MUTATIONAL ANALYSIS OF MATING TYPE INHERITANCE IN SYNGEN 4 OF *PARAMECIUM AURELIA*[1]

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ABSTRACT

Six genic mutations restricting clones to mating type VII (*O*) were isolated in syngen 4, *Paramecium aurelia*. The only three extensively tested were neither allelic nor closely linked. A second type of mutation, allelic to one of the *O* restricted mutants, was also found. Clones homozygous for this mutant gene were selfers, producing both *O* and *E* (VIII) mating types, but only when they were progeny of mating type *E* parental clones. While all seven mutant genes behaved as recessives in monohybrid crosses, clones heterozygous at two different loci often demonstrated an unanticipated phenotype: selfing. The significance of the findings is discussed in relation to mating type determination and the evolution of mating type systems.

THE determination of mating type in most stocks of *Paramecium aurelia* is dependent upon nuclear differentiation (see PREER 1968 for review). The spectrum of nuclear mutations affecting mating type provides important evidence for the genic basis of nuclear differentiation (BUTZEL 1955; TAUB 1963). Mutational dissection of selected biological systems is common currency in the methodology of genetics (*cf.* BENZER 1967; EMERSON 1918). Such mutational analyses in *P. aurelia* are greatly facilitated by autogamy, a self-fertilization triggered by starvation in cells having undergone a stock-specific, minimum number of cell divisions since their last fertilization. Autogamy yields homozygosis at all loci; thus breeding to bring about expression of recessive genes is an efficient, one-step process. F_1 and F_2 generations from some stocks may be scored within two weeks of any initial cross.

Mating type reactivity precedes autogamy in young clones and is also triggered by starvation. Reactive cells of one mating type are capable of agglutinating with reactive cells of complementary mating type, owing to surface specifications called mating type substances (METZ 1954). This agglutination leads to pairing of conjugants.

The distribution of mating types following fertilization is dependent upon a

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developmental process. Newly fertilized cells produce two macronuclei and two micronuclei. The first cell division gives rise to two cells, each of which has one macronucleus and two micronuclei, numbers that are maintained throughout vegetative growth. The progeny of each of these first division products all have macronuclei of a common origin and comprise a caryonide which is usually pure for one mating type (SONNENBORN 1938). All the descendants from a single mating pair comprise a group called a synclone. In spite of genotypic identity, wild-type co-conjugant clones or, indeed, sister caryonides, may express different mating types. Cell lineage studies demonstrated that the macronucleus is the controlling element behind the expression of mating type differences (SONNEBORN 1938, 1947, and 1954).

Syngen 4 of *P. aurelia* is characterized, in part, by its two mating types, VII and VIII. These mating types are remarkably homologous, respectively, to the odd numbered and even numbered mating types of other syngens against which they can be tested (SONNEBORN 1957). Therefore mating types may conveniently and properly be referred to as the odd numbered (*O*) and even numbered (*E*) types.

Most syngens of *P. aurelia* exhibit one or another of two systems of mating type inheritance. Both are founded on the aforementioned basis of genically identical nuclei which are determined for one or the other mating type early in their development, before the first cell division. In the Group A syngens (1, 3, 5, 9, 11, and 14), *O* parents give rise to both *O* and *E* F_1 caryonides with the same frequency as *E* parents do. That is, determination is random in Group A. In the Group B syngens (2, 4, 6, 7, 8, 10, and 12), *O* parents typically give rise to *O* F_1 caryonides and *E* parents to *E* F_1 caryonides, with rare exceptions. Determination is thus highly nonrandom in Group B. The persistence through sexual reproduction of mating type in Group B is dependent upon a cytoplasmic determining agent produced by parental macronuclei which directs the determination of filial macronuclei (SONNEBORN 1954). The expressed mating type of one generation is sometimes not the same type as the cytoplasmic determining factors detected by the next generation (TAUB 1963).

In this communication, several new mutations affecting mating type are reported. Unpredicted interactions between these genes were observed. The relations of the results to a theory of mating type development and to the evolution of mating type are discussed.

