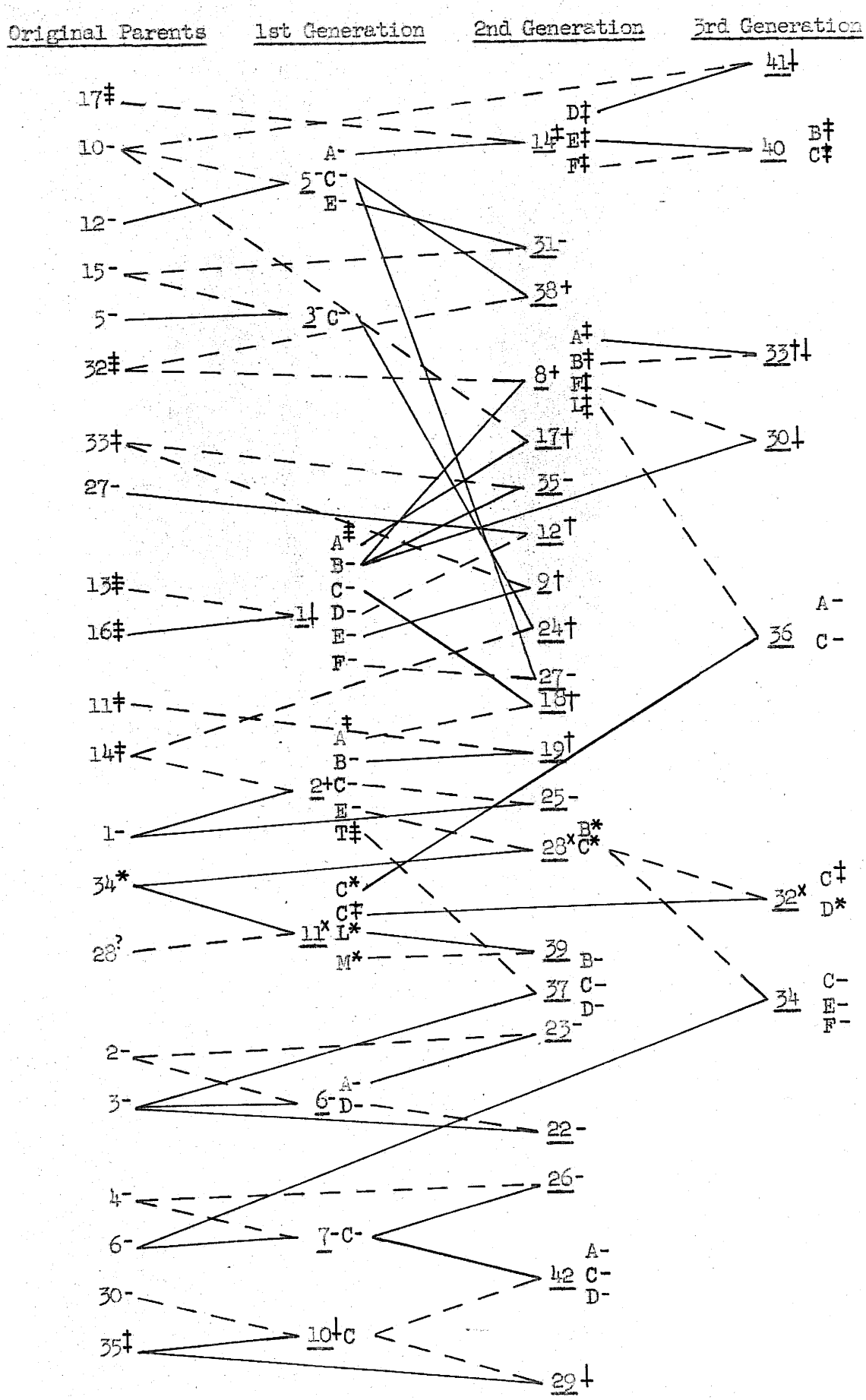
* - E_2 bird.

† - Family segregating for E_1 and negatives.

⊥ - Family segregating for E_3 and negatives.

– - Family composed of all negative offspring. This symbol also represents individual negative birds.

Insufficient numbers of offspring were obtained in certain families to determine the segregation patterns. In those cases, the offspring are listed and their types indicated.


Original Parents
1st Generation
2nd Generation
3rd Generation
17‡
10-
12-
15-
5-
32‡
33‡
27-
13‡
16‡
11‡
14‡
1-
34*
28?
2-
3-
4-
6-
30-
35‡
5 C-
3 C-
1‡
2+C-
11x
6-
7-C-
10‡C
A-
E-
A‡
B-
C-
D-
E-
F-
A‡
B-
E-
T‡
C*
C‡
L*
M*
A-
D-
14‡
8+
17†
35-
12†
9†
24†
27-
18†
19†
25-
28x
31-
38+
39
37
23-
22-
26-
D‡
E‡
F‡
A‡
B‡
F‡
L‡
B*
C*
41‡
40
33‡
30‡
36
32x
34
42
29‡
B‡
C‡
A-
C-
C‡
D*
B-
C-
D-
C-
E-
F-
A-
C-
D-

of alleles. Typing with the sub-reagents E_2 and E_3 suggested that E_2 and E_3 were controlled by alleles, at least in the one family investigated, while the reactions observed with sub-reagent E_3 indicated that E_3 and its absence were controlled by a pair of alleles. Although no sub-reagent could be made to distinguish E_1 in combination with the other subtypes, matings involving only E_1 gave results consistent, on the whole, with the assumption that E_1 and its absence were also inherited as if controlled by a single allelic pair.

Absorption analyses demonstrated that the regularity observed in the genetic data was misleadingly simple. Superficial absorption studies might have suggested that the antigens detected by the crude Reagent E were unrelated, but overabsorptions and reabsorptions showed that the antigens were similar; E_1 and E_3 cells removed activity for E_2. Furthermore, not all E_2 cells were alike, nor were E_3 cells all alike. The system was apparently based on two sets of variables: 1) the antigens represented by the same subtypes thought to be identical by the typing reaction, were in fact a collection of related antigens, and antigens thought to be different by the typing reactions were also related; and 2) the antibody populations in the reagents were definitely complex and represented a series of related rather than distinct specificities.

A similar situation has been encountered in the Rh complex. (The Rh terminology used in any particular connection is that of the author whose work is being discussed.) Two systems, one involving antigens related to the C antigen and the other related to the D antigen have proven to be very complex both because of the variety of antigens and because of the differences among sera used to test for them. Each serum apparently gives different sets of reactions. The C antigens [reviewed by Race et al. (27)] comprise a series known as C, C^W, C^u, c^V and c. Each of these is inherited regularly (36, 71) as if it were produced by the action of a single gene. Most anti-C sera have been found to be "anti-C + C^W." The identification of C^W cells depends upon the fact that they react with some anti-C sera and not with others. Cc and $C^W c$ cells can remove all activity from an "anti-C + C^W" serum. Since each can remove activity only for itself from an artificial mixture of anti-C and anti-C^W, the claim is made that in the former case, anti-C and anti-C^W specific sites are on the same

molecule. Anti-c sera were found which were "anti-c + c^V"; cc and c^Vc cells can remove all activity from these sera. Some anti-C sera were also found to be "anti-C + c^V"; Cc and c^Vc cells can remove all activity. C^u was also found to react with only certain anti-C sera but was different from C^W. It was suggested that anti-C^W in an "anti-C + C^W" serum was due to a non-specific response to immunization with C cells, and anti-c^V in both "anti-c + c^V" and "anti-C + c^V" sera was due to a non-specific response, in the former case to c cells and in the latter to C cells. More definitive proof that C^W was qualitatively a distinct antigen was obtained (72) when a case of hemolytic disease was found to have been induced by a C^Wc fetus in a Cc mother.

In the first report of the finding of the D^u antigen (73) the similarity between D^u and C^W was pointed out. D^u was detected by virtue of its reaction with only several of many anti-D sera. Anti-D sera reacting with D^u cells were supposed to be "anti-D + D^u." Twelve different kinds of D^u cells were distinguished (52, 74). Each type gave a particular pattern of reactions to the sera available, but different types gave different combinations of reactions to the battery of testing fluids. That the variability was not due to quantitative effects was indicated by certain discrepancies between titers of sera and the reactions they gave with different D^u cells (75, 76). The close relationship of D^u to D was shown by the removal of all activity from "anti-D + D^u" sera by both types of cells and the elution of anti-D from D^u cells when they had been treated with an anti-D serum (52). Anti-D has been produced by a D^u person (77).

On the whole each specific type of D^u was inherited regularly, in that D^u bloods of persons related to the D^u propositus gave identical reactions (74). Familial studies also indicated that D^u was controlled by a single gene and inherited as an allele at the D locus (52, 71, 76). Two cases are known however, in which inheritance of D^u was not regular. In one (52), three sisters were shown to have identical D^u types. One of the sisters, however, had a daughter who had a completely different D^u type (the father was dd). The authors suggest that this may be a case of variable expressivity of a blood group gene, even though it is recognized that blood group genes are not supposed to be subject to the effects of external or internal environment. The second case (76) seemed to represent the spontaneous appearance of D^u in a family. A child received a

chromosome designated as $\underline{cD^uE}$ from its mother, whose chromosomal type was believed to be $\underline{CDe/cDE}$. The suggestion is made that this may represent a mutation. It is also pointed out in the same paper that in certain instances there is a difference in the serological reactions of two D^u members of the same family. The conclusion the authors draw from their observations is that the reactions being investigated are between anti-D antibodies and a series of modified forms of the D antigen.

