AN IMMUNOLOGICAL AND GENETIC ANALYSIS OF ANTIGEN 5 IN *PARAMECIUM AURELIA*, SYNGEN 2¹

JANE E. COOPER²

Biology Department, University of Pennsylvania, Philadelphia, 19104

Received December 26, 1967

SINCE the enunciation of the operon theory by JACOB and MONOD (cf. their review, 1961), a fascinating variety of models for regulation of gene action has been proposed. Although most of these were first applied to bacterial systems (cf. reviews by AMES and MARTIN 1964; and HOROWITZ and METZENBERG 1965), the models of induction and repression have been extended to maize (reviewed by McCLINTOCK 1965), *Neurospora* (e.g. HOROWITZ, *et al.* 1961) and perhaps *Drosophila* (LEWIS and LEWIS 1963).

The investigations reported here are concerned with the immunological relationships between members of the antigen 5 system in *Paramecium aurelia*, syngen 2, and with the control of the action of the alleles which determine these antigens. The regulatory mechanism contains at least two components: one apparently "turns off" sensitive alleles and is completely linked (and perhaps identical) to the structural locus; a second reduces the production of the antigens controlled by sensitive alleles, but is not linked to the structural locus.

Antigen 5 is a nonimmobilization antigen detected by precipitation in gel. It was found in the cilia of *Paramecia* by PREER (1959a) and was first isolated from syngen 4 animals by JONES (1964) using low pH, ammonium sulfate precipitation, and DEAE cellulose chromatography. She found it to be located primarily in the body fraction and secondarily in the cilia, using the methods of PREER and PREER (1959) to separate cilia and trichocysts from the bodies. JONES reported that antigen 5 is under the control of a single genetic locus, and that there are at least two different alleles present in the syngen 4 stocks investigated. JONES was not able to study the expression of antigen 5 in F₁ hybrids because syngen 4 animals go into autogamy spontaneously before enough fissions have occurred after conjugation to permit immunological assay (postautogamous animals are homozygous at all loci—cf. BEALE 1954).

JONES found that stock 47 produces an amount of the antigen varying from normal quantities to no detectable antigen (less than 10% of the normal amount). She has not been able to bring about consistent antigen 5 production in lines from this stock. However, later studies by PREER (personal communication) indicate that the "negative" lines from stock 47 actually produce a very

¹ This research was supported by graduate fellowships from the National Science Foundation to the author, and by the U.S. Public Health Service Cellular Physiology Training Grant, administered by the Biology Department of the University of Pennsylvania.

² Present address: Biology Department, The Pennsylvania State University, Delaware County Campus, Chester, Pennsylvania, 19013.

low concentration of antigen 5. The control mechanism involved in variable production of antigen 5 within a single stock is not understood at present.

Spectrophotometric studies by JONES (1964) indicate that antigen 5 is probably a protein. Sedimentation equilibrium studies by STEERS (personal communication) indicate a molecular weight of 32,000. Amino acid analyses indicate a minimum molecular weight of approximately 28,000, assuming one methionine residue per antigen molecule (STEERS, personal communication). These data imply that at least most of the antigen molecule is protein in nature, although the possibility remains that the molecule is a conjugated protein. These data also indicate that the molecule is a monomer. STEERS (personal communication) finds that reduction and alkylation of antigen 5 does not affect its molecular weight, so that there can be no disulfide-linked subunits in the molecule.

Our immunological survey of antigen 5 in syngen 2 stocks indicates a series of alleles at the antigen 5 structural locus. Genetic studies reveal an apparent "dominance" of the allele from stock 3 over several others. For example, the F_1 hybrids of a cross between stock 3 and stock 7 produce the stock 3 antigen 5 ($Ag5_3$) but no detectable antigen of the stock 7 variety ($Ag5_7$). If this phenomenon is not due to the structural gene itself, it must be the result of a regulator gene closely linked to the structural locus.

The second regulatory locus is not linked to the structural locus; it contains two alleles according to the data presented below. One allele reduces the amount of antigen 5 produced by sensitive systems and the other has no effect, i.e. antigen 5 is produced in normal amounts by all systems containing it.

MATERIALS AND METHODS

The syngen 2 stocks which have been used are listed with their source in Table 1. The standard techniques of handling *Paramecium* cultures have been described by SONNEBORN (1950). The culture fluid used in these experiments was an extract of ground desiccated grass (0.15%), buffered with 0.10% $Na_2HPO_4 \cdot 12H_2O$, inoculated one day before use with *Aerobacter aerogenes* (PREER and PREER 1959). Mass cultures were grown from isolated individuals as described by STEERS (1962), and the *Paramecia* were harvested using the method of PREER and PREER (1959).

