THE INHERITANCE OF THE IMMOBILIZATION ANTIGENS OF *PARAMECIUM AURELIA*, VARIETY 2*

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(With One Text-figure)

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INTRODUCTION

Investigations of the inheritance of the immobilization antigens of *Paramecium aurelia* have disclosed a system of nuclear and cytoplasmic interaction that bears on the general problem of cellular differentiation (Sonneborn, 1950*b*). The work described here extends these studies to variety 2.

When paramecia are exposed to antisera prepared against whole or homogenized paramecia, they will be immobilized if they are of the same serotype as the injected animals, or will be unaffected if they belong to another non-cross-reacting serotype. Sonneborn & LeSuer (1948) have shown in variety 4 that animals with the same genome can manifest several serotypes, although only one at any particular time.

When animals of one stock but of different serotypes are crossed, the exconjugant clones retain their original antigenic types. Such results, which have been obtained in variety 1 and variety 4, mean that among animals having the same genotype, the antigenic type actually shown by an animal is determined by the cytoplasm (Sonneborn, 1948; Beale, 1952). Often two stocks differ in their capacity to manifest a certain antigen. This difference in ability to produce the antigen has been found to be under the control of a single gene (Sonneborn, 1948). Also, when antigenic types that are serologically closely alike, yet separable using the proper sera, are present in two or more stocks, it has been shown that genes at a single locus determine which of the alternate serotypes can appear in a cell (Sonneborn, 1950*b*; Beale, 1952).

The present investigation has demonstrated that the inheritance of immobilizing antigens in variety 2 follows a pattern similar to that already shown for other varieties; single genes control the ability to manifest an antigen and the specificity of an antigen. The cytoplasm, in combination with the environment, determines which antigen of all the antigens a *Paramecium* is capable of producing is actually manifested at any particular time.

In the course of the investigation, some variety 2 animals were encountered which should have been homozygous for all loci, yet were of a serotype ordinarily manifested only by a heterozygote. This phenomenon has been shown probably to be caused in most

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instances by deviations from the normally expected pattern of behaviour of individual nuclei during nuclear reorganization. A few other such unusual F_2 clones were, in addition to being of a hybrid serotype, apparently homozygous at another locus. These lines have been shown probably to be the result of intranuclear abnormalities.

MATERIALS AND METHODS

A. Materials

The following stocks of *P. aurelia*, variety 2, collected in the U.S.A., were used: 1 from Strickersville, New York; 7 from Pinehurst, North Carolina; 21 from Woodstock, Maryland; 30 from Coates Pond, Maryland; 35 from Moscow, Indiana; and 35 from Blairstown, New Jersey.

Several homozygous lines derived by autogamy from various crosses were also employed. *d* 30-1 was derived from stocks 30 and 35. It originated from a hybrid of these two stocks which was then put through autogamy. Autogamous animals were then crossed to the 30 parent. This general procedure was repeated with the F_1 animals of this cross, until a line was obtained, *d* 30-1, which had mainly stock 30 genes. It also had a gene for temperature sensitivity from stock 35, animals homozygous for the gene being incapable of multiplication at a temperature of 31° (see below). *d* 28-1 was derived from a cross of *d* 30-1 and stock 28, and, in addition to being temperature-sensitive, could also manifest serotype E. *d* 1-1 was similar to *d* 28-1 except that stock 1 was used as a parent in place of 28. *d* 30-2 originated from a cross of stocks 7 and 30, was temperature-resistant, and was capable of producing the 30-C antigen. *d* 30-3 lines included several F_2 segregants derived from a cross of *d* 30-2 with *d* 30-1. *d* 30-3 animals therefore had genes from stocks 7, 30 and 35. They were temperature-sensitive, could manifest serotype E, and could possess either 30-C or 7-C antigen, the particular type of C depending on the individual clone.

B. Culture

Stocks and all clones derived from them were kept at temperatures from 12 to 31° C., as the experiment required, in a baked lettuce medium or, less frequently, in a 0·075% Cerophyl infusion (Cerophyl tablets or powder obtained from Cerophyl Laboratories, Kansas City, Kansas, U.S.A.) inoculated with the bacterium *Aerobacter cloacae*. Cerophyl medium was prepared by dissolving 11·3 g. of Cerophyl in 1 l. of distilled water, boiling slowly for 3–4 min., and then filtering through cotton. The filtrate was added, with 17 g. Na_2HPO_4, to 15 l. of distilled water and autoclaved for 30 min. Sonneborn's review of methods in the biology of *Paramecium* should be consulted for the details of culturing and for other methods mentioned below (Sonneborn, 1950*a*).

