INHERITANCE OF ANTIBODY RESPONSE

I. SHEEP RED CELLS

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Summary

The heritability of antibody response to sheep red cells (S.R.C.) was found to be $0{\cdot}2836 \pm 0{\cdot}1475$ at 3 days and $0{\cdot}1097 \pm 0{\cdot}1165$ at 6 days. These estimates are subject to environmental variation of measurement which was found subsequently but could not be corrected for. An example of non-genetic, non-environmental error of measurement is illustrated experimentally and the consequences of this type of error are discussed.

I. INTRODUCTION

An approach to the study of the inheritance of the amount of antibody produced in response to injections of antigens has been made by Sang and Sobey (1954) and Sobey (1954). Where an apparent single antibody response to tobacco mosaic virus (T.M.V.) was measured in rabbits, the ability to produce antibodies in different amounts was found to be highly heritable ($h^2 = 0{\cdot}87 \pm 0{\cdot}09$), but the response to bovine plasma albumen (B.P.A.), a complex antigen demonstrated to be producing several antibody responses, showed no heritability ($h^2 = 0{\cdot}09$). As the heritability of a character is an estimate of the fraction of all the variance of that character which is due to genetic differences, a smaller heritability could be due either to a lower genetic variance or to a greater non-genetic variance, or to both. Measurements of response to T.M.V. and to B.P.A. were made in each case with samples of the same serum from the same bleeding, thus one would not expect any differences in environmental variation. Why, then, should the heritability of response to B.P.A. be so much lower than that to T.M.V.?

The response to T.M.V. was measured by the "equivalence zone" technique which involves testing the supernatants for the presence of antigen and antibody after precipitation is complete. Where more than one antibody is concerned in the reaction, the equivalence zone is likely to be absent when the different responses are markedly dissimilar, or wide where they are less dissimilar. Both these phenomena were occasionally encountered and the response determined by interpolation (Sobey 1954), indicating that all animals do not react similarly to T.M.V. and in some cases appeared to produce more than one antibody response. Their occurrence was, however, too infrequent to mask the high heritability to T.M.V.

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The response to B.P.A. was measured by the "optimal proportions" method, and where measurements involving more than one antibody response are made using this technique, it seems quite probable that the index of response, i.e. the first tube to flocculate, will be of the antibody in highest concentration whose optimal coincides with that antigen-antibody ratio. With multiple antibody responses one might, *a priori*, expect more than one optimal point in a series of antigen dilutions. These were rarely found, but the arguments put forward by Elek (1949) suggest that they are not necessarily an invariable occurrence. Further, if flocculation is a second stage of the precipitin reaction, and less specific than the first stage of actual combination of antigen and antibody (Boyd 1947; Topley and Wilson 1948), initiation of flocculation by the antibody in highest concentration could quite conceivably mask any secondary optima. This inadequacy of the measure of response could result in an apparent lack of genetic variability. It is impossible to determine which antibody response among those present is being measured in any one animal, and this could result in a similar measure of response being determined for different antibodies in different animals.

The haemolytic titre, which is used in the work to be described, is probably open to limitations similar to those envisaged for the optimal proportions technique, where more than one antibody response is concerned. By serial dilution of the antiserum, the antibodies with lower responses will quickly become too low in concentration to contribute towards haemolysis, and the end-point giving the measure of response will be that of the antibody which is in highest concentration. On this view, the different antibody responses are very unlikely to be additive in respect of the end-point. The consequences of this, as with the optimal proportions method, would be that in no instance would total antibody response be measured. The measure would actually be that of the antibody in highest concentration, and since the same antibody would not necessarily be present in greatest amount in all individuals, it would not measure the variation of a single specific antibody response either. This could result in an apparent lack of genetic variability.

In order to check this point, experiments were designed to measure the heritability on a second complex antigen and to examine possible reasons for low heritability, if this were found.

II. Experiment 1

(*a*) *Materials*

In all, 120 pairs of albino mice were chosen at random from the second generation of a line of mice selected for sensitivity to oestrogen. These constituted the parents and varied between 19 and 30 weeks in age with an average of 26 weeks at the time of testing. Of these, 114 pairs produced litters, and four offspring, two of each sex where possible, were kept from each litter at the time of weaning. The offspring varied between 19 and 25 weeks of age with an average of 22 weeks at the time of testing. The antigen selected was sheep erythrocytes (S.R.C.) and all the S.R.C. used were obtained from the same sheep.