Extraction and purification of antigen 5: The initial extraction procedure was that described by PREER (1959a) for the immobilization antigen. Packed *Paramecium* cells were resuspended in four times their volume of salt alcohol (one part 0.9% NaCl and one part 30% ethanol) and allowed to stand at 2°C for one hour. The supernatant fluid was removed and its pH adjusted to 2.0 by the addition of 0.2N HCl; denatured protein was removed by centrifugation. The supernatant fluid was neutralized with 0.2 N NaOH; denatured protein was again removed by centrifugation. The supernatant fluid, containing the antigen 5, as well as the immobilization antigen (antigen 4) (PREER and PREER 1959) and antigen 9 (JONES 1964) was retained.

Most of the antigen 4 was removed by adding an equal volume of saturated ammonium sulfate solution (adjusted to pH 7.5) to the extract (PREER 1959a). Essentially all of the antigen 5 and antigen 9 was precipitated between 60% and 90% saturation; this cut was made using solid ammonium sulfate (JONES 1964).

Antigen 5 was separated from antigen 9 and other impurities using DEAE cellulose chromatography as described in detail by JONES (1964). The proteins were eluted with an NaCl gradient; the eluate was collected in 3 ml amounts. The protein content of each fraction was approximated by determining the optical density at 280 m μ and 260 m μ (WARBURG and CHRISTIAN 1941). The separation of the two proteins was monitored with a technique of double

TABLE 1

Source of syngen 2 stocks used

Stock No.	Source
1	Strikersville, New York
3	Baltimore, Maryland
4	Counterfeiter's Lodge, New York
7	Pinehurst, North Carolina
8	Halethorpe, Maryland
9	Buffalo, New York
11	Johns Hopkins Univ., Botanical Gardens, Md.
12	Staunton, Virginia
13	Baltimore, Maryland
28	Woods Hole, Massachusetts
30	Coats Pond, Maryland
34	New Haven, Connecticut
35	Moscow, Indiana
53	Twin Lakes, near Bloomington, Indiana
197	Germany
B197	Trichocyst mutant of 197, isolated by G. H. Beale

diffusion in agar (see below) described by PREER and PREER (1959). Two sera were used: one specific for antigen 5, and a second serum which contained antibodies against antigen 5 and antigen 9. Only those fractions containing antigen 5 or antigen 9 alone were pooled and lyophilized. The purity of the antigen 5 thus isolated was corroborated by standard techniques of ultracentrifugation (SCHACHMAN 1957) and disc electrophoresis (ORNSTEIN and DAVIS 1962).

Preparation of antisera: Sera were prepared against purified antigen 5 from stocks 3, 7, 9, 11 and 28. The undialyzed eluate from DEAE chromatography contained 1.5–2.0 mg/ml of antigen 5 dissolved in 0.01M tris buffer (pH 7.3) and approximately 0.6M NaCl. 3 ml of this eluate were homogenized with 3 ml Freund's complete adjuvant. 1 ml doses of the homogenate were injected subcutaneously into an adult male albino rabbit at weekly intervals for six weeks. Ten days after the last injection the rabbit was bled from the marginal ear vein.

Stock-specific antisera were prepared by removing antibodies that reacted with antigen 5 stocks other than the one against which the serum had been prepared. The heterologous antigen was mixed with the serum and the mixture was allowed to stand overnight at 2°C. Antigen-antibody precipitate was removed by centrifugation at $25,000 \times g$ for three minutes.

Immunological technique: All immunological studies were performed by means of the technique of double diffusion in agar described by PREER and PREER (1959). The theory of this technique has been discussed in detail by PREER (1956) and by PREER and TELFER (1957). Antigen solutions and antisera on opposite sides of an agar plug (in a 1.7 mm I.D. capillary tube) diffuse toward each other and subsequently form a zone of precipitation. The position of the zone, or band, p , is equal to the distance from the antigen-agar interface to the band divided by the total length of the agar plug. Band position is determined in part by the ratio of antigen concentration to antibody concentration. When antigen and antibody concentrations are approximately in their equivalence ratio, the resulting band position, called the equivalence position, reflects the diffusion coefficient of the antigen molecule (NEFF and BECKER 1957). The position of the precipitin band in the agar is measured after a day with a binocular dissecting microscope equipped with an ocular micrometer, using a total magnification of 15–20 $\times$.

Assay of small cultures for type of antigen 5 present: Stocks were tested for presence or absence of antigen 5 which reacts with a serum (designated P247) made against the purified antigen 5 from stock 3 (Ag5₃). Ten lines from each stock were grown in about 150 ml of inoculated culture fluid in 250 ml Erlenmeyer flasks until the medium was "clear." Paramecia

were centrifuged in an oil testing centrifuge at $700 \times g$, using pearshaped 120 ml tubes whose "stem" capacity was 0.2 ml. Each flask yielded about 0.02 ml of packed cells. The *Paramecia* were resuspended in ten times their volume of salt alcohol and allowed to stand at 2°C for one hour. After centrifugation at $25,000 \times g$ for 3 minutes, the supernatant fluid was removed and set against P247 in gel diffusion tubes. Although the supernatants contain a number of different antigens, only a single zone of precipitation was formed because the serum was specific for antigen 5.