C. Genetic techniques

Mendelian ratios occur among conjugating pairs, the particular ratio depending on the genotypes of the two parental clones (see Beale, 1954, for details). Self-fertilization in unpaired animals, autogamy, produces homozygosity at all loci. Thus a clone heterozygous at a particular locus will yield a 1:1 ratio of lines homozygous for the two alleles.

A gene for temperature-sensitivity, discovered by Preer (unpublished), was used as a marker gene in many crosses. Paramecia that are homozygous for the *t* gene when placed

at 31° divide from two to six times, become sluggish, and then die. To test for the presence of the *tt* genotype, single animals were placed in each of six depressions with three drops of Cerophyl fluid. Three of the depressions were placed at 31° C., while three, serving as controls, were placed at 27° C. Every 24 hr. a single animal was transferred from the previous 24 hr. depression into a new depression with fresh medium. At the end of 4 days both temperature-resistant and temperature-sensitive lines will have under-gone 8–12 fissions at 27° C. In contrast, the temperature-sensitive lines, in the same length of time, will have divided only 2–6 times at the higher temperature. Any line which did not show a fission rate of at least two fissions a day in two of the three depressions at 27° C. was discarded as a poor line and, therefore, considered untestable.

In order to ascertain the origin of the cytoplasm of an exconjugant, either mating type or the killer character (see Beale, 1954) was followed.

D. Serological techniques

The details of obtaining and handling sera were the same as those described by Sonneborn (1950*a*). The serotype of a culture was determined by placing samples into the appropriate dilutions of different specific antisera. The tests were read after 2 hr. at 27° C., unless otherwise noted, to determine in which antiserum immobilization had occurred. The paramecia were then designated as being of the same serotype as were the original animals used in the preparation of the particular antiserum—e.g. the A serotype if the immobilizing antiserum was A antiserum. For a more complete description of serotype diagnosis see Sonneborn (1950*a*).

RESULTS

As has been reported elsewhere (Finger, in the Press), eight serotypes have been found in variety 2 stocks. These serotypes, designated by the letters A through H, were titred against homologous and heterologous antisera.

A. The E Serotype

Not all stocks could manifest all of the serotypes. Thus, early in these studies it was found that serotype E could be manifested by some stocks but not by others. In other varieties of *P. aurelia*, as pointed out above, the ability to manifest a particular antigen has been shown to be under the control of a single locus, the gene determining this ability being dominant to its allele. It was of interest to determine whether a similar genic control existed in variety 2. Furthermore, if this were the case, such a gene might be useful as a marker in following more complex genetic control of immobilizing antigens.

Preliminary work on stocks 53 and 30 by Preer indicated that serotype E could arise only in animals having a specific gene for that serotype, the gene *E*. In addition, it was found that the transformation to serotype E occurs rapidly when a culture of non-E animals of the appropriate genotype is placed directly at 31° C. from 37 or 12° C., often within 24 hr. Lines brought from 27 to 31° C. would frequently require several days or even months for the transformation.

A single gene has also been found by the author to control the appearance of serotype E in stock 28 and hybrids of stock 28. Stock 28, able to manifest E, was crossed with *d* 30-1,

which is unable to manifest serotype E. Similarly, *d*28-1, capable of manifesting E, was crossed with d1-1, lacking this ability. All parental stocks and their descendants were maintained at 17° C. prior to being tested for transformation to serotype E following growth at 31° C. The seventeen parental lines were of either serotype B or G.

When animals of complementary mating types become joined, conjugation (reciprocal fertilization) generally ensues. However, cytogamy (self-fertilization) or some other nuclear process not involving cross-fertilization may occur in pair members. To select only true hybrid animals for analysis the *t* gene was followed. In all F_1 pairs where conjugation was indicated by the marker gene, both exconjugants manifested serotype E. Data for the F_1 generation and the F_2 generation by autogamy are presented in Table 1. The 1:1 ratios encountered in the autogamous F_2 lines show that a single gene difference is involved.