MATERIALS AND METHODS

Stocks: Stocks of *P. aurelia* are most conveniently maintained as homozygotes, since periodic autogamy makes maintenance of heterozygotes technically more difficult. Syngen 4, stock 51 (kappa free) from the SONNEBORN collection at Indiana University was the wild-type source of the mating type mutants to be described. Two marker genes were employed in genetic analyses. The temperature-sensitive lethal gene, *ts111* (BEISSON and ROSSIGNOL 1969) was initially carried by derived stock d4–85, which also carried genes *k* and a^{29} which determined kappa loss and serotype, respectively. To decrease unnecessary heterogeneity, d4–85 was first crossed to stock 51 and F_2 segregants carrying *ts111* and *K*, rather than *k*, were selected. The presence of the a^{29} gene versus its stock 51 allele, a^{51}, was not ascertained in these segregants. The pawn gene, *pwA*

(Kung 1971), from stock d4–94 was also sometimes employed as a marker. Stock d4–109 is a "high reactor" (Austin, unpublished). When cultures of this stock reach mating type reactivity, they vigorously maintain that state for a much longer time than do wild-type cultures. For this reason they were a convenient source for standard mating types, called testers.

Culture techniques: A 0.25% infusion of Cerophyl rye grass (Cerophyl Laboratories, Kansas City, Mo.) was inoculated one day in advance of use with *Klebsiella aerogenes.* A single paramecium will divide in this culture fluid to produce 10^3 cells (10 fissions) in 0.5 ml within 72 hours at 27°C. Serial daily isolations were made in pyrex glass depression slides. If greater quantities of paramecia were required, test tube or flask cultures were initiated. Daily fission rates could be regulated by limiting the amount of fresh culture fluid provided (Preer 1948). Culture methods have been described in detail by Sonnenborn (1950, 1970).

Crosses: To obtain parental lines for crosses, the following procedure was used. A subclone was carried by serial isolation until ensuing starvation induced autogamy, as detected by Dippell's (1955) temporary acetocarmine-fast green stain. An autogamous 0.5 ml culture was transferred to a test tube and fed the following volumes on three successive days: 6 ml, 6 ml, and 12 ml. Twenty-four hours after the last feeding the tube of cells became mating type reactive. While these cells represented a mixture of caryonides, Group B mating type inheritance (see beginning of paper) insured that almost all cells would be the same mating type. Furthermore, all cells were relatively closely related, having originated from a common ancestral cell some 17–20 cell divisions earlier. This relatedness favored homogeneity of cytoplasmically located mating type determining agents.

Appropriate crosses were made observing two precautions. First, marker genes were utilized to reveal whether the cytogenetic processes of conjugation and autogamy had proceeded normally. Second, conjugating pairs were observed from the time they were nearly ready to separate and periodically until separation occurred. Usually the interval between the beginning and end of separation is a matter of a minute or less. Longer intervals are referred to as delayed separation. The extent of delayed separation is a rough measure of cytoplasmic exchange between mates (Sonneborn 1954). Wild type pairs with delays of one to two hours or more regularly undergo sufficient exchange to transfer effective amounts of the cytoplasmic determining agent(s) and thus to affect the pattern of mating type inheritance in Group B (Sonneborn 1954; Nanney 1957; Butzel 1968). Consequently one could detect pairs having unusual distribution of markers or having undergone massive cytoplasmic exchange as indicated by delayed (one or two hours) separation of mates at the end of conjugation.

Phenotype tests: To test any culture for mating type it was divided into three aliquots. To the first, reactive cells of known *O* type were added; to the second, reactive *E* cells. The third remained unmixed as a control. If the culture being tested was mating type reactive, a reaction between the culture and its complementary tester could be seen immediately and recorded. If that culture had not yet exhausted its food and therefore was not yet reactive, the mixture of tester and tested cells would feed until the supply of bacteria was depleted. This feeding rendered the reactive testers nonreactive, but subsequent mild starvation yielded simultaneous reactivity of tester and the unknown culture mixtures. If the culture to be tested had ceased to be reactive owing to excessive starvation, the cell mixture could be fed, left overnight, and observed next day. The first two procedures were more satisfactory than the last.

One of three diagnoses may be made for mating type. Most simply, a caryonide may be classified *O* or *E* on the basis of reacting with only *E* or *O* testers, respectively. Sometimes cells of both mating types arise within a caryonide. When these lines begin to starve, mating may be observed without the addition of testers. Such caryonides are called selfers (*S*). Selfers can be identified in two ways: mating within a caryonide and/or mating with both types of testers. In practice some selfers are difficult to identify. Some of these caryonides contain a very low percentage of reactive cells of one of the two mating types, and mating is exhibited only early in clonal history and/or briefly in a reactive period. Other selfers are more balanced in the proportion of the two mating types present at one time, showing an abundance of pair formation alone or in mixture with both testers. Careful observation throughout any reactive period and at more than one point in clonal history was necessary to rule out the possibility that any culture

that first tested as one type might not later show the other type as well. Among wild-type cells grown in bacterized culture, selfer caryonides are uncommon (NANNEY 1957).