The similarities between the C and D systems, and that described in the present study of Reagent E lie in the diversity of similar antigens and the multiplicity of antibody specificities. Even antigens which ought to be identical on genetic grounds are found to be different (cf., in this study, the differences observed among E_2 birds of the same family).

The same general kind of observation has been made in the human M-N system. Variants of both M (78, 79) and N (80, 81) are known which react with only certain reagents and not with others. The new specificities have been designated M_2 and N_2, respectively. Little has been done to determine the relationships of M_2 to M and N_2 to N because both M_2 and N_2 are very rare types [e.g., N_2 has only been found eight times among 80,000 individuals (68)].

It would seem that a more reasonable explanation than that offered by Race and others for many of the observations made in the studies on the C and D antigens would be that the different sera contain antibodies of several specificities. There is no reason to suppose that because C and C^W can remove all activity from a given serum ("anti C + C^W") or because D and D^u can remove all activity from "anti-D + D^u", there are specific sites represented by anti-C and anti-C^W, or anti-D and anti-D^u, which are combined on one antibody molecule. As Landsteiner has pointed out (53), a multiplicity of antibodies may be evoked by one specific antigen. "Anti-C + C^W" sera would then contain a population of antibodies of specificities varying around those of anti-C and anti-C^W. C and C^W, being related, even if qualitatively different, may absorb the several antibody types, though with not necessarily the same efficiency. (More C^W than C cells are needed to exhaust a given serum.)

The same situation prevails in studies which have been interpreted as indicating that linkage or complexing occurs among antibodies of different specificities [e.g.,(82, 83, 84)]. Knowing that a given antiserum

may, and probably does, contain a mixture of antibody specificities, it becomes difficult to designate its actions by a unit symbol. As investigations of the serum proceed, it is found that antigens once thought to be identical, by virtue of their reactions with the serum, are different. From that finding realization is arrived at that the antiserum does not represent a unit specificity. This was the sequence of events in the discovery that anti-A of humans was composed of qualitatively different fractions. Recently, although the human B antigen has not yet been shown to occur in more than one distinguishable form (as has the A antigen), Owen (85) has confirmed that human anti-B is composed of qualitatively different fractions.

We see then, that the genetic data do not establish the unitary nature of an antigen. Anomalies may arise and be compounded by both the variation among related antigens and the multiple specificities represented by any one antiserum. A problem for the immunologist will be to devise more sensitive methods for distinguishing quantitative from qualitative differences, for small qualitative differences reacting with antibodies of related but different specificities may easily simulate quantitative differences detected by antibodies of only one specificity. The geneticist, on the other hand, may have to face the prospect that as the detectable qualitative differences become finer, genetic data will become more difficult to analyze, and anomalies may appear more frequently.

VI. ISOIMMUNE SERA

Twenty pigeons were cross-immunized in pairs, each pigeon being injected with washed erythrocytes from the other pair-member. The birds to be paired in these injections were chosen at random; the only selection exercised was for size, because large birds were preferable in order to obtain maximum amounts of serum, should any antibodies appear. All the normal sera were tested for the presence of isoagglutinins against all of the cells used in the study. None of the normal sera agglutinated any of the cells tested.

Three birds produced antibodies after one course of injections (see Sec. IIA3 for a description of the courses of injections), and six others produced antibodies after two courses. The titers of most of the sera remained rather low, even after one or two more courses of injections after the initial appearance of antibody. Only three pigeons, whose sera contained antibody detectable at a dilution of 1/100 or greater, were bled in quantity.

One month after bleeding, the titer of the stored, frozen serum was unchanged for two of the sera. The third, which had given an initial titer of 2,000 did not react with the homologous cells even at 1/16. Although other cells did react at this dilution, further investigation of this serum was discontinued.

Eight months after the last injection, a blood sample was taken from one of the two birds whose sera were used in typing. The titer for the homologous cells was observed to be 24. Reagent H was made from the serum of this bird. Serum from the other pigeon (from which Reagents C and RC were made) reacted to a dilution of 1/12 with the homologous cells twenty months after the last injection.

A. Reagent RC

1. Serology

Typing studies with the crude serum of pigeon 11, which had been injected with the red blood cells of pigeon 13, indicated a definite division of the flock into positives and negatives. The serum was diluted 1/100 for these tests. When the first typing of offspring was begun, it was noticed that six matings wherein both parents were negative produced 16 positive and 14 negative progeny. When the typing dilution was dropped to 1/16,

most of the anomalies disappeared. This effect of dilution will be discussed in connection with the genetics of antigen C.

Very little serum was obtained from pigeon 11. For this reason, even though the serum was shown to contain qualitatively different fractions (Table 26), the unabsorbed serum was used as a typing reagent.

Table 26

Fractionation* of Pigeon Antiserum 11 (Reagent C)

Cells	Serum Unabsorbed	Serum absorbed by cells: 7	8	16	17	13
7	6	0	0	4	4	0
8	6	0	0	4	4	0
16	6	0	0	0	0	0
17	6	0	0	0	0	0
13	6	0	0	5	4	0

* Pigeon antiserum 11 absorbed once at 1/16, by each of the positive cells listed. Tested at a further dilution of 1/32.

When the supply of this reagent was exhausted, pigeon 11 was reimmunized with cells of the same donor, pigeon 13. The serum so obtained was designated RC (Reimmunized C). While most cells tested reacted alike to Reagents C and RC, occasionally certain cells negative to one were positive to the other. An absorption analysis of RC was performed with cells from ten birds (Table 27), including three of the four cells used in the analysis of C. With regard to C, 8 and 13 had been shown (Table 26) to be identical (they removed activity for each other). With regard to RC,however (Table 27), 8 left agglutinins in the serum for 13. The positives 16, 17, 10R, 29M and 14U left in activity for both 8 and 13, but removed activity for 30K. 30K removed activity for all those cells, suggesting that 30K should be identical with the cells tested. But the reagents prepared by absorbing with these cells behaved differently. Absorbing with 30K removed all activity for 8, but absorbing with the others did not do so. The table therefore reveals the presence of four types of positives, and suggests that the behavior of one is anomalous.

Table 27

Fractionation* of RC, the Reimmunized Serum of Pigeon 11

	Serum absorbed by cells:									
Cells	24M	19P	16	17	10R	29M	14U	30K	8	13
24M	0	0	0	0	0	0	0	0	0	0
19P	0	0	0	0	0	0	0	0	0	0
16	3	3	0	0	0	0	0	0	0	0
17	3	3	0	0	0	0	0	0	0	0
10R	3	3	0	0	0	0	0	0	0	0
29M	3	3	0	0	0	0	0	0	0	0
14U	1	1	0	0	0	0	0	0	0	0
30K	2	2	0	0	0	0	0	0	0	0
8	3	3	1	1	t	1	1	0	0	0
13	4	4	4	4	3	4	4	4	3	0

* The serum was absorbed at 1/10 and tested at a further dilution of 1/2.

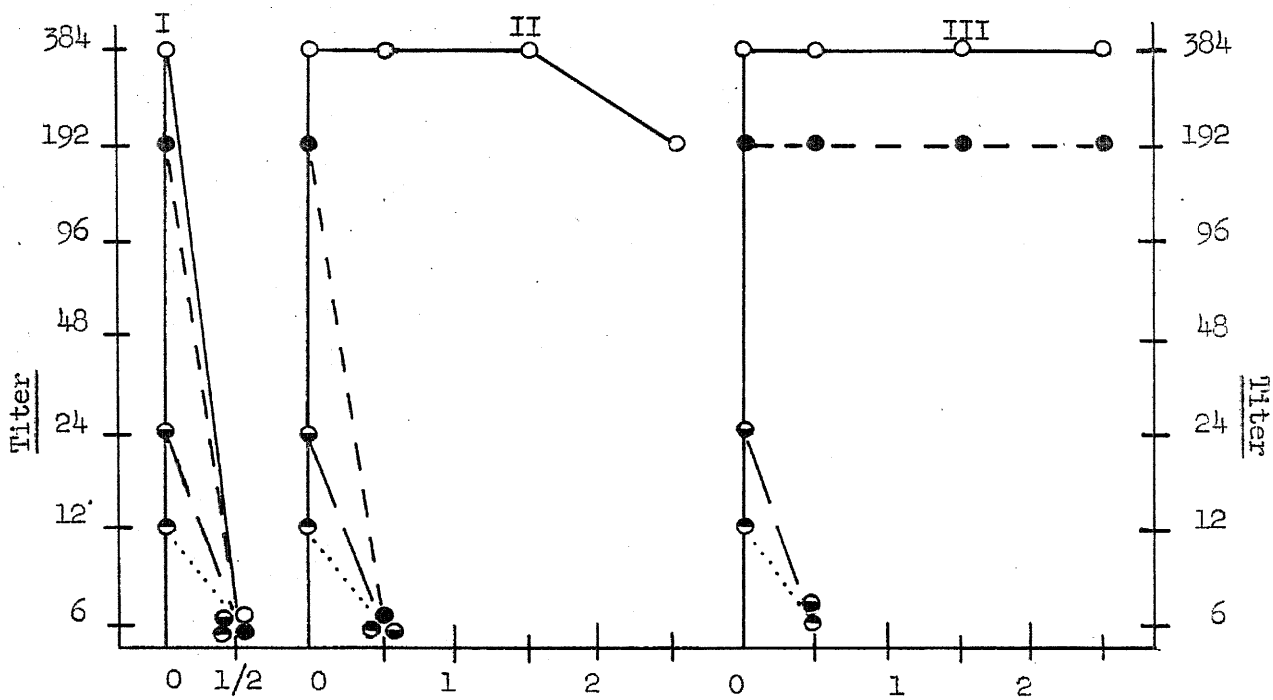
The same results were obtained when either one or two absorptions were performed. Absorptions by 24M and 19P, negatives to RC, serve as dilution controls.