(*b*) *Methods*

Sheep blood was collected from the jugular vein into dextrose-citrate (3 per cent. disodium citrate, 3 per cent. dextrose in distilled water, 1·5 ml to 10 ml blood), stored at 4°C, and always used within 6 days of collection. Before use, S.R.C. were washed three times and suspended in Ca-Mg saline (Mayer *et al.* 1946).

S.R.C. was injected into a tail vein using a ½-in., 26 gauge needle and a tuberculin syringe. All injections were of 0·5 c.c. The method of bleeding was adapted from one devised by Dr. J. Bullen of Cambridge (personal communication). Prior to bleeding, the body temperature of the mice was raised until their tail veins became distended. They were then lightly anaesthetized and the tip of the tail (about 1 cm for mice not previously bled, and 2 or 3 mm for others) amputated with a pair of sharp scissors. The stump was quickly lowered into a labelled Wasserman tube contained in a larger glass tube under a negative pressure of 10 cm Hg. Tubes containing blood were allowed to stand at room temperature overnight, the serum pipetted into clean tubes, and centrifuged to remove any residual cells, pipetted into storage tubes, and stored at −20°C. This method allows mice to be bled without mortality at the rate of one every 2 min, with an average yield of 0·25 ml serum per bleeding.

Guinea pig serum was used throughout as a source of complement. Guinea pigs were bled by cardiac puncture, the blood allowed to clot at room temperature over 3 hr, and the serum removed, centrifuged, and stored in 2-ml amounts at −20°C. Complement was made up of the pooled serum from 20 male animals selected by previous testing for their capacity to yield high-titre complement. Three M.H.D. were used in all antibody titrations. Complement was never stored for periods exceeding 4 weeks and repeated titrations showed no decrease in titre during this period. Material thawed but not used on the day of test was discarded.

Serum was inactivated at 56°C for ½ hr on the day of test. Using a pipette drawn to deliver 30 drops per ml, two drops of serum were serially diluted in two-fold steps in precipitation tubes containing two drops of Ca-Mg saline. One drop of complement and one drop of 3 per cent. S.R.C. were then added, and the tubes incubated in a water-bath at 37°C for ½ hr, then placed in the refrigerator at 4°C overnight, and read. Antibody response was measured as the log of the haemolytic titre, obtained by taking the tube numbers as response values (cf. Carlinfanti 1948). The following classes of response were recorded: 5, complete haemolysis; 0, no haemolysis; and the intermediate classes 4, 3, 2, and 1 were graded according to the degree of haemolysis in the tube and the cell deposit at the bottom of the tube. Each response was plotted on a separate card, and the 50 per cent. end-point determined by drawing a regression line through the end-points from the last 5 value to zero. Where, in very low responses, no 5 value was recorded, the regression line was interpolated back to the 50 per cent. end-point. Response values intermediate between whole numbers were taken to the nearest 0·25 of a unit.

Naturally occurring agglutinins to S.R.C. have been found in mice by Lewis and Loomis (1928) and Stern and Davidshon (1954) but no reports of natur-

ally occurring haemolysins have come to our observation. To investigate their possible presence in our mouse stocks, 80 mice were bled prior to injection and their serum tested by the routine method. None were found.

TABLE 1

DIFFERENCES IN RESPONSE DUE TO SEX

Mean Response at	Parents				Offspring			
	♀♀	♂♂	D.F.	t	♀♀	♂♂	D.F.	t
3 days	2·203	1·732	226	1·96*	3·457	0·895	356	12·38***
6 days	8·000	7·255	211	3·49***	8·217	7·296	331	3·44***

* $P<0·05$.
*** $P<0·001$.

In order to determine the experimental error of the measurement of haemolytic titre, 57 mice were injected with 0·25 per cent. S.R.C. given in a single dose of 0·5 ml. The mice were bled 6 days later and their sera tested in duplicate for antibody response. The response value in each tube was determined by one operator and recorded by another; a first reading of each response was made prior to the duplicate reading, so that in no case were two readings on any one serum made consecutively. The repeatability was determined by a

TABLE 2

EFFECT OF AGE ON RESPONSE

Response to Age	Parents		Offspring	
	♀♀	♂♂	♀♀	♂♂
Mean age in weeks	25·83	26·34	22·78	19·71
Regression of response on age				
3 days	−0·259±0·023	−0·019±0·064	−0·928±0·24	0·085±0·17
6 days	−0·1667±0·02	−0·034±0·02	−0·485±0·18	0·914±0·33

correlation of the duplicate estimates. This gave a value of $r = 0·99 \pm 0·13$. In view of this high degree of repeatability, single measurements were considered adequate.