Temperature sensitivity tests were most conveniently made using the replica plate described by SONNEBORN (1970). Replicates were made into fresh culture fluid; duplicates were placed at 27°C and 35°C. Readings were made 24–36 hours later. Early readings on temperature-sensitive clones at 35°C showed moribund, vacuolated, spherical cells. Prolonged exposure to high temperature led to lysis.

The pawn character was identified by the inability of *pwA/pwA* cells to respond to increased concentrations of Na^+ or K^+. A number of cells were transferred by pipette from culture fluid to DRYL's (1959) Na^+ rich solution while under microscopic examination. Wild-type cells react to this transfer by a period of vigorous backward swimming; pawn homozygotes swim directly forward into the salt solution. Alternatively, this swimming pattern could be observed in the culture fluid in which the clone was growing by adding several drops of 200 mM KCl which brings the medium to a concentration of about 20 mM for KCl. This method was quicker than, and as dependable as, the first. Differential diagnostic tests were best made on growing, rather than stationary, cultures.

Mutagenesis and selection: Masses of vegetative cells from a clone old enough to undergo autogamy (20 fissions) were treated for forty minutes at room temperature with MNNG (N-methyl-N'-nitro-N-nitrosoguanidine, K & K Laboratories, Inc., Plainview, N.Y.). The MNNG was first dissolved in a small volume of 95% ethanol (KIMBALL 1970) and brought to twice the desired final concentration with DRYL's solution in such a way that the paramecia were not exposed to a concentration of ethanol greater than 2%. Equal volumes of the MNNG solution and concentrated cells were added to depression slides. No visible toxicity of the mutagen was noted during the 40 minutes' treatment. The mutagen was diluted by a factor of 1.5×10^{-3} from the cells by repeated low speed centrifugation and resuspension. Still further dilution resulted when the cells from the last pellet were fed a volume of culture fluid calculated to give one to two fissions' growth. Treated cells were slower to exhaust their food supply than were controls. Upon examination after starvation both treated and control cultures exhibited high percentages of autogamous cells. This fertilization provided an opportunity for mutations which arose in any micronucleus a few cell divisions earlier to become homozygous in both macro and micronuclei. Since it is the macronuclear genes that are expressed, new mutant phenotypes could be selected.

The selection technique was devised by TAUB (1963). In Group B, exautogamous cells are almost always the same mating type as their preautogamous parents. Mutations affecting the mating type system may change this pattern of inheritance. If this happens, the mutant may express the minority mating type in mass exautogamous cultures. Potential mutants may then be selected because, upon mild starvation, their different mating type permits pair formation with the majority mating type present.

RESULTS

Mutagenesis: All mutant lines were derived from three experiments. In the second and most successful of these, 1.4×10^4 *E* mating type cells were treated with 50 μg/ml MNNG, followed immediately by growth to 4.2×10^4 cells in 20 ml of culture fluid at which point starvation induced autogamy. In a control culture, 6.4×10^4 cells underwent autogamy in the same volume of fluid. In order to ascertain the percentage survival, 100 autogamous cells were isolated from each group. Only 43 experimental isolates survived and grew; all control isolates survived.

A search for mating type mutants was made as follows. Experimental and control autogamous groups were each concentrated by centrifugation from 20 ml each to a fraction of 1 ml and resuspended in 100 ml of culture fluid. Each group was distributed in 0.5 ml portions to 200 depressions. Mating was observed in 50

experimental and 112 control depressions over a period of time which should have included the entire reactive phase. To estimate rates of change of mating type resulting from autogamy in the two groups, one must take into account the differential survival (see preceding paragraph) which resulted in different initial numbers of viable cells per depression for the controls (320) and for the experimentals (90). Furthermore, mating pairs may arise in depressions as a result of change in the direction of mating type determination in cells descended from only one or more than one initial autogamous cell. Therefore if $F =$ the frequency of depressions in which mating pairs are observed, then