Table 1. *The inheritance of the ability to manifest serotype E*

F_1 generation

Cross Stock which can show E × stock which cannot show E		No. of pairs both members showing E	No. of pairs neither member showing E
Stock 28, type G × d30-1, type G		7	0
*d*28-1, type G × *d*1-1, type B		31	0
	Totals	38	0

F_2 generation by autogamy

Origin of F_1 clone		Lines showing E	Lines not showing E
Stock 28 × *d*30-1		121	130
*d*28-1 × *d*1-1		78	59
	Totals	199	189

It was observed, however, that not all serotypes appearing in *d*30-1 and *d*28-1 would readily transform to E. Animals of serotypes A, B, C and G can transform to E. On the other hand, animals of an unknown serotype, not one of these four, are unable to change to E even though descended from animals known to possess the *E* gene. It was therefore of some importance in these crosses and in crosses using the *e* locus as a marker to know the serotype of a clone prior to its being placed at 31° C. Fortunately, the four serotypes that can transform to E are among the most common of the serotypes in variety 2. Furthermore, when animals of the unknown serotype were found in the progeny of the crosses, animals of the more common serotypes were generally present in the same culture, and, therefore, the line as a whole could be classified as having *e* or *E*.

In clones homozygous for the *t* gene, it might be expected that the temperature-sensitive trait would interfere with the detection of the *E* gene. Fortunately, however, the transformation to serotype E occurs within 36 hr. after a line is placed at 31° C. During this interval at 31° C. the full effect of the *tt* genotype is not manifested. Temperature-sensitive animals will have normal mobility and can be used in immobilization tests.

B. The C Serotype

A serotype found in different stocks, although reacting similarly to a serum prepared against one of the stocks, may sometimes be distinguished from stock to stock by small but reproducible titre differences. In variety 4, in addition to controlling the ability to manifest a serotype, genes control this specificity of the immobilizing antigen. To com-

pare further variety 2 with the other varieties, the inheritance of stock-specific serotype C was studied. These studies were also carried out for two other reasons: (1) to determine the variability of the serotype of hybrid animals under the same environmental conditions and under changing conditions; (2) to repeat and investigate fully using marker genes the phenomenon discovered by Preer (unpublished) in crosses of the C serotypes: the persistence of a hybrid serotype in presumably homozygous animals.

(1) *Titration and stability*

Serotype C has been found in many stocks, but stocks 7, 21, 30 and 85 were the only ones whose C's were titred against several C antisera in order to find workable differences. The C serotypes found in the four stocks were distinguishable from each other by their reactions to three antisera (Table 2). The genetic analysis was restricted to 7-C and 30-C.

Table 2. *Titrations of serotype C*

Serum no.	Prepared against: Stock	Prepared against: Serotype	Immobilization titres of serotype C Stock: 30	85	7	21
F3	*d*30-1	C	6, 8	5, 7	4, 7	4, 5
P9	7	C	5, 8	4, 6	6, 9	2, 4

The figures in the body of the table represent the lowest concentration of the serum that will immobilize the animals in 2 hr. at 27° C. (the first figure given), and the lowest concentration that will retard the animals (the second figure). The figures represent concentrations of serum as follows:

ml. diluent serum / ml. serum	12·5	25	50	100	200	400	800	1600	3200	6400
Dilution no.	1	2	3	4	5	6	7	8	9	10

The animals manifesting the 30-C used in all the experiments below originated from *d*30-2 clones. These clones were chosen because their 30-C serotype was extremely stable at 17, 27 and 31° C., unlike the C serotype of the original stock 30. To provide an environment in which both serotype 7-C and 30-C had approximately equal opportunity for expression, clones were placed at 17° C. with enough culture fluid to permit a fission rate of 0·5, 1 or 1·5 fissions per day.

(2) *The F_1 generation*

The F_1 hybrids were studied in some detail to determine the part played by the cytoplasm in the determination of the serotype after the introduction of new genes and the constancy of the expression of these genes under the same environmental conditions.

When 7-C and 30-C animals are mated the exconjugants may be 7-C, 30-C or mixed (see below). It could be determined whether lines pure for a parental serotype were actually hybrids, without deriving an F_2 generation, by introducing the *T* and *E* genes as markers. True exconjugant clones could therefore be distinguished from clones derived from animals which had undergone cytogamy or macronuclear regeneration by any one or a combination of several of the following ways: (1) *T* transfer, (2) *E* transfer, (3) F_1 mixing, and (4) segregation of 7-C and 30-C serotypes or *E* and *T* genes within a clone following autogamy.

Diagnostic immobilization tests for the two parental serotypes were read after 15 min. at room temperature. Serum P9 immobilized 7-C animals in a final dilution of 1:50, in

which concentration 30-C animals were only retarded. F3 serum, in the same length of time and concentration, immobilized animals of serotype 30-C and only retarded the swimming of 7-C animals. Samples of all clones tested were exposed to each serum separately. Control tests against parental C serotypes were always set up along with the experimentals and gave remarkably consistent results. In spite of the high degree of cross-reactivity between 7-C and 30-C serotypes, homologous animals were always immobilized in serum which only retarded animals of the heterologous serotype.