In order to choose the best time for injection to bleed the mice, time-response curves, using a selected dose of 0·01 per cent. S.R.C. given in a single intravenous injection, were determined. The time response of 20 animals to this dose is shown in Figure 1. From these data it was decided to bleed at 3 and 6 days.

The frequency distribution of response for 3- and 6-day responses for both sexes is given in Figure 2. The 6-day responses are normally distributed; the distributions of the 3-day responses have an apparent bi-modality owing to the large number of animals which failed to respond.

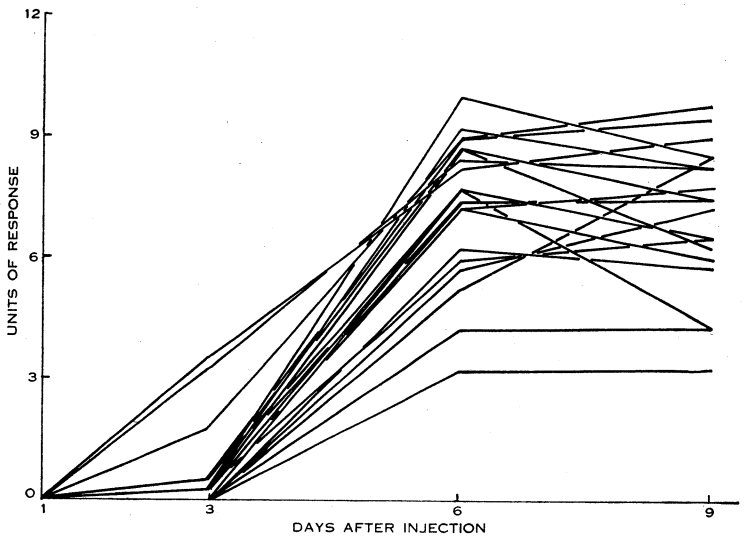

Fig. 1.—Time response curves of 20 mice to a single injection of 0·5 ml of 0·01 per cent. S.R.C.

(*c*) *Results*

Before combining all the data it was thought advisable to examine the effect of sex and age on the response of individuals, as the mice used differed considerably in age, and response was measured in both sexes. The difference in response of the two sexes is shown in Table 1 and is marked at both 3 and 6 days. Table 2 shows that the effect of age on response in the parents is marked in females at both 3 and 6 days, the degree of response falling off with increasing age. The effect of age in the males is negligible. In the offspring, which were younger when tested, the effect in females is apparently much stronger, but in males there is an increase in response with increasing age at both 3 and 6 days. It is possible that the ability to respond reaches a peak and falls to an asymptote, the peak being reached much earlier in females. The data examined here are not adequate to decide this point. A similar sex difference of response was found by Gorer and Schutze (1938) to the antigens of *Salmonella typhimurium* and *S. enteritides.*

III. Experiment 2

(*a*) *Measurement of Heritability*

In Table 3 the mean responses of parents and offspring are shown. As would be expected the female offspring which were younger than their female parents when tested have a somewhat higher response at 3 days and a slightly

higher response at 6 days. The males which were also younger than their male parents had a markedly lower response at 3 days and about the same at 6, suggesting that the rather low regression of response on age found at 3 days is more correct than the higher one at 6 days which in any event has a high standard error.

The responses were corrected to constant age and sex and regressions of offspring on mid-parent were calculated using corrected figures. These regressions were $0{\cdot}2836 \pm 0{\cdot}1475$ at 3 days, and $0{\cdot}1097 \pm 0{\cdot}1165$ at 6 days, giving a rather low heritability at both times of sampling. The correlation between the response of a mouse at 3 days and its response at 6 days was found to be $r = 0{\cdot}565 \pm 0{\cdot}068$. The heritability of response to S.R.C. is intermediate between that found for response to T.M.V. ($h^2 = 0{\cdot}87$) and B.P.A. ($h^2 = 0{\cdot}09$), being nearer that of B.P.A.