Absorption of RC by 8F, the positive parent of 30K, indicated that the behavior of 8F was not anomalous; it left activity in the serum for 8 and 13.

That a quantitative effect is probably not involved in the analysis of RC is shown by the fact that the same results were obtained with the cells listed in Table 27 whether one or two successive absorptions were performed. Not enough is known about the behavior of different negatives in absorption to exclude completely the possibility of a quantitative effect. However, a comparative absorption study (Fig. 6A) definitely indicated that qualitative differences existed among the positives. Absorption by 13, the homologous cells, removed all activity; absorption by 28B left in activity only for 13 and absorption by 10C left in activity for 13 and 28B. Cells of pigeon 11 (the recipient) and 7C, a negative, did not affect the activity of the serum in tests with any of the positives (Fig. 6B).

It would have been interesting to type with the sub-reagents which could have been made by use of the information obtained in Table 27. Unfortunately, such a program was technically impossible; the quantity of

Figure 6A

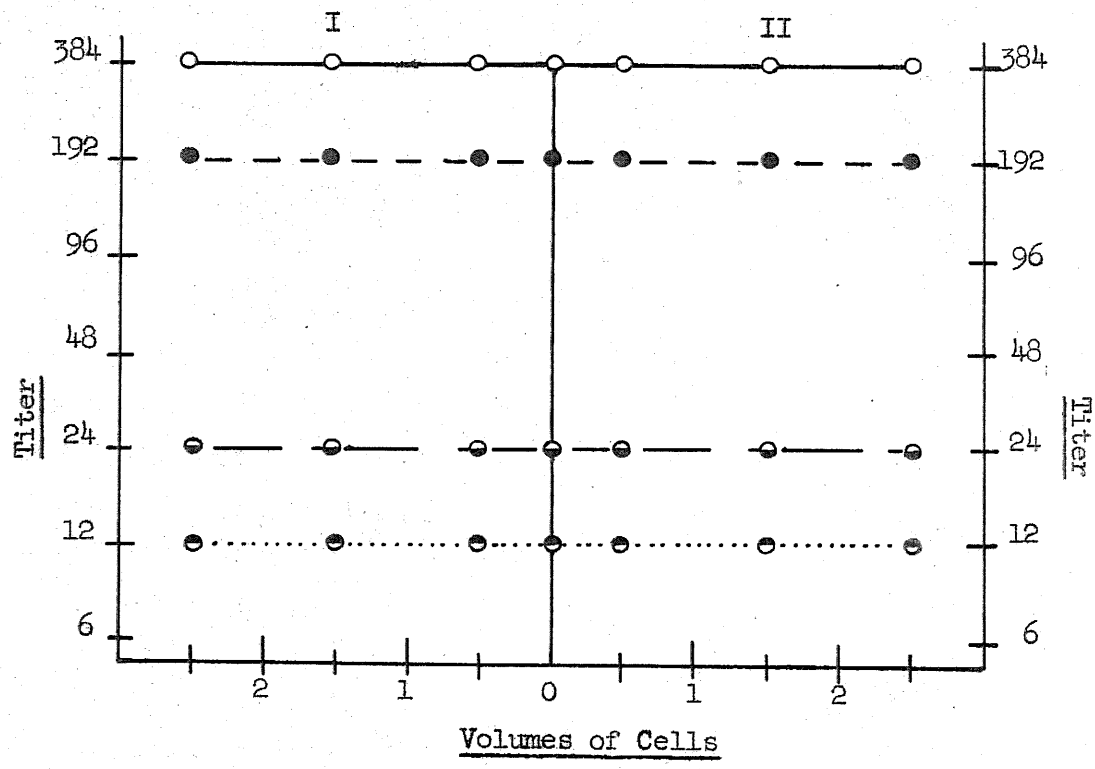


Reagent RC, at 1/6, absorbed by positive cells. Points represent the titer on a logarithmic scale after absorption by the indicated volumes of cells.

I - absorbed by cells 13
II - absorbed by cells 28B
III - absorbed by cells 10C

○ - cells 13
● - cells 28B
◒ - cells 42C
◓ - cells 10C

Figure 6B



Reagent RC, at 1/6, absorbed by negative cells. Points represent the titer on a logarithmic scale after absorption by the indicated volumes of cells.

I - absorbed by cells 11
II - absorbed by cells 7C

○ - cells 13
● - cells 28B
⊖ - cells 42C
⊖ - cells 10C

serum obtained was just sufficient to type all the birds available, and to perform the few absorptions mentioned.

2. Genetics

Typing with C and RC proved to be very inconsistent with regard to the typing reaction itself and to the genetic results obtained. Many cells which had been positive to Reagent C at 1/100 were later found to be negative at 1/20 (or 1/16). With both reagents, weakly reacting positives varied from positive to negative and back to positive again, in successive tests. Strongly reacting positives (grade "3" or higher) usually gave reproducible reactions. The inconsistencies, however, were sufficiently numerous to prove a serious hindrance to genetic analysis of the system.

Table 28 illustrates the most obvious difficulty encountered in analyzing the data. Seven negative x negative crosses produced a total of 19 positive offspring. Some of the negative parents, and many negative offspring, retyped at a dilution of 1/8, proved to be weakly positive. Some of the parents in the negative x negative matings were therefore not truly negative. In fact these matings were similar to those that produced anomalous offspring in the studies on Reagents A and E. Reagents E and RC,however, also recognized qualitative differences.

Table 28

Inheritance of C

Number of Matings	Type of Mating	Offspring	
		+	0
8	0 x 0	0	60
7	0 x 0	19	34
2	+ x 0	26	0
11	+ x 0	55	45
3	+ x +	11	0
2	+ x +	8	6

Typing performed with Reagent RC at 1/16.

A suggestion that the weak positives, as a class, differed qualitatively from the strong positives is seen in Figure 6A. Pigeon 10C (negative at 1/16, weakly positive at 1/8) removed activity for its weakly

positive offspring 42C (positive at 1/16) but did not decrease the activity of the serum toward the strong positives. The anomalies observed, then, probably arise from quantitative variations among the weakly positive cells.

Eight matings of negatives produced 60 negative progeny. None of the members of these families were rechecked with the reagent at the lower dilution, but in Figure 6B, it is seen that 7C, a negative of this group, did not remove activity for even the weak positive 10C.

The genetic analysis of Reagent RC therefore involves negatives, weak positives (including those cells which react weakly at 1/16, and those which react weakly at 1/8 and do not react at 1/16), and at least two types of qualitatively different strong positives. The weak positives are qualitatively different from both types of strong positives but may exhibit quantitative variations among themselves. If three different antigens detected by Reagent RC are controlled by independent genes, then we may reasonably expect a large preponderance of positives in the crosses of positives x negatives which produce some negative offspring. On the other hand, if the antigens are related, and are controlled by alleles, the progeny of such crosses will be produced in a positive to negative ratio of 1:1.

Eleven matings of positive x negative (Table 28) in which ten of the positive parents were unrelated, produced 55 positive and 45 negative progeny. Two positive birds produced 26 positive offspring and no negatives when mated to negatives. These observations suggest that the different cellular specificities detected by Reagent RC may be controlled by alleles (Figure 7).

B. Reagent H

1. Serology

Variability in typing reactions like that noted with Reagent RC was also encountered with pigeon antiserum 6E (Reagent H), produced in response to injection of the red blood cells of pigeon 9D. The first absorption analysis performed, with cells all reacting positively to the antiserum, indicated the serum to be very complex; at least six different specificities were observed among eleven positive cells. With the small quantity of serum available, no attempt was made to prepare sub-reagents. No further studies were performed on the serum, except for its use as a typing tool.

Figure 7

Summary of Matings Involving Antigen C

A dotted line drawn from a pigeon number to a mating number (underlined) indicates the bird to be the female parent. A solid line indicates the male parent.

Symbols and Code

○ - All members of the family were negative.

● - All offspring were positive.