$$F = \sum_{k=1}^{n} x^k .$$

Here $x =$ frequency of depressions in which one cell changed; x^2, two different autogamous cells changed in the same depression, etc. The number of viable cells in the inoculum is represented by n. By manipulating this expression algebraically and assuming that $x^n \simeq 0$, F is closely approximated by

$$F \simeq \frac{x}{1-x} .$$

Solving for x, x^2, x^3, etc., the frequency in which depressions arose containing one exceptionally determined autogamous cell, two such cells, three such cells, etc., can be calculated. The frequencies of the different classes of depressions, times the number of depressions, times the type or class of change (1, 2, 3, etc.) equals the numbers of cells changed. Since the numbers of viable cells used to initiate the two sets of depressions are known, rates of change can be calculated. In the experimental group the rate was 0.35%; in the controls, 0.27%. This control rate approximates the background level of nonmutational change. Interestingly, both estimates were considerably lower than NANNEY's 1–2% (1951). This difference may be attributed to a lowered sensitivity of the present technique, some change in the behavior of the stock under the conditions here employed, or a fundamental change in the stock.

The selection technique was based upon the formation of pairs, thus permitting immediate initiation of genetic analysis. From the experimental group, 46 initially viable pairs were selected, each taken from a different depression (and therefore of independent origin). Exconjugants were isolated into separate depressions and cultured, resulting in 35 fully viable experimental pairs of clones, i.e. synclones. Twenty-four control synclones were similarly isolated, all of which lived. Mating type tests were carried out on subcaryonides isolated from the clonal cultures. On the assumption of clonal uniformity (which closely approximates the facts), the results of the tests were taken to indicate the mating type of both the caryonide and the clone.

Most pairs gave rise to two exconjugant clones of complementary mating type, a pattern typical of wild-type cells (Table 1). Other patterns were also seen, particularly that in which both exconjugant clones were E. These and other combinations are, however, within the range of expression sometimes exhibited by

TABLE 1

Mating type distribution of exconjugants isolated in mutagenesis experiment

	Mating type of co-conjugant clones					
Group	*O-E*	*O-O*	*E-E*	*S-S*	*O-**	*E-**
Control	23	1	0	0	0	0
Experimental	26	0	7	1	1	0

* One clone failed to become reactive.

wild type (SONNEBORN 1954; NANNEY 1957; BUTZEL 1968) and not in themselves proof of mutation.

Exconjugant *E* clones were maintained in daily isolation until autogamy was observed in a sample of a starving leftover culture. If any clones had been heterozygous for recessive genes limiting the expression of mating type to *O*, 50% of their exautogamous progeny would express *O* in marked contrast to homozygous wild-type *E* clones where < 1% are likely to change mating type. As a preliminary tests, small groups of autogamous cells from each F_1 clone were fed, grown, and allowed to starve to mating type reactivity. Twelve groups showed some degree of exautogamous mating type heterogeneity: they conjugated within their groups without the addition of tester cells. When isolations were made from these same twelve autogamous cultures, five sets of isolations (from the only five cultures that gave rise to extensive mating in the preliminary test) showed segregation of mating types. Some were *E* and some, non-*E*. These five lines were designated e, f, g, h, and i, tentatively considered to be mutants, and were selected for further analysis.

Monohybrid crosses: In the homozygous condition, four of the five putative mutant genes restricted mating type to *O* (lines f, g, h, and i) even though the heterozygous parents and non-mutant F2 sister lines were type *E*. This sort of mutation also arose in two other experiments (lines a and c). Relatively low yields of mutants in these experiments may be attributed to less rigorous screening. The remaining putative mutant line expressed a phenotype whose determination has not previously been ascribed to a single gene. In line e, starvation brought on not just the potential to mate if challenged with cells of complementary type, but intracaryonidal mating. Cells descended from a cell with a single macronucleus expressed different mating types. Such caryonides are designated selfers (*S*).