A clone was considered mixed when both 7-C antiserum and 30-C antiserum had an immobilizing effect in the appropriate dilutions. If only some of the animals of a subculture were immobilized by each of the two sera, the clone may have been made up of animals some of which were 7-C and some 30-C, or some of the animals may have been mixed and the remainder of a type other than C. To distinguish between these alternatives in such cases, the immobilization tests were read at 15 min. intervals. If the number of animals immobilized increased with increased time, the culture contained both 7-C and 30-C animals; if the number remained constant, both antigens were present in individual animals. The basis for this method lies in the ability of a C antiserum to immobilize heterologous C cells after a sufficiently long period of time in concentrations that would only retard the paramecia after brief contact.

Most of the true (see above) exconjugant clones were either of the mixed type, with both members of a pair tending to be mixed (44 pairs of a total of 104 pairs in one cross), or one member mixed and the other a non-C serotype (29 pairs in the same cross). In some cases one exconjugant was mixed, and its partner was of a parental serotype. In such cases the 7-C serotype was as prevalent as the 30-C, although 30-C is the more stable serotype in intrastock crosses. In the few pairs where mixing was undetected in either side, an exconjugant usually retained its original serotype.

Thus animals of the same genotype under the same environmental conditions may express different phenotypes. An exconjugant possessing the genes controlling 7-C and 30-C serotypes may manifest either the mixed serotype, the 7-C serotype, the 30-C serotype, or, of course, some serotype other than C.

(*a*) *The role of the cytoplasm.*: As has been mentioned above, most F_1 clones are of the mixed serotype. This result is in marked contrast to the results of crosses between C and non-C animals. Exconjugants in these crosses retained the original parental serotypes (see below). Thus, as in other varieties, the cytoplasm apparently permits the expression of an introduced gene only when the cytoplasm is in a suitable condition or 'state' as determined by other genes (cf. Beale, 1952).

Although the cytoplasm allows both alleles to be expressed in a hybrid animal, the cytoplasm of the two parents may exert an additional, although transitory, influence on the progeny apart from this overall effect. This influence of the cytoplasm on the F_1 serotype may be detected by comparing the titres of mixed F_1's with the titres of the parents from which they derived their cytoplasm. As late as the tenth fission after conjugation the cytoplasm may still exert its influence in many clones. This lag is detected as a strong homologous reaction, identical with the reaction shown by the parental line when exposed to homologous serum, coupled with a heterologous reaction only slightly stronger than that demonstrated by the parental line.

In a very small percentage of exconjugant clones mixing may be detected as early as the

second post-conjugation fission, while in other clones a pure C serotype may persist for at least twenty fissions. Not only may the parental serotype become mixed shortly after conjugation, but changes from one C type directly to the alternative pure C type may occur within six fissions after mating. Apparently, then, the effect of the parent's cytoplasm in determining the kind of C manifested in descendants must be either an impermanent one or an effect not easily recognizable by immobilization tests with these sera.

(*b*) *Stability and variation in hybrid animals.* In the original group of experiments titration of the F_1's of hybrid serotype showed that the majority of the clones were immobilized only in serum more concentrated than that needed to immobilize the parental clones. Whenever a mixed clone reacted in this fashion, though, the titres against the two parental antisera were not always lowered to an equal degree. Such mixed animals generally reacted most like the 7-C parent. However, when the crosses were repeated a year later almost all of the mixed F_1 clones were immobilized in the same dilutions of sera as were the parents. Perhaps very slight extra- or intracellular environmental shifts may play a part in determining the serotype hybrid cells may possess, for instance, by altering the kind or amount of antigen synthesized (cf. Sonneborn & Balbinder, 1953).

Once a hybrid animal has transformed to the mixed C serotype, the transformed animal is quite stable. The phenotypes of F_1 clones were followed by testing samples every six or seven fissions after conjugation until about twenty fissions were reached. The greater proportions of transformations of clones kept at 17 and at 27° C. were to the mixed type. Of ninety-three lines that were mixed at the seventh or thirteenth post-conjugation fission 78% were still mixed after twenty fissions. 30-C lines were more likely to remain 30-C (11 of 38 lines) at the twentieth fission than were 7-C lines (9 of 56 lines).