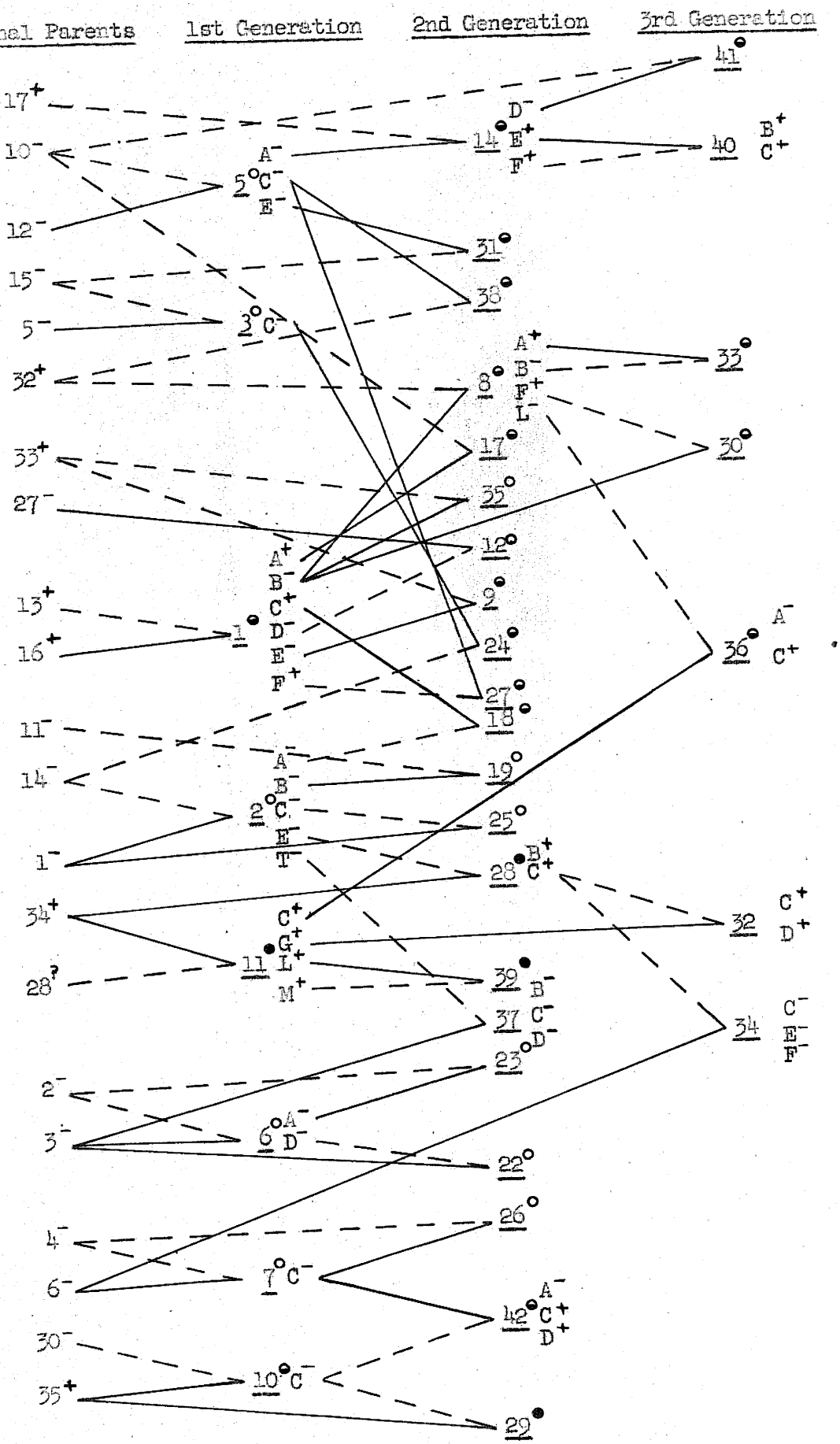
⊖ - Some offspring were positive, some were negative.

\+ - The individual pigeon was positive.

\- - The individual pigeon was negative.

Typing was done with Reagent RC.

Insufficient numbers of offspring were obtained in certain families to determine the segregation patterns. In those cases, the offspring are listed and their types indicated.


Original Parents
1st Generation
2nd Generation
3rd Generation
17+
10-
12-
15-
5-
32+
33+
27-
13+
16+
11-
14-
1-
34+
28?
2-
3-
4-
6-
30-
35+
A- C- E-
A+ B- C+ D- E- F+
A- B- C- E- T-
C+ G+ L+ M+
A- D-
7 C-
10 C-
A+ B- F+ L-
D- E+ F+
B+ C+
A- C+
C+ D+
C- E- F-
A+ B- C+
41
40
31
38
32
8
17
35
12
2
24
27
18
19
25
28
11
39
37
23
22
26
42
29
1
36
30
32
34

2. Genetics

Inconsistencies in reactions and in the genetic analysis occurred in the study of Reagent H, as they had in the study of Reagent RC. There was wide variation in the reactions of cells; some negatives at a 1/16 dilution of the reagent were weakly positive at 1/8, and negatives when crossed gave rise to positive progeny (Table 29).

Table 29

Inheritance of H

Number of Matings	Type of Mating	Offspring	
		+	0
15	0 x 0	0	124
6	0 x 0	18	24
11	+ x 0	44	43
1	+ x +	1	5

Typing performed with Reagent H at 1/16.

That Reagents H and RC differed is seen from the fact that pigeon 11, the recipient which produced RC, was positive to Reagent H. Many cells were RC-positive, H-negative, while others were RC-negative, H-positive.

As was pointed out in the discussion of Reagent RC, familial typing with an antiserum which contained many independently determined antigens might be expected to detect an excess of positive progeny in matings of positives x negatives which produced both types of offspring. The two classes were produced in equal numbers (Table 29) which is suggestive that reactivity to Reagent H is controlled by a series of alleles (Figure 8).

C. Discussion

The Reagents RC and H have each been shown to be composed of qualitatively different fractions. Typing studies indicated, however, that reactivity to each of these reagents was transmitted to the progeny of any particular bird as if a positive vs. negative alternative were controlled by alleles. Some uncertainty was attached to the characterization of weakly reacting cells, because over a period of about one year

Figure 3

Summary of Matings Involving Antigen H

A dotted line drawn from a pigeon number to a mating number (underlined) indicates the bird to be the female parent. A solid line indicates the male parent.

Symbols and Code

○ - All members of the family were negative.

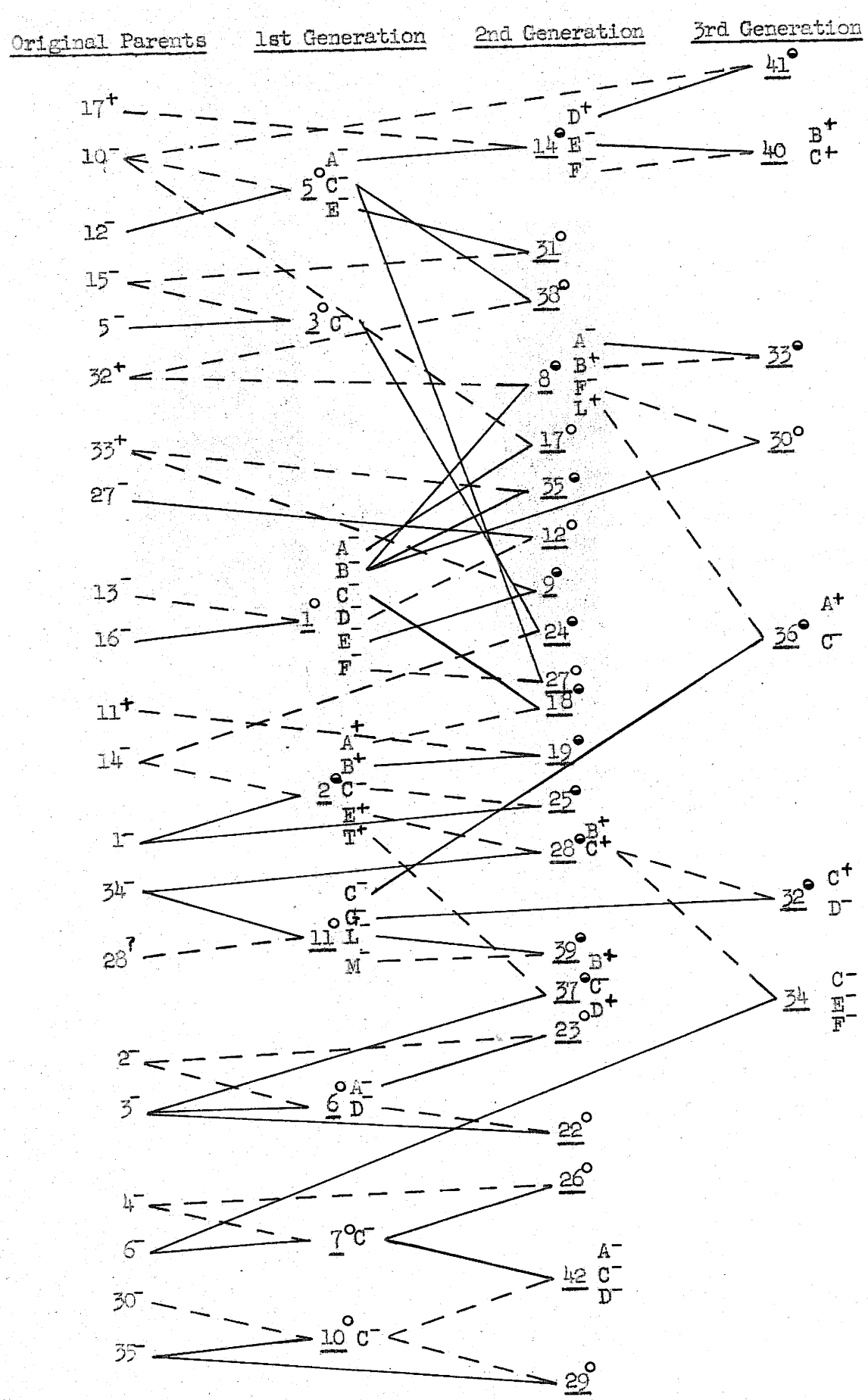
● - All offspring were positive.