Lines resulting from crosses or autogamy were assayed with sera specific for each of the parental types of antigen. Serum concentration was adjusted so that the precipitin band, if formed, would be near the equivalence position ($p = 0.6$) for the antigen.

Genetic crosses: Sexually reactive animals were obtained by daily pouring the top inch of the 27°C tube cultures into a tube containing three volumes of fresh culture fluid (SONNEBORN 1950) until dense populations were obtained. These animals were then kept in the dark at 12°C for at least 24 hours. Complementary mating types were mixed and placed in the dark at 17°C ; 18–20 hrs later, conjugating pairs were washed and isolated. Exconjugants were isolated and maintained at 27°C as separate clones representing the F_1 generation.

Members of these F_1 lines were induced to undergo autogamy, using the methods outlined by SONNEBORN (1937). Postautogamous animals were isolated and maintained as separate lines.

RESULTS

Expression of antigen 5 in syngen 2 stocks: Sixteen syngen 2 stocks have been assayed for production of antigen capable of cross-reacting with a serum (P247) made against the purified antigen 5 from stock 3 (Ag5_3); the results are shown in Table 2. Formation and intensity of precipitin bands by extracts from ten lines of a stock are indicated by +, ++, or +++ (+++ is an opaque band; + is translucent and presumably represents a lower concentration of antigen-antibody complex). "Var" signifies that detectable precipitin bands (of the intensity indicated) were formed by some, but not all, of the extracts from lines from that stock—a phenomenon similar to that reported for stock 47 of syngen 4 by JONES (1964). If no detectable band was formed by any of the ten lines tested, it is indicated by "neg."

The fact that no cross reaction has been detected in stocks 12, 13, 35, 197 and B197 may mean either that no antigen 5 is formed, or that the antigen formed is so different (qualitatively and/or quantitatively) that no cross reaction is

TABLE 2
Production of antigen 5 (as detected by P247) in syngen 2 stocks

Stock	Reaction	Stock	Reaction
1	+++*	13	neg.
3	+++	28	+
4	+(var)	30	+++ (var)
7	++	34	+(var)
8	+++ (var)	35	neg.
9	+	53	++ (var)
11	+++	197	neg.
12	neg.	B197	neg.

* Precipitin band intensity indicated by +, ++ or +++ (see text).

visible. Later studies have shown that stock B197 produces a reduced amount of antigen 5—less than 20% of the amount produced by stock 3. The antigen can be detected in most lines from stock B197 by diluting P247 at least 1:4. Antigen 5 does not form a readily visible band at p values below 0.3 (when the antibody is in excess by a factor of about eight; each doubling of antibody concentration decreases p about 0.1). The fact that it is necessary to dilute serum P247 in order to detect $Ag5_{B197}$ could mean either: 1) that the antigen 5 produced is qualitatively different from $Ag5_3$, but is produced in normal amounts, or 2) that stock B197 actually produces less antigen 5. The latter is the case. Mass cultures of stock B197 produce about one-eighth as much antigen 5 per ml of packed cells (determined spectrophotometrically after column chromatography) as other stocks. In addition, when P247 is absorbed with enough $Ag5_{B197}$ to remove all antibodies against $Ag5_{B197}$, reactivity with the homologous antigen, $Ag5_3$, is also removed. In other words, $Ag5_{B197}$ molecules are capable of reacting with most, if not all, of the available precipitating antibody in P247.

Stock B197 apparently arose from stock 197 by means of a mutation at a single genetic site, the trichocyst mutation (PREER 1959b). This site is not linked to the antigen 5 locus (see DISCUSSION). Therefore, stocks 197 and B197 are presumably isogenic at all other loci, and it is probable that stock 197 also produces a reduced amount (hence undetectable with full-strength P247) of antigen 5, although no further studies have been made on this stock. Stocks 12, 13 and 35 have not been investigated beyond the preliminary assay with P247, so no definitive statement can be made regarding antigen 5 production in these stocks. In other words, these stocks may also produce an antigen so qualitatively and/or quantitatively different from $Ag5_3$ that no antigen could be detected with full-strength P247.

The studies with stock B197 indicate that syngen 2 stocks may be divided into at least two quantitatively distinct classes: 1) that in which the antigen, when present, is produced in the amounts found in stock 3 (taken as "normal")—including stocks 1, 4, 7, 8, 9, 11, 28, 30, 34 and 53; and 2) that in which a reduced amount is produced—including stocks B197 and 197, and possibly other "negative" stocks—12, 13 and 35. It is also possible that stocks 12, 13 and 35 are members of a third group in which no antigen 5 is synthesized.