When the stability of exconjugant clones of a serotype other than mixed is further compared, the higher frequency of transformations of 30-C to a non-C serotype parallels the results found in control crosses where only 21–28% of 30-C × 30-C exconjugant clones remained C. Mixed clones, on the other hand, rarely transform to types other than C (12 of 93 lines). The mixed animals resemble animals of the 7-C serotype in this respect.

Thus, the similarity of the behaviour of hybrid animals to that of the 7-C parent is indicated by three facts:

(1) Hybrids very frequently transform from the 30-C parental serotype directly to the 7-C serotype.

(2) Mixed animals usually react more strongly with 7-C antiserum than with 30-C antiserum.

(3) The stability pattern of mixed lines is more like that of 7-C than 30-C.

There are several possible explanations for these parallels. The 'hybrid antigen' may be a new antigen with stability characteristics of the 7-C serotype, or it may be simply a mixture of two parental antigens. It is also possible that, since genes which determine the specificity of one serotype may also control the stability of a second, the hybrid antigen and its stability characteristics are a result of interaction between genes at the C locus and genes at other loci (cf. Sonneborn, Ogasawara & Balbinder, 1953).

(3) *The F_2 generation*

Autogamy induced in clones heterozygous for a single pair of alleles determining C should yield equal numbers of 7-C and 30-C lines. Although equal numbers of the two types were found as predicted, many lines showed the mixed character of the F_1 (Table 3). These exceptional lines were studied in detail in order to determine their nature.

Table 3. *The segregation of autogamous F_2 generation lines for C*

Experiment	No. of F_1 clones	No. of lines of each serotype: 30-C	7-C	Mixed	χ^2	P
53-1	2	10	10	0	0	1·00
53-10	4	36	24	22	2·4	0·10-0·20
53-28	19	142	124	97	1·2	0·20-0·30
54-5	5	36	31	2	0·37	0·50-0·70
Totals	30	224	189	121	2·94	0·05-0·10

The values for χ^2 are obtained only by comparing the number of lines of 30-C and 7-C serotype observed with the number expected according to a 1:1 ratio. The number of mixed lines is not included in these calculations.

(*a*) *Distribution of mixed F_2 clones.*

Mixed lines do not occur randomly among lines derived from different F_1 clones. The frequency of the percentage of mixed lines in each clone is shown together with the 95% confidence intervals for each clone in Fig. 1. Although the numbers of lines from each clone are generally small, the distribution is definitely bimodal. Thus, one group of F_1 clones produces mixed lines at autogamy only rarely, while the second group produces mixed lines frequently. This latter group will be referred to hereafter as the clonally mixed group.

(*b*) *Mechanisms for producing mixed F_2 lines.*

Before considering the possible explanations for mixed F_2 lines it should be noted that, among the F_2, clonally mixed lines arising from crosses marked with the *t* gene some were both mixed (the phenotype of a heterozygote) and also temperature-sensitive (the phenotype of a homozygote). The significance of these lines will become evident in the following discussion of possible causes for F_2 mixing. Heterozygosity following nuclear reorganization may be accounted for in several ways:

(i) *Conjugation of animals within an F_1 clone.* If, instead of passing through autogamy, isolated animals had really gone through conjugation, mixing might persist. Isolated exconjugants cannot be distinguished cytologically from animals in autogamy. Because autogamy was induced in most clones at 17° C., the temperature most favourable for conjugation, selfing clones might be expected. However, in clones where the possibility of selfing was ruled unlikely by repeated checking for pairs in discard and original cultures, mixing continued to occur sporadically. Also, when autogamy was induced at 27° C., a temperature at which conjugation rarely occurs, mixed lines still appeared. Therefore conjugation appears very unlikely as a major cause for either clonal or non-clonal mixing. Furthermore, selfing as the sole cause for the clonal class is completely ruled out because, even if every animal in a clone were to mate, the highest proportion of mixed lines that could be expected would be 50%, since the ratio of homozygous to heterozygous exconjugant clones in matings between two heterozygous animals would be 1:1, and as seen in Fig. 1, the frequency often exceeds 50%.