⊖ - Some offspring were positive, some were negative.

\+ - The individual pigeon was positive.

– - The individual pigeon was negative.

Insufficient numbers of offspring were obtained in certain families to determine the segregation patterns. In those cases, the offspring are listed and their types indicated.


Original Parents
1st Generation
2nd Generation
3rd Generation
17+
10-
12-
15-
5-
32+
33+
27-
13-
16-
11+
14-
1-
34-
28?
2-
3-
4-
6-
30-
35-
5 A- C- E-
3 C-
1 A- B- C- D- E- F-
2 A+ B+ C- E+ T+
11 C- G- L- M-
6 A- D
7 C-
10 C-
14 D+ E- F-
8 A- B+ F- L+
17
35
12
9
24
27
18
19
25
28 B+ C+
31
38
23
37 C- D+
22
26
29
41
40 B+ C+
33
30
36 A+ C-
32 C+ D-
39 B+
34 C- E- F-
42 A- C- D-

reactions of some cells were noticed to change from weakly positive to negative while the reverse change occurred in others. Since the change occurred in either direction, it is difficult to see how the reagents could have been at fault. Instead, it would appear to have been the antigens on the cells that changed; and it might be further assumed that the quantity rather than the quality was the variable factor, for the "adult" antigens once fully developed on the blood cells are generally believed to be a permanent characteristic of those cells.

Because of the absorption studies performed with these reagents, it is believed that the gradation in intensities among individuals observed in typing was due to the different reactions of different antigens. It has been shown (31, 33), that a continuous series of reaction intensities may occur when either an isoimmune serum or a rabbit antiserum provides the test reagents. In the present study, some "negatives" were shown to be positive by absorption. Even though one genetically negative individual must exist (viz., the individual that developed the antibodies in these isoimmune sera), the determination of how many other true negatives there are in a population requires the use of absorptions as well as direct tests. At any rate, when working with an isoimmune serum we can be certain we are dealing with a segregating character of some sort. This is in contrast to a quantitative character detected by a heterospecific antiserum, in which all "negatives" can sometimes absorb the specific agglutinins.

VII. LINKAGE STUDIES

A. Analyses

The antigens ("positivity vs. negativity" in the case of Reagent A) were all tested for linkage to sex, plumage pattern and eye color. The tests for sex linkage involved crosses in which positive females mated to negative males produced both positive and negative progeny. If the character were controlled by a gene on the sex chromosome, all male offspring in such crosses would be positive and all female offspring negative. Independence of the sex chromosome would be shown if positivity and negativity were distributed alike in the two sexes of offspring. Tests for linkage to the two morphological characters involved matings of pigeons heterozygous for the character and positive (heterozygous) to the particular reagent, with other pigeons recessive for the character and negative to the reagent. (See Levi (86) for a complete review of pigeon genetics.) Complete records were kept on eye color and plumage pattern inheritance.

Not all the possible crosses were made, but those which were tested indicated that each antigen was independent of sex, eye color, and a plumage pattern locus (Table 30).

All tests for linkage between pairs of antigens were of crosses wherein one parent was doubly positive (doubly heterozygous) and the other doubly negative. Offspring were scored as similar to either parental type or as recombinant types. The combination in the doubly heterozygous parent was in each case such as to reveal linkage, if it existed, in the coupling phase.

No linkage was detected between A and E, A and C, or A and H (Table 31). Because of the small numbers of offspring, however, it is not possible to exclude the possibility of weak linkage in those combinations.

The linkage values indicated in Table 31 are all suspect because of the difficulties encountered in typing, especially with Reagents E, C and H. Until better reagents and much larger numbers of offspring can be obtained, the indicated values are to be considered merely suggestive, and not compelling evidence for the existence of linkage. Nevertheless, several analyses suggest that some relationships may actually

Table 30

Linkage Tests

Pair of Characters	Number of Matings	Offspring			
		M.+	M.O	F.+	F.O
Sex-A	2	7	6	5	3
Sex-E_1	5	9	10	15	12
Sex-E_2	1	-	-	2	-
Sex-E_3	4	11	5	5	11
Sex-C	5	11	11	5	11
Sex-H	7	17	19	17	10
		C+	cO	CO	c+
Pattern-E_1	1	3	2	4	3
Pattern-E_3	2	4	3	6	1
Pattern-C	1	4	2	3	2
Pattern-H	2	2	1	1	1
		Tr+	trO	TrO	tr+
Eye Color-E_1	3	6	9	6	4
Eye Color-E_3	1	-	2	1	-
Eye Color-C	3	5	10	5	8
Eye Color-H	5	7	16	11	9

\+ = presence of the antigen.

O = absence of the antigen.

The color patterns investigated were T-pattern ($\underline{C}^T$), checker ($\underline{C}$) and bar ($\underline{c}$), which are alleles. $\underline{C}$ in the table includes both checker and T-pattern.

Orange eye color ($\underline{Tr}$) is dominant over pearl eye color ($\underline{tr}$).

Table 31

Tests* for Linkage Between Pairs of Antigens

Pairs of Antigens	Number of Matings	Offspring		% Recombination
		Parental	Recombinant	
A-E_3	3	13	12	---
A-C	3	13	12	---
A-H	1	6	6	---
E^{**}-C	6	37	4	9.8
E_1-C	2	10	3	23.0
E_3-C***	5	33	1	2.9
E-H	6	25	10	28.6
E_1-H	3	20	5	20.0
E_3-H	3	5	5	50.0
C-H	4	13	7	35.0

* All matings were of double heterozygotes x recessives.

** By "E" is meant reactivity to Reagent E, unabsorbed. The E_1 and E_3 classes are included within each linkage determination for the E^3 complex.

*** One mating was $\underline{E^1c}/\underline{e^1c}$ x $\underline{E^3C}/\underline{e^3c}$. This mating did not constitute a test cross for linkage between E and C but was a test cross for E_3 and C.

exist among the antigens E, C and H. The recombination value between C and H is .35, too high a figure to be reliable as an index of actual linkage, especially in view of the smallness of the sample. Between the E complex and C the approximate crossover frequency is .10, and that between E and H is .29. These figures would suggest a map order of C E H, and approximate distances as follows: $\overset{C}{\underset{}{\quad}}\ \overset{10}{\underset{}{\quad}}\ \overset{E}{\underset{}{\quad}}\ \overset{29}{\underset{35}{\quad}}\ \overset{H}{\underset{}{\quad}}$.

It is unfortunate that no three-point test crosses could be made.

In discussing the genetic relations among the E subtypes (Section VC) the possibility was suggested that $\underline{E^1}$ might be allelic to $\underline{E^3}$. But in Table 31, the recombination frequencies of each with C are found to differ markedly. Families 8 and 38, in which the female parent was pigeon 32 (type E_1E_3), were not included in the calculations determining the crossover frequencies between $\underline{E^1}$-C and $\underline{E^3}$-C listed in Table 31, because the E_3 offspring may have also been E_1.

If one considers the one E-negative offspring of pigeon 32 (Table 24) to be a valid E-negative, then pigeon 32 was of the genotype $\underline{E^1e^3}/\underline{e^1E^3}$ and the recombination frequency between $\underline{E^1}$ and $\underline{E^3}$ in these matings was about 5%. If one then assumes that the reciprocal crossover, which would not be detectable with the reagents at hand, may have occurred with equal frequency, this gives a recombination value of 10% between $\underline{E^1}$ and $\underline{E^3}$. Pigeon 8F, which, when mated to a negative produced one supposed E_1 offspring as well as E_3 and negative progeny (see mating 30, Table 24), would actually represent the crossover class expected. Its genotype would be $\underline{E^1E^3}/\underline{e^1e^3}$. The one E_1 offspring of pigeon 8F would indicate a crossover frequency of 0.11, in mating 30. Within this family (offspring of pigeon 32), and considering only E_1 and E_3, the linkage data between $\underline{E^1}$ and $\underline{E^3}$ are remarkably consistent, and suggest that the responsible genetic factors may recombine with reasonable frequency. If so, the chromosome map suggested would be as follows:

C E_3 E_1 H

3 10 20

35

All the E_1 offspring of pigeon 32 were C-negative. This is reasonable, because there were only seven E_1 progeny and the $\underline{E^1}$-$\underline{C}$ recombination value is estimated at .23. Pigeon 32 can then be considered to be of the genotype $\underline{E^1e^3c}/\underline{e^1E^3C}$, and its recombination offspring 8F as $\underline{E^1E^3C}/\underline{e^1e^3c}$. The double crossover frequency predicted in the absence of interference would be .003 (.10 for the $\underline{E^1}$-$\underline{E^3}$ interval and .03 for the $\underline{E^3}$-$\underline{C}$ interval - .10 x .03 = .003). Interference would preclude the appearance of a double crossover. But the one apparently E_1 offspring of 8F was C-positive. Any conclusion concerning the relationships of $\underline{E^1}$ to $\underline{E^3}$ and $\underline{E^1}$ to $\underline{C}$ is thus seen to be highly uncertain.