That the *O*-restricted and *S* phenotypes were genically determined was proven by further crosses of these lines to wild-type *E* cells. In all cases using *O*-restricted mutations, the *E* parent carried the marker gene *ts111* (temperature-sensitive lethal) or *pwA* (pawn). In some crosses one parent was marked with *ts111* and the other with *pwA*. With few exceptions F_2 clones were initiated by isolation from only cultures exhibiting more than 95% autogamy. Data from crosses and subsequent autogamies were accepted only if the markers segregated acceptably in the F_2 generation. Marking was particularly important in crosses involving the selfer line since a mixture of a selfer with a clone expressing one mating type

TABLE 2

Cumulative F_2 mating type distributions resulting from monohybrid crosses of mutants to wild-type mating type E

Line	a	f	g	c	h	i	e
Mutant phenotype	*O*	*O*	*O*	*O*	*O*	*O*	*S*
Cumulative autogamous	*O:S:E*	*O:S:E*	*O:S:E*	*O:S:E*	*O:S:E*	*O:S:E*	*O:S:E*
F_2 segregations from *E* F_1's	84:1:92	96:2:108	33:2:38	16:0:16	16:2:21	29:0:32	0:30:28
χ^2	0.45	0.95	1.57	—	1.26	0.15	0.07
$P_{1:1}$	.50	.32	.21		.26	.69	.80

S are included among *E* in the χ^2 tests of columns a through i since they were clearly not *O*-restricted and therefore probably wild type. The χ^2 tests are for agreement with the theoretical 1:1.

did not insure pairing between them. Because selfer lines carried a recessive gene (*ts111*) that their intended mates did not, exconjugant pairs which tested as wild type in temperature-sensitivity tests were probably products of the desired cross. Eventual marker segregation completed the proof of the cross. Pairs of exconjugants, both of which were temperature-sensitive, were the result of selfing and discarded. In the mating mixtures, large numbers of wild-type *E* cells in proportion to selfers increased the probability of recovering the desired pairs.

Most commonly, both co-conjugant F_1 clones of a synclone were *E*, as determined by testing one caryonide from each clone (non-sister caryonides). Sometimes all four caryonides from each synclone were tested, with the compatible result. When the *E* clones (presumptive heterozygotes) underwent autogamy and isolations were made, F_1 clones yield F_2 ratios consistent with the 1:1 expectation for a pair of mendelian alleles (Table 2). A consistent skew toward *E* is noted among all six groups of F_2 progeny arising from the crosses involving lines with the *O*-restricted phenotype. This variation is in the direction expected if occasional isolations were made of non-autogamous individuals or individuals which underwent macronuclear regeneration (SONNEBORN 1947).

Allelism tests: Two mutant genes may have one of three relations: allelic, non-allelic and linked, or non-allelic and unlinked. The first and third possibilities are considered in detail in Table 3. Since two *O*-restricted cells cannot be crossed by conventional methods, *E* heterozygotes for one mutation (hypothetically designated *m2*) must first be constructed. These cells could be mated to other homozygous mutant lines carrying hypothetical *m1* (allelic) or *m3* (non-allelic) genes. The phenotypes of resulting generations depend upon whether synclones resulting from either cross maintain the *m2* or the $m2^+$ allele (50% chance for either one) from the *E* parent. Regardless of whether the two genes are or are not allelic, the two co-conjugant clones resulting from pairs maintaining the $m2^+$ allele from the *E* parent would both be *E*. (This prediction is based on empirical evidence noted earlier and in Table 1: simple heterozygotes for mating type mutations are often *E*. Exceptions to this trend, and their implications, will be discussed later.) These clones would be heterozygous for $m1/m2^+$ or $m3/m3^+$ and exhibit a 1:1 segregation of mating type following autogamy. Hence these syn-

TABLE 3

O-restricted mutations: anticipated results of crosses between homozygotes for one gene and heterozygotes for another

Assumption	Cross	F1 Synclonal genotype	F1 Co-conjugant phenotypes	F2 By autogamy Genotype	F2 Phenotype
				m1/m1	*O* } 100%
Allelism	*m1/m1* (*O*)	*m1/m2*	*O-O* 50%	*m2/m2*	*O* }
	×				
	m2/m2+ (*E*)	*m1/m2+*	*E-E* 50%	*m1/m1*	*O* 50%
				m2+/m2+	*E* 50%
Non-allelism	*m3/m3 m2+/m2+* (*O*)	*m3/m3+ m2+/m2+*	*E-E* 50%	*m3/m3 m2+/m2+*	*O* 50%
	×			*m3+/m3+ m2+/m2+*	*E* 50%
	m3+/m3+ m2/m2+ (*E*)	*m3/m3+ m2/m2+*	*E-E* 50%	*m3/m3 m2+/m2+*	*O* }
				m3+/m3+ m2/m2	*O* } 75%
				m3/m3 m2/m2	*O* }
				m3+/m3+ m2+/m2+	*E* 25%