(ii) *Occurrence of non-autogamous animals in autogamous cultures.* In crosses made early in this study, F_1 clones were assumed to be in autogamy if they were isolated from cultures, samples of which showed 100% of the animals in nuclear reorganization (20–40 animals per sample). On computing confidence intervals, it is found that 95% of the cultures from which such samples were taken would be expected to have no fewer than 83% of all the animals of the culture actually in autogamy. Thus, non-autogamous animals, i.e. F_1 animals, could occasionally be included among lines assumed to be homozygous for all genes. Inclusion of vegetative animals could, therefore, account for some of the mixing

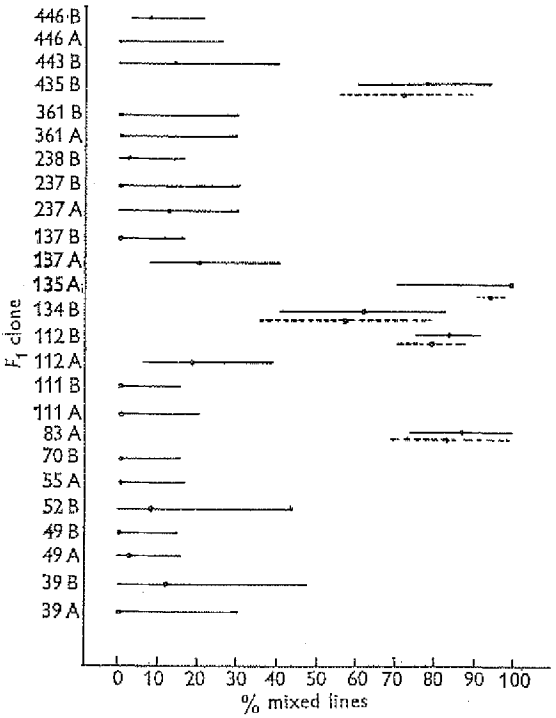

Fig. 1. Mixing in F_2 lines originating from different F_1 clones. 95% confidence intervals. Dotted lines represent confidence intervals for the same clone represented by the unbroken line immediately above. The dotted line is the confidence interval corrected for any mixed lines due to whatever mechanism is causing mixing in clones with less than 50% mixed lines. Such a correction is based on the assumption that the same mechanism is operative in the clones producing a high percentage of mixed lines.

of both classes, but only a small fraction of the clonal class. In addition, it should be noted that the segregation of marker genes in the clonal class of mixed lines rules out the explanation in lines with *tt* genotype.

(iii) *Hemixis.* The breakdown of the macronucleus during *vegetative* life, termed hemixis by Diller (1936), may, in some instances, give a cytological picture of the macronucleus similar to that found at autogamy. But, because there are no micronuclear changes, a hybrid cell that has been through hemixis will retain heterozygosity in both kinds of nuclei. This heterozygosity would be expected at all loci. Since most of the lines isolated as autogamous in all experiments had anlage when stained, hemixis could not have occurred in these lines. Furthermore, the appearance of the recessive phenotype of a marker gene

carried by a mixed animal would eliminate hemixis as the cause of mixing in such lines. As mentioned above, this did actually occur in some mixed clones of the class 2 type; cells were mixed and temperature-sensitive.

(iv) *Macronuclear regeneration.* During autogamy haploid nuclei identical in genic make-up fuse to form a syncaryon, which in turn give rise to the new micronuclei and macronucleus. Since the macronucleus controls the phenotype (Sonneborn, 1946), i.e. is the somatic nucleus, the fact that its origin is the same as that of the micronuclei ensures that the genome of the micronucleus will be reflected in the action of the macronucleus. But should anlage formation be inhibited, and the fragments of the disintegrating old macronucleus regenerate and form the new macronucleus, then the clone derived from a cell with such a macronucleus would have characters determined by the old micronuclei and macronucleus. Such macronuclear regeneration would cause the retention of the mixed phenotype even in the presence of homozygous micronuclei.

The stage of nuclear reorganization at which a sample was stained for autogamy, which was usually immediately following macronuclear breakdown, did not permit a clear-cut distinction between autogamy and macronuclear regeneration. Later, single F_1 animals in nuclear reorganization were permitted to divide twice and then all the fission products but one, the cell used to carry on the line, were stained. In this way the macronuclei of the animals of every line tested for serotype in the F_2 generation could be classified as originating either from the newly formed syncaryon or from a fragment of the old F_1 macronucleus.

Macronuclear regeneration in one set of crosses was found in 4 % of all lines in which the old macronucleus had broken down and accounted for all or most lines associated with otherwise segregating F_2 lines. This macronuclear regeneration was found to be one, if not the only, cause for the non-clonal class of mixed lines. The cytological evidence on involvement of macronuclear regeneration in clonal mixed lines is not decisive. In the single instance where macronuclear regeneration was detected in large numbers within lines originating from a single F_1 clone, there were no survivors to be tested for serotype. It proved impracticable to attempt completely to eliminate macronuclear regeneration as an explanation for some of the mixed lines in the clonal class, because of the rarity of the clonal occurrence and the high inviability during and following autogamy. However, the segregation of a marker gene rules out macronuclear regeneration as the cause for all of the mixed lines of the clonal type. Eleven per cent of such mixed lines were also temperature-sensitive. In any macronuclear regenerating line *all* loci would be expected to retain their F_1 condition—mixed serotype and temperature resistance.