Results of qualitative analyses of heterologous reactions between the antigen 5 from stocks 3, 11, 30, 7, 9 and 28 and sera other than P247 are presented in Table 3. These results indicate: 1) that the antigens from stocks 3 and 11 are very similar; 2) that $Ag5_{30}$ is more closely related to them than $Ag5_7$ is; and 3) that $Ag5_9$ and $Ag5_{28}$ are the least like $Ag5_3$ of the syngen 2 antigens studied with several sera. In addition, some cross reaction was seen between syngen 4 antigen 5 (from stocks 51 and 29.8) and sera against antigen 5 from syngen 2 stocks (see Table 3).

Table 4 presents a summary of the qualitative and quantitative classification of antigen 5 from the sixteen syngen 2 stocks investigated, based on similarity to the antigen 5 from stock 3. There are at least two quantitative classes and at least five qualitative differences in antigen 5 in syngen 2.

TABLE 3

Cross reactions of antigen 5 from stocks of syngens 2 and 4

Precipitating antigen source		Immunizing antigen source*					
Stock	Syngen	3 (P247)	1 (P248)	7 (P261)	9 (P250)	28 (P263)	51 (P236)†
3	2	<u>++++</u> ‡	+++	+++	+	+	neg
11	2	<u>+++</u>	<u>+++</u>	+++	+-	+-	neg
30	2	+++	+++	++	+-	+-	neg
7	2	++	+	<u>+++</u>	+	++	neg
9	2	+	+	<u>+-</u>	<u>++</u>	+-	neg
28	2	+-	+-	+	neg	<u>++</u>	neg
51	4	+++	+++	++	neg	+	<u>+++</u>
29.8	4	+++	neg	neg	neg	+	<u>++</u>

* Serum number is indicated in parentheses below stock number.

† P236 was obtained from PREER.

‡ Band intensity is indicated by +- -- +++; homologous reactions are underlined.

Genetic crosses: Since qualitative differences in the immunological reactions of Ag5₃, Ag5₇, Ag5₉, and Ag5₂₈, and a quantitative difference (in terms of a reduced amount of antigen produced) in Ag5_{B197} had been found, an analysis of the nature of their genetic control was possible. Stocks were crossed as follows: 3 × 7, 3 × 9, 3 × 28, 7 × 9, 9 × B197, and 3 × B197. In most of the crosses, gene markers other than the antigen 5 allele were followed concurrently as a means of detecting the occurrence of either cytogamy or selfing.

In all crosses involving stock B197, the occurrence of aberrant trichocysts (the consequence of a recessive allele (PREER 1959b)) has been followed. The trichocysts are readily observable with a phase contrast microscope after the *Paramecia* have been crushed beneath a coverslip. In crosses involving stock 3 (but not B197), the ability to synthesize an immobilization antigen other than G has been taken as an indication of successful nuclear exchange, since stock 3 synthe-

TABLE 4

Classification of syngen 2 stocks according to antigen 5 production

Quantitative classification	Qualitative classification*				
	I†	II	III	IV‡	V
Normal	3	..	7	9	28
	11	..	..	..	..
Variable§	1	30	53	4	..
	8	..	..	34	..
Reduced	B197	..	..	..	..
	197	..	..	..	..
Negative¶	12, 13, 35	..	..	..	..

* In order of decreasing similarity to Ag5₃.

† 3, 11 and B197 have been shown with several sera to be very similar or identical.

‡ The classification of stocks 4 and 34, relative to stocks 9 and 28 is not clear at present.

§ Lines produce either normal amounts or undetectable amounts.

¶ Tested only with full-strength P247 (see text).

sizes only the G type under the usual conditions of culture (PREER, personal communication). In the cross between stock 7 and stock 9, the presence of kappa in one, but not both of the exconjugants indicates that selfing has not occurred, because only the stock 7 parent contains kappa before the cross; however, only the analysis of antigen 5 establishes that cytogamy has not occurred (see Table 5).

1. *Crosses between stocks producing normal amounts of antigen: 3×7 , 3×9 , 3×28 , 7×9* : The expression of antigen 5 in exconjugant (F_1) lines is shown in Table 5 for the crosses indicated. When animals of both parental types are grown together for several fissions before assaying, or extracts from the two parental stocks are mixed together, both parental types of antigen are detectable in the mixture. In the crosses involving stock 3, only $Ag5_3$ was detectable in the F_1 clones, indicating a "dominance" of stock 3 over the other parental type. Neither cytogamy nor selfing occurred in the clones tested, since both exconjugant lines produce the same kind of antigen 5 ($Ag5_3$), and manifest a serotype other than G (characteristic of the non-3 parent). The 3×9 cross has been done in both "directions" with regard to mating type, i.e. $3^{III} \times 9^{IV}$, and $3^{IV} \times 9^{III}$. The results are the same in both cases, and have been combined in the table.