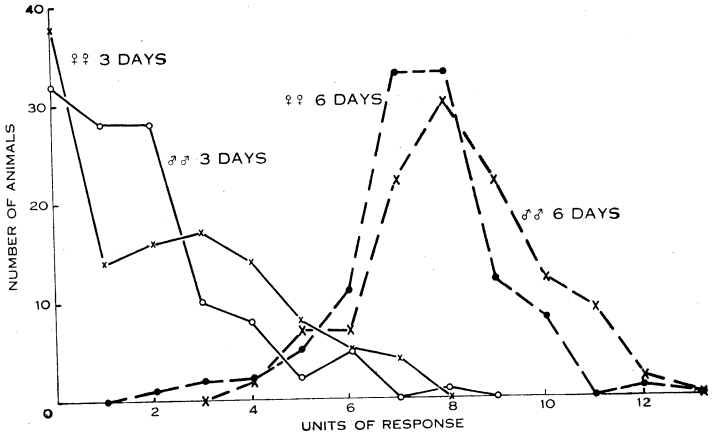

Fig. 2.—Frequency distributions of 3- and 6-day responses of male and female mice to 0·01 per cent. S.R.C. given intravenously.

Besides the inadequacies of measurement discussed in the introduction which, if real, would mask the genetic variation, there are sources of environmental variation affecting measurement which, while not affecting the genetic variation *per se* will increase the non-genetic variation and the error, giving a lower measure of heritability. Of such sources of variation in the present study, those considered of possible importance were measurements made on different days, and the use of S.R.C. of different ages. To determine the significance of the variability introduced by these factors the following factorial experiment was designed. The use of S.R.C. from different sheep was also included although it was not a source of variation in the work just described.

(*b*) *Materials*

More anti-serum was required than could be obtained from mice, and for convenience rabbit anti-S.R.C. serum was used. Three rabbits were each given two intravenous injections of 1 c.c. of 1 per cent. S.R.C. spaced by 7 days. They

were bled from the marginal ear vein 7 days after the last injection. After clotting at room temperature for 12 hr the serum was pipetted off, centrifuged, and stored at – 20°C.

S.R.C. were obtained from four different sheep. For the purpose of the factorial design of the experiment, the timing of bleeding was so arranged that on two different days, blood of the ages 0, 2, 4, and 6 days would be available from each sheep. Guinea pig serum was used throughout as a source of complement.

TABLE 3

MEAN RESPONSES OF PARENTS AND OFFSPRING

Mean response at	Parent ♀♀	Offspring ♀♀	D.F.	*t*
3 days	2·203	3·457	286	4·813***
6 days	8·000	8·217	268	0·998
	Parent ♂♂	Offspring ♂♂		
3 days	1·732	0·895	296	4·33***
6 days	7·255	7·296	272	0·163

*** $P<0·001$.

(*c*) *Methods*

The serum from each rabbit was divided into 32 samples and each sample allocated a random number from Fisher and Yates' tables (Fisher and Yates 1953). The samples from each animal were then randomly divided into eight groups of four samples. Four groups of samples were randomly allotted to each of the two different days on which testing was to be made. On each day the four groups of samples were allocated at random to the four different sheep, and the samples in each group were similarly allocated to the four groups of cells of different ages, against which they were to be tested; so that on each of 2 days the serum from three rabbits was tested against red cells of 0, 2, 4, and 6 days of age from four different sheep. The analysis of variance of this factorial experiment is given in Table 4.

(*d*) *Results*

As shown in Table 4 the main factors, other than differences between rabbits which introduce significant variation are differences in age of S.R.C., and unspecified differences associated with the day on which a test was made. First order interactions between rabbits and days, and between sheep and S.R.C. of different ages, also introduce a statistically significant variation. The former, while not of any great importance in the present analysis, would have a more marked effect where more rabbits were being compared over a wider range of days. The interaction between sheep and S.R.C. of different ages is shown

in Figure 3. Although response increases with increased age of S.R.C. with a linear trend, the S.R.C. from different sheep show considerable heterogeneity.

The data on which the heritability was estimated were, unfortunately, not suitably recorded to allow a re-estimate of heritability in the light of these findings. It is considered very unlikely, even had this been possible, that the estimate would have reached $h^2 = 0{\cdot}5$. This is still much lower than that found to T.M.V. and it was decided to examine a multiple antibody system of known constitution in relation to quantitative measurements of response. To this end the following experiment was designed.