The relationship between $\underline{E^3}$ and $\underline{C}$ is more consistent. Pigeon 32 produced eighteen offspring, nine of which were E_1, C-negative, the other nine being E_3, C-positive. Excluding the progeny of pigeon 32, all other test crosses involving E_3 and C yielded one apparent recombinant (E_3-negative, C-positive) out of a total of thirty-one progeny. It would have been very desirable to have retested the apparent recombinant to determine if it was truly E_3-negative, but it died before this could be done. There is no doubt, however, that a very close relationship does exist between

$\underline{E}^3$ and $\underline{C}$. We cannot decide on the basis of the available data between the possibility that E_3 and C are factors of the same antigen and the possibility that E_3 and C are distinct antigens whose causative genes are closely linked.

C and H, in Table 31, are seen to yield a recombination value of .35. The data are not significantly different from those to be expected of independent segregation. But both independence and distant linkage are in direct opposition to the observation that pigeon 32, heterozygous for both $\underline{C}$ and $\underline{H}$, produced eighteen offspring, none of which were doubly positive or doubly negative.

A true and accurate determination of linkage is dependent on reliability of the typing procedure, and on the classification of large numbers of offspring. These studies, which do not fulfill either criterion, are to be considered only as indicating that genetic relationships, as yet undefined, do exist among the antigens detected by the E reagents and Reagents RC and H.

B. Discussion - The Relationship Between Antigens E_3 and C

Only one case of linkage between a blood group gene and a morphological character is known to the writer. Sawin et al. (87) presented evidence showing that the A blood group of rabbits was linked to the character "brachydactyly". That linkage was not found with the very small number of markers utilized in the present study is not surprising, since pigeons may have as many as forty pairs of chromosomes. (See Makino (88) for a list of the chromosome numbers which have been proposed for the pigeon at various times).

Because of the anomalous absorption results and occasional uncertainties in the typing reactions, any description of relationships among antigens E, C and H is subject to strong reservations. An observation worthy of discussion, however, is that of apparent linkage between E_3 and C, and the appearance of one evident crossover. But this crossover could represent an artifact, and there may have been other crossovers, undetected because of other artifacts. In spite of the probability of artifacts, the linkage data, together with the observation that all E_3 offspring of family 8 were C-positive and all E_1 offspring C-negative, are suggestive of a close genetic relationship between these antigens. Antigens E_3 and C are different, as evidenced by the occurrence of pigeons typed as E_3-negative, C-positive or E-positive, C-negative. The combination E_3-positive, C-

negative has not been encountered.

Since genetic independence is excluded, the only relationships we need consider are linkage or allelism of the causative genes. Allelism would suggest that the antigenic specificities involved are the properties of a single antigenic molecule. In spite of the meagerness of the data, and the hesitation in accepting the results obtained as conclusive, we see, then, that this study provides another case to be included in the debate over linked genes vs. multiple allelic explanations of immunogenetic complexes.

The debate had its origin in the suggestion by Fisher [cited by Race(89)]that in the Rh system three antigens occurred in pairs (C vs. c; D vs. d; and E vs. e). With the recent discovery of anti-f (37), the system has been extended to include four pairs of antigens. The members of each pair were interpreted as being controlled by alleles. Previous to Fisher's suggestion, all the Rh reactions given by any one blood had been thought to depend on the presence of one pair of alleles, the different combinations of reactions being due to the occurrence of multiple alleles at the Rh locus. For example, Wiener and Landsteiner (90) had proposed a series of three alleles to explain the inheritance of certain groups of reactions. Variants have been found which caused those believing in the Fisher interpretation to add alleles at certain loci (e.g., $\underline{C}^{W}$, $\underline{D}^{U}$, $\underline{c}^{V}$) and the followers of the Wiener school to postulate new alleles at the Rh locus. [For a history of Rh studies and development of the debate, see Race and Sanger (4). Wiener's view is briefly but adequately described in Wiener and Wexler (42)].

In essence, the disagreement involved the answer to the question: does one gene produce an antigen with only one specificity or can one gene produce an antigen with many apparent specificities? Race and his coworkers would answer affirmatively to the former and negatively to the latter; Wiener would be of the reverse opinion. Landsteiner (53) suggested that if a blood is agglutinated by two different sera of different specificities, it may have two different antigens, or one antigen, with which antibodies in each serum react. If there were two antigens, they might be separable genetically; if there were only one, it could not segregate. The English workers have never observed crossing over among the postulated three loci; they maintain that the three genes are very closely linked. On the basis of statistical analyses they have concluded

that there are three most common chromosomes in the European white population (CDe, cde and cDE). All other Rh types are believed to have arisen by crossing over among these and other primary crossover types.

The inheritance of blocks of "antigenic factors" as units has been reported several times. The discovery of a new antiserum (anti-S) which subdivided the MN blood groups (91) led to the observation that there were less S-positive types among N persons than among M (92). Familial studies suggested that S was linked to M and N. Suggestions were offered that the S specificity could be a mutation that changed M to MS and N to NS, or that S could be closely linked to M and N. The latter hypothesis was favored by Sanger (93). Discovery of anti-s (94) was interpreted by the English workers as favoring the linked gene hypothesis (93). Wiener (42, 95), however, has suggested that if the M-N-S antigens were due to linked genes, the frequency of S among M and N persons should have reached equilibrium. This would have prohibited the discovery of the relationship in the general population. As an alternative, Wiener proposed a system of four alleles controlling the unit antigens MS, Ms, NS, and Ns.

Two additional antigens, recently investigated, are thought to be linked to the M-N-S system. One (Hu), when first discovered in 1934 (96), was noted to react only with Negro N or MN bloods. The second (He) was found to exist practically only in West Africans (97). All He-positive bloods were type N, and most were also type S. Familial data indicated that He was inherited with NS or Ns as a unit. The authors suggest that He and Hu may be alleles but critical data to support this supposition are lacking.

The unitary inheritance of several antigenic specificities has been demonstrated in chickens (7), ducks (20) and cattle (6). In the cattle study, as many as eighty alleles were identified, accounting for the inheritance of twenty-one blood factors in various combinations in the B system. Twenty-two alleles in various combinations of seven blood factors controlled the inheritance of the C system.

The multiple allelic theory of the inheritance of complex antigenic specificities is based on the hypothesis that each allele produces an antigen which is related to but chemically distinct from the antigens produced by the other alleles. The reactions observed

in which different antigens cross-react with different reagents are believed to result from the multiplicity of antibodies with related affinities in such reagents. Many antigens have "factors" in common; that is, a reagent produced against one member of the allelic series will cross react with related antigens produced by other alleles.

The writer is inclined to believe, but cannot offer proof, that the E subtypes and C are common factors in a group of related antigens. Their relationship would be the more interesting to study however, if it could have been shown that a genetic crossover had in fact been observed, for as has been stated previously, blood group "genes" postulated to be linked have never been shown to recombine.

VIII. KAHN ANTIGEN

In 1952, when Dr. Burnet visited this Institute, he pointed out that a typing tool for an inheritance study in pigeons existed in the use of alcoholic extracts of tissues, particularly commercial serological Kahn antigen. Stone and Burnet (98) had noted that vaccinia (cowpox) virus would agglutinate the cells of certain pigeons and not of others. The viral activity could be separated from the hemagglutinating activity of the virus preparation, and it was shown (99, 100) that lipid extracts and phospholipids could produce the same phenomenon.

The procedure in these tests was to add one drop of a 2% cell suspension to two drops of a dilution of Kahn antigen in a round-bottomed standard agglutination test tube. Dilutions tested ranged from 1/10 to 1/4,000. Readings were of the agglutination pattern formed by the cells as they settled to the bottom of the tube. In a positive reaction, the cells formed an even layer covering the bottom of the tube, while in a negative reaction the cells rolled down and formed a compact button at the bottom of the tube.

At first some cells gave positive reactions, but when they were rechecked they had become negative. Some cells were consistently negative. It was noticed that many cells gave positive reactions in the saline controls. This may have indicated that a non-specific "stickiness" existed, but the characteristic was confined to tests in saline. When the same tests were performed using rabbit antisera and pigeon isoantisera, positive agglutination patterns were given only by cells known to be positive to the reagent. "Sticky" cells never gave evidence of agglutination when the saline controls were centrifuged.

The same tests were repeated using an extract of chick allantois which had been infected with vaccinia, with similar results.

Both extracts, Kahn and vaccinia, were checked with several samples of chicken cells. Some of the cells gave clear positive reactions and at no time did any give positive saline reactions.

IX. DEVELOPMENTAL STUDIES

A. Experimental Findings

Embryos and squabs were bled and typed at various stages of development. The typing reactions were carried out at the same reagent dilution and in the same manner as had been done when adults were tested.

Embryos were bled in the following manner: After removal of the shell over the air sac, the allantois was wet with warm isotonic sodium citrate solution. The blood vessels which then became obvious were pinched with a small tweezer. Citrate was dropped very slowly on the cut vessels, washing the blood into a citrate-filled receptacle. By rupturing most of the visible blood vessels in turn, 0.1 to 0.2 ml. of packed red blood cells could be obtained from 10-day or older embryos.

Newly-hatched squabs were bled by cutting a toe and dipping the foot into a tube of citrate solution. Squabs one week of age and older were bled by incising the brachial vein.