The F_1 data from these crosses do not imply selective exclusion of one type of nucleus during conjugation (as in *Tetrahymena pyriformis*, cf. NANNY 1963), since all exconjugants express one marker from each parent. However, until an F_2 generation, obtained by inducing autogamy in the F_1 animals, had been analyzed, the possibility remained that these animals were not heterozygous at the antigen 5 structural locus. Lines from the 3×7 cross were induced to undergo autogamy; 37 postautogamous lines produced $Ag5_3$; 38 produced $Ag5_7$. (All of the postautogamous lines are able to produce an immobilization antigen other than G.) These results indicate that both antigen 5 alleles are indeed present in F_1 animals. In addition, the band positions with 1/3 P247 show an interesting difference in frequency distribution (see Figure 1) for the two parental types: the p -value frequency distribution for $Ag5_3$ bands has only one peak as would be expected; but the distribution for $Ag5_7$ bands is clearly bimodal. The significance of this finding will be discussed later. The mean p value, with its standard error, is given for each of the antigens.

Stocks 7 and 9 are both recessive to stock 3. When they are crossed, antigen 5 of both parental types is detected in each of the six F_1 clones. This result, of course, shows that cytogamy has not occurred. It also indicates that there is no dominance between the antigen 5 alleles of these two stocks.

TABLE 5
Antigen 5 expression in the F_1 generation of four crosses

Cross	Antigen 5 expressed	Serotype expressed	Number of exconjugant lines
3×7	$Ag5_3$	C	18
3×9	$Ag5_3$	E	48
3×28	$Ag5_3$	E	34
7×9	$Ag5_7 + Ag5_9$	not tested	6

FIGURE 1.—The frequency distribution of p values for $\text{Ag}5_7$ bands and $\text{Ag}5_3$ bands in F_2 individuals obtained by autogamy after the cross: stock 3 $\times$ stock 7. The mean, with its standard error, is in parentheses after the legend for each type of antigen.

2. *Crosses between stocks producing different amounts of antigen:* $9 \times B197$; $3 \times B197$: $\text{Ag}5_{B197}$ is produced in reduced amounts (see above) and is serologically distinguishable from $\text{Ag}5_9$. Thus it is possible to determine whether the quantitative difference is controlled by the same genetic locus as the qualitative difference by analyzing the F_2 generation of a cross between stock B197 and stock 9. The trichocyst mutation was used as an additional marker. In the F_1 generation, the 20 exconjugant lines possessed normal trichocysts as expected. Analysis showed the antigen 5 produced to be quantitatively and qualitatively like that of stock B197. This result indicates that the B197 qualitative and quantitative alleles are dominant to those in stock 9 (see DISCUSSION).

One hundred postautogamous lines were analyzed for antigen 5. Specific antisera were used to distinguish $\text{Ag}5_9$ and $\text{Ag}5_{B197}$; p values of less than .60 with $1/4$ P247 indicated reduced amounts of antigen. Of the 48 lines producing $\text{Ag}5_9$, 24 produced normal amounts ($(p = .66 \pm .10$ (mean band position with 95% confidence limits)) and 24 produced reduced amounts ($p = .50 \pm .12$). Of the 52 lines producing $\text{Ag}5_{B197}$, 24 were quantitatively normal ($p = .64 \pm .11$) and 28 were reduced, giving "trace" reactions (a diffuse band, not accurately measurable, except that p is usually less than .4). A χ^2 test indicated that $P > .50$ for a 1:1 ratio of parental (normal $\text{Ag}5_9$ + reduced $\text{Ag}5_{B197}$) to recombinant (reduced $\text{Ag}5_9$ + normal $\text{Ag}5_{B197}$) types. Therefore, the locus which regulates the amount of antigen 5 produced is not linked to the structural antigen 5 locus.

Because some variability had been observed in the production of antigen 5 in both normal and reduced lines, ten postautogamous lines were followed through

TABLE 6

Constancy of antigen 5 production in "reduced" and "normal" postautogamous lines from the cross: stock 9 × stock B197, determined with 1/4 P247

Line	Assay number				Mean value (95% confidence)	Type of antigen 5*
	1	2	3	4		
1	.25	.48	.50	.44	.48 ± .06	Ag5 ₉
2	trace†	neg	neg	neg		Ag5 _{B197}
3	.67	.68	.68	.60	.66 ± .07	Ag5 _{B197}
4	.70	.63	.69	.68	.68 ± .05	Ag5 ₉
5	.61	.57	.66	.64	.62 ± .06	Ag5 _{B197}
6	.70	.72	.70	.61	.68 ± .09	Ag5 ₉
7	trace†	.57	.60	.56	.58 ± .02	Ag5 ₉
8	.65	.70	.65	.67	.67 ± .04	Ag5 ₉
9	.60	.64	.66	.66	.64 ± .05	Ag5 _{B197}
10	.59	trace†	.56	.52	.56 ± .02	Ag5 ₉

* Determined at the time of the initial assay.