IV. Experiment 3

(*a*) *Materials*

Human red cells of groups A and B together with two sources of cells of group AB, referred to as AB_1 and AB_2, and two sources of anti-A (a_1, a_2) and anti-B (b_1, b_2) were kindly supplied by Dr. R. J. Walsh of the Red Cross Blood Transfusion Centre.

Table 4

ANALYSIS OF VARIANCE OF FACTORS AFFECTING THE MEASUREMENT OF HAEMOLYTIC TITRE

Source of Variation	D.F.	Mean Square
Rabbits (*R*)	2	227·14****
Sheep (*S*)	3	0·42
Ages of S.R.C. (*A*)	(3)	
Linear	1	8·81**
Quadratic	1	0·40
Cubic	1	0·35
Days (*D*)	1	1·87**
First order interactions		
$R \times S$	6	0·09
$R \times A$	6	0·05
$R \times D$	2	1·23*
$S \times A$	9	1·37***
$S \times D$	3	0·07
$A \times D$	3	0·14
Error	57	0·26

* $P = 0{\cdot}05$-$0{\cdot}01$. *** $P < 0{\cdot}001$.
** $P = 0{\cdot}01$-$0{\cdot}001$. **** $P << 0{\cdot}001$.

(*b*) *Methods*

a_1 and b_1 were mixed and are referred to as ab_1, and similarly a_2 and b_2 were mixed and referred to as ab_2. ab_1 and ab_2 were then each divided into three volumes; the first was left unabsorbed, the second absorbed with cells of group B, and the third absorbed with cells of group A. Each of these six groups of antiserum was then made up in four strengths, 1 (undiluted), ½, ¼,

and ⅛. Each of these 24 lots of antisera was then tested for its ability to agglutinate both AB_1 and AB_2 cells. The whole process was then repeated making a total of 96 agglutination estimations.

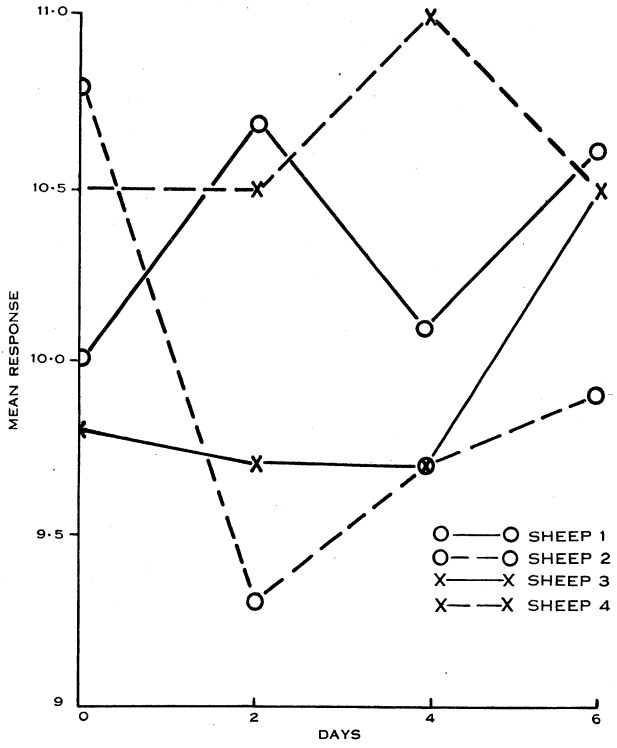

Fig. 3.—First order interaction showing the heterogeneity of mean responses to S.R.C. of different ages from different sheep.

The titre was converted to a log scale by reading tube numbers as response values. Four grades of agglutination were scored, namely 3 complete agglutination, 0 no agglutination, and 2 and 1 intermediate degrees of agglutination. The 50 per cent. end-points were determined by a discriminant analysis (Claringbold, Sobey, and Adams, unpublished data). The analysis of variance, using these end-points, is given in Table 5.

(*c*) *Results*

The titres of the ab_1 and ab_2 sera are significantly different, as would be expected, but their analogous behaviour in respect to treatment is borne out by the absence of significant interactions. The titre of a_1 was higher than b_1 and that of a_2 higher than b_2; as both differences are of a similar order, the data can be pooled. Hereafter, and in Table 5, the mean titres of b_1 and b_2, a_1 and a_2, ab_1 and ab_2 are referred to as *b*, *a*, and *ab* respectively. When *ab* serum

is absorbed with B cells the *a* antiserum left has a titre which is exactly the same as that of unabsorbed serum. On the other hand, when *ab* serum is absorbed with A cells the remaining *b* antiserum has a titre well below that of whole serum. (Fig. 4 and Table 5.) This result was found at all dilutions and shows that the titre of *a* antiserum has been measured throughout and the presence of *b* has gone undetected.