(v) *Fusion of non-sister haploid nuclei.* If during autogamy the gamete nuclei that fused were derived from different meiotic products—and therefore non-sister haploid nuclei—half the progeny of a clone undergoing such a reorganization would be heterozygous and half homozygous for one or the other of the two alleles. Thus, fusion of non-sister haploid nuclei could be responsible for mixing following autogamy. Lines in which mixing occurred in more than 50 % of the F_2 lines from a single F_1 clone, though, could not have arisen solely as a consequence of non-sister fusion if the nuclei united randomly. It is possible that the few mixed lines appearing in the non-clonal class of mixed lines could be so derived.

(vi) *Extra set of chromosomes.* Heterozygosity at the *C* locus could be due to polyploidy. If the mixed lines were due to polyploidy, segregation of the parental serotypes and high inviability would be expected to occur following subsequent autogamies. Segregation

would occur because the chromosome(s) carrying the extra *C* gene would be distributed randomly at nuclear division to the newly formed nuclei and some of those nuclei would then be expected to become homozygous for one of the alleles. The clone with such a nucleus would then be pure for a parental serotype. High inviability would result because of disturbances in distribution of the chromosomes at the reduction divisions.

No clonally mixed lines survived autogamy. This finding, by itself, does not support the existence of extra chromosomes, because typically autogamy in variety 2 lines will often kill most or all the cells of a clone. More convincing evidence that extra chromosomes may cause the clonal type of mixing comes from comparing the amount of inviability of the exconjugants of crosses of these mixed lines with parental animals with the inviability of crosses between pure C segregants and the same parental clones. The segregating clones used were either normal F_2 segregants (or lines which had been mixed as 'F_2's' and had segregated just prior to being crossed). In the crosses of the clonally mixed lines, inviability was more than three times as high as in crosses of F_2 7-C segregant $\times$ F_2 30-C segregant (4 pairs surviving out of 54 isolated compared with 45 pairs of 64).

(vii) *Other chromosomal aberrations.* Mortality found in crosses with mixed lines would also be expected from clones in which macronuclear regeneration or nuclear aberrations other than polyploidy had arisen. For example, if non-disjunction had occurred at meiosis, certain loci could be duplicated, e.g. the *C* locus. The fertilization nucleus would be tetrasomic at this locus and would possess both C^7 and C^{30} if the cell in which non-disjunction occurred were a hybrid. Aneuploid nuclei usually do not survive as well as normal nuclei in the formation of gametes (Kimball & Gaither, 1955). Again, such chromosomes would be expected to assort randomly following autogamy and occasionally some lines pure for C should segregate.

Two characteristics of mixed lines of the clonal class should be noted. The lines have a very low fission rate when carried in daily isolations in depression slides and survive for long periods of time only in mass cultures. Secondly, occasionally such mixed lines change in mass cultures, and then the entire clone becomes one of the parental serotypes. Whether this occurs during conjugation or following autogamy is not known. The failure of any of these mixed animals to survive autogamy in daily isolation suggests that this change may occur during vegetative reproduction. If so, then possibly this change is really a transformation to one of the parental serotypes even when both alleles are present in the macronucleus. The manifestation of a pure C serotype in the presence of both C^7 and C^{30} genes for at least twenty fissions is known to occur in F_1 clones, as had been mentioned. Of course, there are alternative explanations, such as a reversion to homozygosity through a loss of a chromosome or gene, etc.

Another chromosomal anomaly that could account for heterozygosis would be unequal crossing-over in a hybrid animal. Also, crossing-over within a complex locus could produce one recombinant chromosome with both *C* alleles. No inviability would be expected with crosses of animals whose syncarya contained chromosomes with *C* loci duplicated by either of these methods of crossing-over. Neither would segregation follow later autogamies.

It would appear, then, that several mechanisms acting concordantly or separately, such as conjugation of animals within an F_1 clone, the occurrence of non-autogamous animals in generally autogamous cultures, hemixis, fusion of non-sister nuclei, and macronuclear regeneration, could give rise to the non-clonal class of mixed lines. There is only evidence

for the operation of one, macronuclear regeneration. As for the clonally mixed lines, none of the above-mentioned mechanisms acting alone could account satisfactorily for these lines, although the combination of several acting independently would provide a reasonable account. Finally, a mechanism apart from those responsible for clonally mixed lines as a whole must be the cause for the temperature-sensitive mixed lines. It appears likely that some intranuclear aberration, such as aneuploidy, may reasonably explain their occurrence.