† "Trace" indicates a pale, diffuse band in the upper portion of the gel (p is usually less than .4); such bands are not accurately measurable and their position was not included in the determination of mean values.

about thirty fissions; samples were assayed at eight-fission intervals, or about every three days. The results, including mean values with 95% confidence limits for each line, are shown in Table 6. The mean value with 95% confidence limits for all assays of all "normal" lines (3, 4, 5, 6, 8 and 9 in Table 6) is $.66 \pm .04$. The mean value with 95% confidence limits for ten assays of "reduced" lines (1, 2, 7 and 10) which yielded measurable bands (i.e. excluding "trace" results) is $.53 \pm .05$. The data indicate that antigen 5 production is sufficiently constant within the postautogamous lines to permit their assignment to the quantitative classes indicated in Table 6.

As has been noted, stock B197 produces less than 20% of the amount of antigen 5 produced by stock 3. The two antigens cannot be distinguished qualitatively. A cross between stock 3 and stock B197 showed that the F_1 individuals produce less antigen 5 than the stock 3 controls (see Table 7). (The trichocyst mutation was used as an additional marker, and all F_1 clones were found to have normal

TABLE 7

Antigen 5 production in twenty-two F_1 clones from the cross: stock 3 × stock B197

Type of extract	Number of extracts	Band position, p , with 1/4 P247
F_1	4	Negative (see text)
F_1	18	.47 ± .09*; +
Stock 3	2	.59† (.58, .60); +++
Stock B197	2	Trace
Stock 3 + Stock B197 (1:1)	2	.50† (.50, .50) +

* Mean, with 95% confidence limits.

† Average of the two values in parentheses.

trichocysts.) The four negative results may represent cases in which the antigen has been destroyed before the assay, a phenomenon that has occasionally been observed in crude extracts by this author and by JONES (personal communication). The negative results may also indicate that antigen 5 production is variable in these heterozygotes. Such variability has been reported in syngen 2 (above) and in syngen 4 (JONES 1964). F_1 clones have been assayed only once because they tend to go into autogamy spontaneously before enough animals can be accumulated for a second assay. For this reason, there is no further information about the "negative" F_1 clones at present. The control test in which stock B197 and stock 3 extracts are mixed in a 1:1 ratio most nearly parallels the results with extracts from most of the F_1 clones.

After autogamy in the F_1 clones, the quantitative difference in antigen 5 and the trichocyst type were observed in 43 postautogamous lines. Full strength P247 was used for the assay for antigen 5 because it gives "negative" results (no visible band) with the amount of antigen usually synthesized by stock B197, and gives positive results with the "normal" amount produced by stock 3. The quantitative alleles segregated 23 normal to 20 reduced ($\chi^2 = 0.21$; $0.70 > p > .50$). The quantitative Ag5 and trichocyst loci failed to show linkage, that is they were in parental association in 19 lines and were recombined in 24 lines.

DISCUSSION

Expression of antigen 5 in syngen stocks: Extracts from stocks 4, 8, 30, 34 and 53 do not always produce precipitin bands. Their antigen 5 expression is termed "variable." As in the case of variability in stock 47, syngen 4 (JONES 1964), the expression of antigen 5 is inconsistent both between and within lines from these stocks. Subsequent work by PREER (personal communication) indicates that "negative" clones of stock 47 actually produce reduced amounts of antigen. It is possible that the "negative" lines in variable syngen 2 stocks are also synthesizing reduced amounts which are not detectable with the assay method used.

In stock B197, the detectability of antigen 5, even with dilute antiserum, is somewhat variable; however, in no case has a clone from this stock produced a normal amount of the antigen insofar as this can be detected with serum P247. One may, therefore, consider the antigen 5 production in stock B197 to be reduced; elaboration of this point is to be found in the discussion of the genetics of the system (see below).

Genetic analysis: In the three crosses between stock 3 and other stocks producing a normal amount of antigen, stocks 7, 9 and 28, the only detectable antigen 5 produced by F_1 lines is that of stock 3. Both parental antigens are detectable in mixtures of parental extracts. These results are independent of the parental mating types: the cross between stock 3 and stock 9 has been done "in both directions" with respect to mating type; in both cases only Ag5₃ is produced by the F_1 clones. Furthermore it is clear that both alleles are present in the F_1 , for normal 1:1 segregation of the two parental types is found after autogamy. It must be concluded that the stock 3 genome, including the allele for antigen 5

(symbolized by v^3) in some way blocks the expression of antigen 5 of the other parental type, i.e. v^3 is dominant to v^7 , v^9 and v^{28} .

The data indicate that the structural determinants for $Ag5_3$ and $Ag5_7$ are alleles, as are those for $Ag5_9$ and $Ag5_{B197}$. In the absence of contradictory evidence, the genetic model described below will treat all structural genes as alleles at a single locus, v .