The stage of development at which embryos were bled is not known with complete accuracy. The egg was placed in an incubator sometime during the day it was laid, and it was kept there, being turned twice a day, until the day it was bled. Embryo development was recorded only in terms of the age of the egg in days.

The developmental study was incidental to the main purpose of this investigation. Eggs were, on the whole, too valuable to be destroyed for typing embryos. When a mating was to be discontinued, its last pair of eggs was incubated for the developmental study.

1. "Antigen" A

Positivity to Reagent A developed between the tenth and fourteenth days of development in ovo. The reactions observed with cells of fourteen day embryos were generally weak, while those of fifteen day embryos were much stronger. A maternal effect was excluded, because two A-negative females (mated to A-positive males) laid eggs from which strongly reacting A-positive cells were obtained.

The red blood cells of two A-positive embryos were pooled and the pool used to absorb an aliquot of Reagent A. The absorbed Reagent A agglutinated cells 13 at about the same intensity as did Reagent A unabsorbed, but activity was removed for the red blood cells of two newly-

hatched A-positive squabs.

The effects noted when squabs were tested with Reagent A at various intervals were quite diverse. Reactions could: 1) remain unchanged from hatching on; 2) become stronger, usually attaining maximum intensity by about fourteen days after hatching; 3) become weaker, and sometimes disappear completely (those reactions which disappeared were usually weak at hatching); or 4) be strong at hatching, get weaker in about seven days and begin to become stronger in about fourteen days. Statements 3) and 4) are made on the basis of titer studies done weekly on certain squabs, from hatching to thirty days.

These observations may suggest that there are at least two categories of distinct antigens which react to Reagent A, one characteristic of embryonic cells and the other of post-embryonic cells. The various reaction changes would on this basis be associated with both the times of disappearance of the embryonic antigens and appearance of the post-embryonic antigens and with the rates at which these occur. Both time and rate may vary in different pigeons. A more accurate description of the changes undergone by these two categories of antigens would be possible if enough embryonic cells could be obtained to make reagents specific for embryonic and post-embryonic cells.

2. "Antigen" E

No embryonic cells reacted with any of the E reagents. In fact, all newly hatched squabs were also negative. Very often within one week, and always within two weeks after hatching, the reaction reached maximum intensity in those squabs in which E did develop. E_2 and/or E_3 activity developed at the same time.

Two squabs which reacted weakly to sub-reagent E_2 and strongly to E_3 at fourteen days of age displayed a remarkably divergent subsequent development for these antigens. Within another fourteen days, the E_3 reaction had disappeared and the E_2 reaction had intensified in one of these squabs. The other lost the E_2 reaction and became E_3. Other squabs remained consistently E_2 or E_3.

3. Antigen C

The cells of one 14-day embryo gave a weak reaction with Reagent RC. Some of the C-positive squabs were negative at hatching while others were weakly reactive. Maximum intensity of reaction was

reached in one to two weeks. It seemed that stronger levels of eventual reaction were reflected in earlier detection of the antigen. This, as was indicated in the development of A, suggested differences among pigeons as to the time at which production of the antigen was initiated. Alternative suggestions could involve rate of production as well, or, again as in A, both rate and time.

4. Antigen H

All embryos and all newly-hatched squabs were negative to Reagent H. Maximum intensities of reactions were reached,in positive squabs, in two to three weeks.

5. General Aspects

The developmental studies left no doubt that A differed from the other antigens. The developmental histories of E, C and H, however, were closely similar. Almost all reactions of E, C and H, while attaining maximum intensity in from one to three weeks, had become noticeably weaker in four weeks. No explanation can be offered for this curious behavior.

B. Discussion

The developmental study will be discussed mainly in relation to the results reported by Miller (101) in studies of the time of appearance of species-specific antigens in offspring of the hybrid Columba livia x Columba guinea crossed inter se or to livia.

Miller found that antigens specific to guinea were detectable as soon as sufficient blood could be obtained for testing, at about 72 hours incubation of the egg. The blood of some embryos lacked some of the antigens, but their parents could be shown to be heterozygous for the genes producing the antigens in question. In general, all the antigens could be found on embryonic cells, but the reactions were weak. As older embryos were typed, it was found that the reactions approached the agglutination strength and titer characteristic of adult cells. Cells from A- and F-positive newly-hatched squabs tended to be weaker in agglutination and titer than either embryonic or adult cells.

Antigens distinguishing individuals within a species have been demonstrated to be present on erythrocytes early in embryonic life in humans (102), rabbits (103) and chickens (104). Briles (104) also discovered that certain other chicken antigens developed only some days after hatching, and came to full expression slowly. In contrast,

Andresen (13) found that the Lewis factor (Le^a) was present on the cells of a greater percentage of human infants than adults, indicating that the antigenic specificity was lost during the postnatal development of some individuals.

The studies described above, except Andresen's, were performed with antisera produced by immunization with adult cells. The Le^a antibody was found in a case of hemolytic disease. When fetal or cord bloods were used to immunize rabbits (106), the resulting reagents selectively agglutinated all other cord bloods and blood from several infants below one month of age. Owen (22) has found that chick cells retain activity toward a rabbit anti-chick embryo red blood cell reagent for as long as six weeks. These contrasting phenomena, the disappearance of embryonic sites and the appearance of adult antigenic sites, are analogous to the observations made in this study on the development of antigen A. However, Reagent A was produced by the immunization of a rabbit with adult cells. The anti-embryo cell fraction of the reagent could have been formed as a response to immunization or it may have been merely a normal component of the rabbit serum. It is known that human adults may have fetal hemoglobin inside their red blood cells (see (44) for a review of the different hemoglobins) and the present study suggests that antigenic sites common to embryo and adult may exist on the surface of the adult red blood cells.

No correlation is evident between the time of appearance of an antigen and its species-specific or intra-specific nature.

X. GENERAL DISCUSSION

Throughout the course of these discussions, we have found it necessary to point out repeatedly the uncertainty attached to many of the interpretations, because of difficulties encountered in the typing studies and anomalies noted in the absorption analyses. Absorption anomalies were believed to result from the multiple specificities present in the reagents, but no explanation was given for the fact that typing reactions were sometimes weak and irregular.

In all standard serological work, it is the practice to use only "good" reagents whenever possible; a "good" reagent is one which gives clear positives and negatives and gives a positive reaction with the proper cells at a reasonably high dilution. If such a serum does not exist for the genetic analysis of a particular antigen, repeated attempts are made to procure it, and attempts are made to improve the testing techniques in order to obtain unambiguous results. Andresen states, in "The Human Blood Groups", that only sera showing a strong reaction should be selected as testing fluids, and even then in many cases it is advisable to use two sera for testing. Other factors which may interfere in the reading of a given test include such diverse variables as room temperature, small amounts of metal in the distilled water used to make the saline solution, and the existence of grades of reactivity in different cells. For a full description of the procedures necessary for improving the accuracy of readings and for preparing good antisera, see Wiener (3). Andresen (107) describes, from a medico-legal viewpoint, the errors which may appear when typing for each of the human blood groups.

In the present study, we could not undertake analysis of as many sera as might be needed in order to produce a battery of uniformly good reagents. The few reagents that could be made had to be used. Nevertheless, the antigens described must be real and the systems must exist, for the reagents were duplicated; Reagent A was duplicated by Reagent I; B, D and G were instances of the same or very similar reagents; and E and F were identical. It is equally clear that the isoimmune antisera detected real antigenic differences within the species.

As a result of the findings of this study, it can be unequivocally stated that intra-specific erythrocyte antigen differences exist in pigeons.

Rabbit anti-pigeon red blood cell sera have demonstrated differences in the reactivities of the bloods of different birds, and, although iso-agglutinins could not be found in the normal sera of pigeons, immune antibodies were produced in certain pigeons in response to injection with the cells of other pigeons. The differences detected by several of the typing fluids are inherited in a regular Mendelian manner, the positive reaction always dominant to absence of a reaction. The use of one reagent suggested that inherited quantitative differences occur among the cells of different pigeons. There were also indications that some of the genetic loci involved are complex.

References

1. Landsteiner, K., Zblt. für Bakt. Abt. 1, (1900) 1, 357.
2. Landsteiner, K., Wien. klin. Wschr., (1901) 14, 1132. Cited by Race and Sanger (4).
3. Wiener, A. S., Blood Groups and Transfusion. Third Edition. 1943. C. C. Thomas. Springfield, Ill. 438 pp.
4. Race, R. R., and R. Sanger, Blood Groups in Man. 1950. C. C. Thomas. Springfield, Ill. 290 pp.
5. Stormont, C., Genetics, (1950) 35, 76.
6. Stormont, C., R. D. Owen, and M. R. Irwin, Genetics, (1951) 36, 134.
7. Briles, W. E., W. H. McGibbon, and M. R. Irwin, Genetics (1950) 35, 633.
8. Hamilton, A. S., Am. J. Phys., (1948) 154, 525.
9. Christian, R. M., D. M. Ervin, and L. E. Young, J. Immunol., (1951) 66, 37.
10. Burhoe, S. O., Proc. Nat. Acad. Sci., (1947) 33, 102.
11. Owen, R. D., Genetics, (1948) 33, 623.
12. Stormont, C., Genetics, (1951) 36, 577.
13. Andresen, P. H., Acta Path. et Micro. Scand., (1947) 24, 616.
14. Landsteiner, K., and J. Van der Scheer, J. Immunol., (1924) 9, 213.
15. Landsteiner, K., and J. Van der Scheer, J. Immunol., (1924) 9, 221.
16. Landsteiner, K., Proc. Soc. Expt'l. Biol. and Med., (1931) 28, 981.
17. Irwin, M. R., Proc. Soc. Expt'l. Biol. and Med., (1932) 29, 850.
18. Irwin, M. R., and L. J. Cole, J. Exp. Zool., (1936) 73, 309.
19. McGibbon, W. H., Genetics, (1944) 29, 407.
20. McGibbon, W. H., Genetics, (1945) 30, 252.
21. Burnet, F. M., and F. Fenner, Heredity (1948) 2, 289.