TABLE 4

Segregation of mating types defining double heterozygotes

Cross	a homozygote × g heterozygote	f homozygote × g heterozygote	f homozygote × a heterozygote
Number of synclones	5	4	2
Autogamous F_2 segregation	O:S:E	O:S:E	O:S:E
from E F_1's	30:0:11*	21:0:16†	22:0:19†
	22:0:11‡	34:0:8*	26:0:8*
	11:0:20†	31:0:9*	
	25:1:8*	19:0:22†	
	10:0:11†		

* Deviates significantly at the 5% level from 1:1, but not from 3:1.
† Deviates significantly at the 5% level from 3:1, but not from 1:1.
‡ Does not deviate significantly from either 1:1 or 3:1.
Exact probabilities calculated after WARWICK (1932).

clones are not diagnostic for allelism. The set of co-conjugant clones resulting from pairs maintaining two mutant genes are critical in the allelism test. If alleles *m1* and *m2* were maintained, they should fail to complement and both exconjugant and subsequent exautogamous generations would be 100% *O*. If nonalleles *m2* and *m3* were maintained, complementation should yield a heterozygous (*E*) F_1 phenotype, but exautogamous clones should segregate as 3:1 (*O*:*E*) if the loci are unlinked. Linkage would enrich the *O* class at the expense of the *E* because the development of *E* would depend upon recombination.

The appearance of 3:1, in addition to 1:1, F_2 ratios (Table 4) indicates that genes determining *O* restriction in the three lines tested were non-allelic. The hypothesis of non-allelism was further substantiated by the recovery of double mutants in two combinations, a-g and f-g. These lines did not differ phenotypically from their F_2 single-mutant sister exautogamous clones and were distinguishable only by breeding analysis.

Allelism between selfer and *O*-restricted mutations may be more easily tested since *S* lines contain some *E* cells and therefore crosses can be made in a more direct manner. Anticipated results are depicted in Table 5. If a selfer gene (desig-

TABLE 5

Anticipated mating type results of crosses between O*-restricted and selfer mutants*

Assumption	Cross	F_1 Genotype	Autogamous F_2 Genotype	Phenotype
Allelism	*m1/m1 (O)*			
			m1/m1	*O* 50%
	×	*m1/s*		
			s/s	*S* 50%
	s/s (S-E)			
Non-allelism	*m2/m2 s+/s+ (O)*		*m2/m2 s+/s+*	*O* 25%
	×	*m2+/m2 s+/s*	*m2/m2 s/s*	? 25%
	m2+/m2+ s/s (S-E)		*m2+/m2+ s/s*	*S* 25%
			m2+/m2+ s+/s+	*E* 25%

TABLE 6

Mating type inheritance in crosses between the S *mutant and* O*-restricted mutations*

Cross	a×e	f×e	g×e
Autogamous F_2	O:S:E	O:S:E	O:S:E
segregation from *E* F_1	34:19:17	63:42:0	26:15:20
χ^2	0.17*	4.20†	2.15*
P	.92*	.04†	.36*

* Probability calculated on an expected ratio of 2:1:1.
† Probability calculated on an expected ratio of 1:1.

nated *s*) were allelic to a gene for *O* restriction (*m1*) one would expect the F_1 to be *m1/s* which should yield an F_2 exautogamous segregation of 1:1 (*O*:*S*). Nonallelic genes (*m2* and *s*) would yield an $m2/m2^+ \; s/s^+$ F_1. One might expect this F_1 to be *E*, but phenotypic variability in the F_1 was experienced, as will be discussed. An F_2 segregation of three types of progeny, *O*, *S*, and *E*, each occurring in a frequency of at least 25%, was anticipated. Only experimental evidence on gene interaction would disclose which phenotypic class would be enriched by the 25% double mutant genotypic class.

The F_2 results (Table 6) indicate that the gene determining selfers in line e and the gene restricting line f to *O* are allelic. The observed ratio is not a good fit to the theoretical 1:1. The deficiency in the *S* class may well have been a result of undetected selfing potential. This is a reasonable possibility because selfing is sometimes transient and small in amount (see MATERIALS AND METHODS—*Phenotype tests*). Indeed, although the fit to a 1:1 is poor, the alternative 2:1:1 expected for nonallelism is clearly excluded by the total absence of the *E* class. The 2:1:1 ratios from crosses of lines a × e and g × e fit an hypothesis of non-allelism and epistasis, where the presence of any homozygous *O*-restricted mutation prevents the expression of the selfing gene.