Although we might note only that the v^3 allele exhibits dominance over other alleles such as v^7 , it may be useful to explain the results in terms of repressor loci. According to this view we may assume that a dominant R_1 allele is present in stock 3. It has no action on the v^3 allele, but acts to prevent the expression of the v^7 , v^9 and v^{28} alleles. Stocks 7, 9 and 28 are assumed to contain a recessive r_1 which in no way affects the v locus. Since only the two parental classes appear in the F_2 by autogamy, we must assume close linkage between R_1 and v . For example:

Parentals	$\frac{R_1}{R_1} \frac{v^3}{v^3}$	$\times$	$\frac{r_1}{r_1} \frac{v^7}{v^7}$	
	Stock 3		Stock 7	
	R_1 produces a repressor (but v^3 insensitive)		No repressor produced	
F_1	$\frac{R_1}{r_1} \frac{v^3}{v^7}$			
	Repressor produced by R_1 inhibits v^7			

The F_2 gives only the two parental genotypes because of close linkage between the R_1 and v loci.

The model can be extended consistently to explain the results of crosses involving B197, in which a reduced amount of antigen is produced. For example, in the cross of stock B197 with stock 9, the F_1 shows only $Ag5_{B197}$, so stock B197 must have a dominant allele (such as R_1) repressing the v^9 allele. Furthermore, since stock B197 shows a reduced amount of $Ag5$, and since this reduced amount is present in the F_1 and segregates to all classes in the F_2 , B197 must have a second repressor allele unlinked with the v locus. Thus:

Parentals	$\frac{R_1}{R_1} \frac{v^{B197}}{v^{B197}}$	$\frac{R_2}{R_2}$	$\times$	$\frac{r_1}{r_1} \frac{v^9}{v^9}$	$\frac{r_2}{r_2}$	
	v^{B197} insensitive to			No repressor produced		
	repressor produced by R_1 ;					
	R_2 reduces level of $Ag5_{B197}$					
F_1	$\frac{R_1}{r_1} \frac{v^{B197}}{v^9}$	$\frac{R_2}{r_2}$				
	R_1 repressor completely blocks action of v^9 ;					
	R_2 repressor reduces activity of v^{B197}					

After autogamy, there would be four classes of individuals in the F_2 , if the R_2 locus is not linked to the R_1 - v complex: (all classes are homozygous):

$\frac{R_1}{R_1} \frac{v^{B197}}{v^{B197}} \frac{R_2}{R_2}$	in which a reduced amount of Ag5 _{B197} is produced (Stock B197 parental type)
$\frac{r_1}{r_1} \frac{v^9}{v^9} \frac{r_2}{r_2}$	in which a normal amount of Ag5 ₉ is produced (Stock 9 parental type)
$\frac{R_1}{R_1} \frac{v^{B197}}{v^{B197}} \frac{r_2}{r_2}$	in which a normal amount of Ag5 _{B197} is produced (recombinant type)
$\frac{r_1}{r_1} \frac{v^9}{v^9} \frac{R_2}{R_2}$	in which a reduced amount of Ag5 ₉ is produced (recombinant type)

These four classes of individuals should occur with equal frequencies according to this model. The data support such a model.

The status of the R_2 locus in stock 3 can be determined by analysis of clones resulting from autogamy in F_1 individuals from the cross between stocks 3 and 7. It is reasonable to predict that stock 7, like stock 9, contains the r_2 allele, because Ag5₇ and Ag5₉ are present in approximately the same amounts in the heterozygote resulting from the cross between stock 7 and stock 9. Stock 9 has already been shown to be sensitive to the product of the R_2 locus (see above). If stock 7 contained R_2 , the Ag5₉ synthesis would be reduced in this heterozygote. Therefore, stock 7 is represented by:

$$\frac{r_1}{r_1} \frac{v^7}{v^7} \frac{r_2}{r_2}$$

If stock 3 contains the R_2 allele, there will be three phenotypic classes of individuals after autogamy:

$$\frac{R_1}{R_1} \frac{v^3}{v^3} \frac{R_2}{R_2} \text{ or } \frac{r_2}{r_2}$$

in which normal amounts of Ag5₃ will be produced because v^3 is unaffected by R_2 ;

$$\frac{r_1}{r_1} \frac{v^7}{v^7} \frac{r_2^2}{r_2^2} \quad (\text{Stock 7 parental type})$$

in which a normal amount of Ag5₇ will be produced in absence of any repressors,

and
$$\frac{r_1}{r_1} \frac{v^7}{v^7} \frac{R_2}{R_2} \quad (\text{recombinant type})$$

in which the repressor produced by R_2 will reduce the activity of v^7 so that a reduced amount of Ag5₇ will be produced.

The fact that the band positions of Ag5₃ extracts occur in a normal distribution, but those of Ag5₇ extracts occur in a bimodal distribution after autogamy (see Figure 1) provides some evidence for this model, and, therefore, for the assignment of an R_2 allele to the stock 3 genome.