These data illustrate that with a mixture of two antibodies the titre of the mixture as measured by agglutination is the same as the titre of the component antibody of highest titre.

TABLE 5

ANALYSIS OF VARIANCE

50 per cent. end point titre

Source of Variance	D.F.	Mean Square	*F*
Biological			
Source of *ab*	1	1·17	23**
Source of AB	1	0·03	<1
Treatments	(2)		
$ab \times a$	1	0·03	<1
$ab + a \times b$	1	44·56	873***
Interactions as error	7	0·051	
Experimental			
Replication	1	0·08	<1
Dilutions	(3)		
Linear	1	86·02	331***
Quadratic	1	0·00	
Cubic	1	0·51	2·0
First order interactions	19	0·23	<1
Experimental error	61	0·260	

** $P = 0{\cdot}01 - 0{\cdot}001$.

*** $P < 0{\cdot}001$.

V. DISCUSSION

The heritability of response to S.R.C. is intermediate between that found for response to T.M.V. and B.P.A., being probably nearer that of B.P.A. (Sang and Sobey 1954). It is difficult to see why response to T.M.V. should be so much more highly heritable than to the other two antigens. The experiments carried out to investigate the extent of environmental variation did not indicate that this variation would affect heritability to a significant extent. It is always possible that the genetic variation of response to T.M.V., which appears to be a single response under the conditions used (Sang and Sobey 1954), is, in fact, greater than the genetic variation of response to B.P.A. and S.R.C. However, an obvious cause of discrepancy is the method of measuring response, which the test carried out using human red cells shows may be quite inadequate, measuring only the concentration of a single antibody in a mixture, the measurement

being always that of the antibody in greatest concentration. It would appear, therefore, that the methods used to measure antibody response are unable to detect either the total response of a system of multiple antibodies, or the variation of any one component antibody present in the system. Where a single antibody response is concerned, as in T.M.V., they would appear to be adequate.

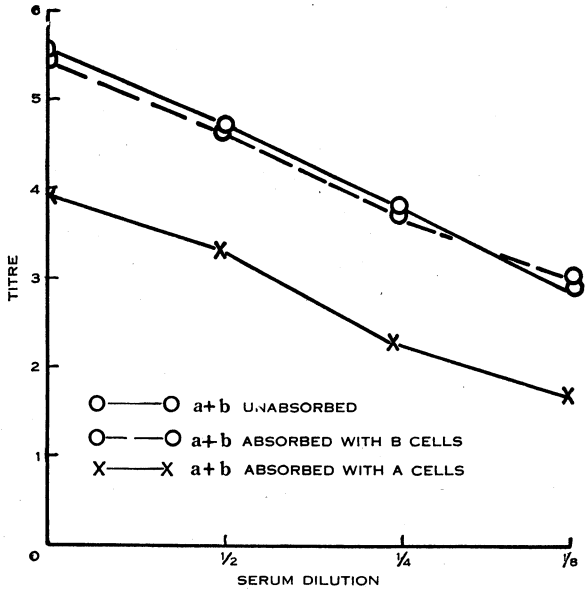

Fig. 4.—Mean titres of absorbed and unabsorbed mixed antisera.

Davidshon and Stern (1954) found differences between inbred lines of mice in their response to S.R.C., but with considerable "within line" variability. This variability would not be expected if the explanation of low heritability of complex antigens put forward here is correct, but since Davidshon and Stern used mice of different age and sex without correction and red cells from different sheep, their results are not comparable with ours. In any event, recent findings by Gruneberg (1954), McLaren and Michie (1954), and Biggers and Claringbold (1954) indicate that the phenotype of inbred lines does not reflect their genetic uniformity.

In conclusion, it is suggested that the present work supports previous contentions that studies concerning the inheritance of antibody response require a detailed knowledge of the complexity of the antigens concerned, and some means of identifying and measuring the different individual antibody responses, or of measuring the total of all the responses present.

VI. Acknowledgment

We are indebted to Dr. P. J. Claringbold for statistical advice.

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