22. Owen, R. D., Personal communication.

23. Irwin, M. R., Genetics and Immunology, chapter in Genetics in the 20th Century. ed. L. C. Dunn. 1951. The MacMillan Company. New York. 634 pp.

24. Irwin, M. R., Evolution, (1953) 7, 31.

25. Wolfe, H. R., J. Immunol., (1942) 44, 135.

26. Hollander, W. F., J. Heredity, (1948) 39, 289.

27. Race, R. R., R. Sanger, and S. D. Lawler, Heredity, (1948) 2, 237.

28. Landsteiner, K., and P. Levine, Proc. Soc. Expt'l. Biol. and Med., (1927) 24, 600.

29. Landsteiner, K., and P. Levine, Proc. Soc. Expt'l. Biol. and Med., (1927) 24, 941.

30. Landsteiner, K., and P. Levine, J. Exp. Med., (1928) 48, 731.

31. Henningsen, K., Acta Path. et Micro. Scand., (1949) 26, 639.

32. Landsteiner, K., and P. Levine, J. Expt. Med., (1928) 47, 757.

33. Stormont, C., Personal communication.

34. Mourant, A. E., Nature, (1945) 155, 542.

35. Race, R. R., and G. L. Taylor, Nature, (1943) 152, 300.

36. Callender, S. T., and R. R. Race, Ann. Eug., (1946) 13, 102.

37. Sanger, R., R. R. Race, R. E. Rosenfield, P. Vogel, and N. Gibbel, Proc. Nat. Acad. Sci., (1953) 39, 824.

38. Race, R. R., R. Sanger, and D. Lehane, Ann.Eug. (1953) 17, 255.

39. Stormont, C., Genetics., (1952) 37, 39.

40. Olson, C. Jr., J. Immunol., (1943) 47, 149.

41. Wiener, A. S., Personal communication to R. D. Owen.

42. Wiener, A. S., and I. B. Wexler, Bact. Rev., (1952) 16, 69.

43. Pauling, L., H. A. Itano, S. J. Singer, and I. C. Wells, Science, (1949) 110, 543.

44. Itano, H. A., Science, (1953) 117, 89.

45. Itano, H. A., Am. J. Human Gen., (1953) 5, 34.

46. Landman, O. E., and D. M. Bonner, Arch. Biochem. and Biophys. (1952) 41, 253.

47. Buchbinder, L., J. Immunol., (1934) 26, 215.

48. Lattes, A., and A. Cavazzuti, J. Immunol., (1924) 9, 407.

49. Landsteiner, K., and D. H. Witt, J. Immunol., (1926) 11, 221.

50. Landsteiner, K., and P. Levine, J. Immunol., (1929) 17, 1.

51. Olbrich, S., and E. Walther, Ztschr. für Immunitäts., (1941) 99, 194.

52. Race, R. R., R. Sanger, and S. D. Lawler, Ann. Eug., (1948) 14, 171.

53. Landsteiner, K., The Specificity of Serological Reactions. Revised Edition. 1945. C. C. Thomas. Springfield, Ill. 310 pp.

54. Henningsen, K., Acta Path. et Micro. Scand., (1949) 26, 769.

55. Fisher, R., Heredity, (1953) 7, 81.

56. Landsteiner, K., and P. Levine, J. Immunol., (1930) 18, 87.

57. Irwin, M. R., and R. W. Cumley, Am. Nat., (1940) 74, 222.

58. Irwin, M. R., Biol. Rev., (1946) 21, 93.

59. Irwin, M. R., Quart. Rev. of Biol., (1949) 24, 109.

60. Irwin, M. R., and L. J. Cole, J. Exp. Zool., (1936) 73, 85.

61. Irwin, M. R., L. J. Cole, and C. D. Gordon, J. Expt. Zool., (1936) 73, 285.

62. Irwin, M. R., and R. W. Cumley, Genetics, (1945) 30, 363.

63. Fox, A. S., Genetics, (1949) 34, 647.

64. Fox, A. S., and T. B. White, Genetics, (1953) 38, 152.

65. Kabat, E. A., and M. M. Mayer, Experimental Immunochemistry. 1948. C. C. Thomas. Springfield, Ill. 567 pp.

66. Boyd, W. C., Fundamentals of Immunology. Second Edition. 1947. Interscience Publishers. New York. 503 pp.

67. Sokolowskaja, I. I., Bull. Acad. Sci. URSS Ser. Biol. (1936) pp 465-489. Cited by Irwin (23).

68. Andresen, P. H., Acta Path. et Micro. Scand., (1947) 24, 539.

69. Bryan, C. R., and W. J. Miller, Proc. Soc. Nat. Acad. Sci., (1953) 39, 412.

70. Miller, W. J., and C. R. Bryan, Proc. Soc. Nat. Acad. Sci., (1953) 39, 407.

71. Lawler, S. D., D. Bertinshaw, R. Sanger, and R. R. Race, Ann. Eug. (1950) 15, 258.

72. Lawler, S. D., and J. J. van Loghem, Lancet, (1947) 253, 545.

73. Stratton, F., Nature, (1946) 158, 25.

74. Race, R. R., R. Sanger, and S. D. Lawler, Nature, (1948) 162, 292.

75. Stratton, F., and P. H. Renton, Nature, (1948) 162, 293.

76. Renton, P. H., and F. Stratton, Ann. Eug. (1950) 15, 189.

77. Argall, C. I., J. M. Ball, and E. Trentelman, J. Lab. and Clin. Med. (1953) 41, 895.

78. Friedenreich, V., and A. Lauridsen, Acta Path. et Micro. Scand. (1938) Supp. 38, 155.

79. Jakobowicz, R. L., L. M. Bryce, and R. T. Simmons, Nature (1950) 165, 158.

80. Crome, W., Dtsch. Ztschr. gerichtl. Med. (1935) 24, 167. Cited by Race and Sanger (4).

81. Friedenreich, V., Dtsch. Ztschr. gerichtl. Med., (1936) 25, 358. Cited by Race and Sanger (4).

82. Dodd, B. E., Brit. J. Exp. Path., (1952) 33, 1.

83. Bird, G. W. G., Brit. J. Exp. Path., (1953) 34, 131.

84. Bird, G. W. G., Nature, (1953) 171, 748.

85. Owen, R. D., In press.

86. Levi, W. M. L., The Pigeon. Second Edition. 1945. R. L. Bryan. Columbia, S. C. 512 pp.

87. Sawin, P. B., M. A. Griffith, and C. A. Stuart, Proc. Nat. Acad. Sci., (1944) 30, 217.

88. Makino, S., An Atlas of the Chromosome Numbers in Animals. Second Edition (First American Edition) 1951. Iowa State College Press. Ames, Iowa. 290 pp.

89. Race, R. R., Nature, (1944) 153, 771.

90. Wiener, A. S., and K. Landsteiner, Proc. Soc. Exp. Biol. N. Y. (1943) 53, 167.

91. Walsh, R. J., and C. Montgomery, Nature, (1947) 160, 504.

92. Sanger, R., R. R. Race, R. J. Walsh and C. Montgomery, Heredity, (1948) 2, 131.

93. Sanger, R., Nature, (1950) 165, 939.

94. Levine, P., A. B. Kuhmichel, M. Wigod, and E. Koch, Proc. Soc. Expt'l. Biol. and Med., (1951) 78, 218.

95. Wiener, A. S., Am. J. Human Gen., (1952) 4, 37.

96. Landsteiner, K., W. R. Strutton, and M. W. Chase, J. Immunol., (1934) 27, 469.

97. Chalmers, J. N., E. A. Ikin, and A. E. Mourant, Brit. Med. J., (1953) No. 4829, 175.

98. Burnet, F. M., and J. D. Stone, Austr. J. Exp. Biol. and Med. Sci. (1946) 24, 1.

99. Stone, J. D., Austr. J. Exp. Biol. and Med. Sci., (1946) 24, 191.

100. Stone, J. D., Austr. J. Exp. Biol. and Med. Sci. (1946) 24, 197.

101. Miller, W. J., Phys. Zool., (1953) 26, 124.

102. Bornstein, S., and M. Israel, Proc. Soc. Expt'l. Biol. and Med., (1942) 49, 718.

103. Keeler, C. E., and W. E. Castle, J. Heredity (1934) 25, 433.

104. Briles, W E., W. H. McGibbon, and M. R. Irwin, Genetics (1947) 33, 97.

105. Ikin, E. A., H. Lehmann, and A. E. Mourant, Brit. Med. J. (1953) No. 4836, 602.

106. Andresen, P. H. The Human Blood Groups. 1952. C. C. Thomas. Springfield, Ill. 114 pp.