At the present time, it is impossible to determine whether stock 3 and stock B197 are dominant over stocks 7, 9 and 28 because of a repressor synthesized by R_1 or because of the antigen 5 itself. Ag5_{B197} is immunologically indistinguishable from Ag5₃, so it is likely that the two antigens would behave similarly in a

repressive capacity as well. While the R_1 locus is strictly hypothetical, the R_2 locus is required by the data because its segregation is independent of the structural ν locus.

The author wishes to express appreciation to DR. JOHN R. PREER, JR., for his guidance and encouragement throughout the course of this work.

SUMMARY

A serological study of antigen 5 production in several syngen 2 stocks of *Paramecium aurelia* indicates that they may be divided into at least two quantitatively distinct classes and at least five qualitatively distinct classes. The genetic control of the synthesis of antigen 5 is mediated through loci with the following characteristics: 1) the structural locus (ν) is responsible for qualitative differences between stocks; there are at least five ν alleles in syngen 2; 2) the R_1 repressor locus, which is linked to the ν locus (and may be identical to it), and at which there are at least two alleles: one (R_1) which completely represses sensitive ν alleles so that no detectable antigen 5 is produced; and a second (r_1) which has no observable effect on ν alleles; and 3) the R_2 repressor locus, which is not linked to the ν locus, and at which there are at least two alleles: one (R_2) which partially represses sensitive ν alleles so that a reduced amount of antigen 5 is produced; and a second (r_2) which has no observable effect on ν alleles.

LITERATURE CITED

- AMES, B. N., and R. G. MARTIN, 1964 Biochemical aspects of genetics: the operon. *Ann. Rev. Biochem.* **33**: 235-258.
- BEALE, G. H., 1954 *The Genetics of Paramecium aurelia*, 1st ed., Cambridge University Press. London, England.
- HOROWITZ, N. H., M. FLING, H. MACLEOD, and Y. WATANABE, 1961 Structural and regulative genes controlling tyrosinase synthesis in *Neurospora*. *Cold Spring Harbor Symp. Quant. Biol.* **26**: 233-238.
- HOROWITZ, N. H., and R. L. METZENBERG, 1965 Biochemical aspects of genetics. *Ann. Rev. Biochem.* **34**: 527-564.
- JACOB, F., and J. MONOD, 1961 Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**: 318-356.
- JONES, A. S., 1964 Description and genetic analysis of a non-immobilizing ciliary antigen of *Paramecium aurelia*. Ph.D. Thesis, University of Pennsylvania.
- LEWIS, H. W., and H. S. LEWIS, 1963 Genetic regulation of dopa oxidase activity in *Drosophila*. *Ann. N.Y. Acad. Sci.* **100**: 827-839.
- MCCLEINTOCK, B., 1965 Control of gene action in maize. *Brookhaven Symp. Biol.* **18**: 162-184.
- NANNEY, D. L., 1963 Irregular genetic transmission in *Tetrahymena* crosses. *Genetics* **48**: 737-744.
- NEFF, J. C., and E. L. BECKER, 1957 Antigen-antibody reactions in agar. III. Rate of change of band migration with antigen concentration. *J. Immunol.* **78**: 5-18.
- ORNSTEIN, L., and B. J. DAVIS, 1962 *Disc Electrophoresis*. Reprinted by Distillation Products Industries, Eastman Kodak Co.

- PREER, J. R. JR., 1956 A quantitative study of a technique of double diffusion in agar. *J. Immunol.* **77**: 52-60. — 1959a Studies on the immobilization antigens of *Paramecium*. II. Isolation. *J. Immunol.* **83**: 378-384. — 1959b Nuclear and cytoplasmic differentiation in the protozoa. *Symp. Soc. Study Develop. Growth* **16**: 3-20.
- PREER, J. R., JR., and L. B. PREER, 1959 Gel diffusion studies on the antigens of isolated cellular components of *Paramecium*. *J. Protozool.* **6**: 88-100.
- PREER, J. R., JR., and W. H. TELFER, 1957 Some effects of nonreacting substances in the quantitative application of gel diffusion tests. *J. Immunol.* **79**: 288-293.
- SCHACHMAN, H., 1957 Ultracentrifugation, diffusion and viscometry. pp. 32-103. In: *Methods in Enzymology, Vol. IV*: Edited by S. P. COLOWICK and N. O. KAPLAN, Academic Press, N.Y.
- SONNEBORN, T. M., 1937 Induction of endomixis in *Paramecium aurelia*. *Biol. Bull.* **72**: 196-202.
- 1950 Methods in the general biology and genetics of *Paramecium aurelia*. *J. Expt. Zool.* **113**: 87-148.
- STEERS, E., JR., 1962 A comparison of the tryptic peptides obtained from immobilization antigens of *Paramecium aurelia*. *Proc. Natl. Acad. Sci. U.S.* **48**: 867-874.
- WARBURG, O., and W. CHRISTIAN, 1941 Isolierung und Kristallisation des Gährungsferments Enolase. *Biochem. Z.* **310**: 384-421.