(4) *Crosses between animals of the same genotype but of diverse serotypes*

One of the most striking principles in antigen inheritance was demonstrated by Sonneborn (1948) in variety 4 when he crossed animals derived from a single homozygous animal but differing in serotype. Exconjugant clones retained their original serotypes. Thus, it was concluded, the cytoplasm governs which antigen (of the many an animal is capable of possessing) is actually manifested.

A similar cytoplasmic control has also been found in variety 2. Here a cross was made between one homozygous line of a non-C serotype (*d* 30-2 and another homozygous line of serotype 30-C (*d* 30-3). The *t* gene was used as a check on cytogamy and selfing. Exconjugants from this cross remained diverse in their serotypes. F_2 generations derived by autogamy were usually of the same serotype as the exconjugant clone. There was no segregation for serotype although *t* segregated normally. This result, then, confirms Sonneborn's observations.

DISCUSSION

A. Persistence of F_1 phenotype through autogamy

These studies have indicated that, in addition to the expected simple Mendelian control of specificity and of the ability to manifest an antigen, *P. aurelia* may show nuclear aberrations not unlike those found in the more classical genetic organisms. On first encountering the phenomenon of the persistence of the hybrid phenotype following a nuclear reorganization designed to induce homozygosity for all genes, interest was stimulated by the possibility that the failure of segregation was due to autonomous cytoplasmic determiners. More recent discoveries, however, make chromosomal abnormalities a more likely possibility. Since the initial discovery, Dippell (1956) has examined several stocks of *P. aurelia*, variety 4, and has found wide variations in chromosome number from stock to stock. Also Sonneborn (1954) has indicated that many radiation-induced mutations in *P. aurelia* are the result of chromosomal aberrations. In addition, recent studies on the genetic effects of ageing have demonstrated cytologically both micronuclear and macronuclear abnormalities (Dippell, 1955). Of particular interest was the occurrence of unequal segregation of clumped chromosomes at division which could be expected to result in chromosome losses and aberrations. Sonneborn & Schneller (1955) have also presented genetic evidence that in ageing clones phenotypes determined by alternate alleles segregate at macronuclear regeneration and at fission. Apparently, then, the sort of intra- and internuclear deviations suggested here as providing reasonable explanations for the genetic deviations encountered may not be of limited occurrence in the life cycle of Paramecium.

B. THE POSSIBILITY OF LINKED GENES IN THE CONTROL OF THE C SEROTYPE

Although the segregation of the parental C serotypes strongly supports the hypothesis that the specificity of the C serotype is controlled by a single pair of genes, the possibility that closely linked loci are involved has not been eliminated. The control of the synthesis of other 'surface' antigens by a series of closely linked genes has been suggested for the Rh group of antigens in man and has been mentioned in connexion with blood cell antigens in cattle (Fisher, 1947; Irwin, 1951). The existence of rare recombinants among the C serotypes would, of course, be difficult to demonstrate conclusively because of the large number of mixed lines—one of the recombinant types expected—occurring regularly, presumably due to causes other than crossing-over between loci. On the other hand, it may be possible to detect those lines unable to manifest C, the alternative recombinant type, by testing large numbers of animals in mass cultures for the presence of C individuals.

SUMMARY

1. The inheritance of antigens which produce immobilizing antibodies has been studied in *Paramecium aurelia*, variety 2. A single gene can control the ability to manifest an antigen. The appearance of one of two alternative cross-reactive antigens is also controlled by a single factor. The stability and titres of the F_1 clones of a cross between two homologous serotypes and the variation in the appearance of the serotype of such genotypically identical clones were examined.

2. The cytoplasm has an influence on the appearance of the immobilizing antigens in exconjugants of crosses between animals of similar serotypes and, especially, diverse serotypes. The influence of the parental serotype in crosses between cross-reacting serotypes may persist for as long as twenty postconjugation fissions, but in the majority of instances disappears within a few fissions. In contrast, when paramecia of the same genotypes but diverse serotypes are crossed, the parental serotype may persist indefinitely.

3. The persistence of a hybrid phenotype in F_2 generation animals presumed to be homozygous is probably due to nuclear abnormalities which ensure the maintenance of a heterozygous genotype, rather than to the action of cytoplasmic particles.

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