Only one combination (f-g, Table 4) showed any sign of enrichment of the *O* class expected for linkage, and this enrichment was not great. The gene controlling *O* restriction in line f was shown to be allelic to the gene controlling selfing in e. Since crosses between lines e and g (Table 6) did not give supportive evidence for linkage (a decrease in the *E* class), the linkage hypothesis was put aside.

These crosses provided the necessary information to assign meaningful symbols to the genes carried by each line. Symbols mtA^O and mtC^O are mutant genes from a and g, respectively; symbols mtB^O and mtB^S, to genes from lines f and e, respectively. The "*mt*" suggests the mating type system on which the genes work. Capital letters arbitrarily designate loci and superscripts descriptively designate alleles. Wild type is designated as a "+" in the allele position. *O*, *S*, and + were chosen rather than Roman numerals because they are more readily distinguishable in reading. This decision was made in recognition of the fact that syngen specificity is lost in the designation. Lines c, h, and i have not yet been tested for allelism.

Interactions between O-restricted genes: Isolation of double mutant stocks, homozygous for two different *O*-restricted genes, permitted obtaining for study (by crossing to homozygous wild type) sets of clones all of which were doubly heterozygous, thereby facilitating study of the double heterozygous phenotype. Without the double mutant stocks, double heterozygotes were restricted to 50% of the F_1 synclones depicted in Table 3. Double heterozygotes produced by the mating of mtA^o/mtA^o mtC^o/mtC^o or mtB^o/mtB^o mtC^o/mtC^o to wild-type *E* clones carrying a marker gene resulted in an unexpected phenotype: selfing. However, selfing was not observed in all synclones or even all caryonides of one synclone; it was found in 0–4 of the 4 caryonides in different synclones. This result may formally be described in either of two ways: in selected genetic backgrounds, mt^+ genes are not completely dominant or non-allelic genes fail to complement completely (i.e., do not give a completely wild-type phenotype). Mating tests of heterozygotes require greater care in timing than do those of homozygotes because their genotype can change so early in clonal history in syngen 4 as a result of the early occurrence of autogamy. Scrupulous care was taken to identify all selfing caryonides. Nevertheless some doubly heterozygous caryonides expressed *E* exclusively. Doubly heterozygous genotypes encouraged, but did not demand, selfing.

In one cross of mtA^o/mtA^o mtC^o/mtC^o to an *E* line wild type for all genes but pawn, 31/52 caryonides selfed while in another cross of the same stocks only 3/20 caryonides selfed. Two of these latter selfing caryonides arose from one exconjugant of a pair showing exceptional distribution of the marker gene. Inquiry into the cause of this particular abnormality made the scope of gene interactions more clear. Both selfing caryonides were wild type for the pawn character, but the two *E* caryonides from the co-conjugant clone, which should have been genotypically identical, were pawn. The pawn exconjugant had not incorporated into its macronuclei the wild type allele that should have been donated by its *O*-restricted mate.

From each of the four exceptional caryonides, 21 autogamous isolations were made, as were 84 isolations from an entirely *E* F_1 synclone that was wild type for the pawn character. Only 2/84 clones survived from the exceptional group. They were phenotypically identical to their *E*-pawn parents and may not have undergone normal cytogenetic processes at autogamy. In the normal group, 73/84 survived autogamy. Mating type segregated as 58 *O*:15 *E* approximately a 3:1 ($\chi^2 = 0.77$; $P_{3:1} = 0.37$).

The death of virtually all F_2 progeny of the exceptional synclone and the F_1 distribution of marker genes suggests cytogenetic error in the initial mating. Another such synclone was earlier observed, but less well analyzed. An hypothesis of unilateral fertiliaztion is compatible with these observations. CHEN (1940) presented cytological evidence in *P. bursaria* for this sort of fertilization. SONNEBORN (personal communication) has evidence for similar phenomena in syngens 4 and 13 of *P. aurelia*, as has HAGGARD (in preparation) in intersyngenic crosses. As in any normal fertilization, the mates would have formed haploid gamete nuclei, two $mtA^omtC^opwA^+$ nuclei in one mate, and two mtA^+mtC^+pwA nu-

clei in the other. Normally, reciprocal fertilization would yield genotypically identical exconjugants. However if one $mtA^{+}mtC^{+}pwA$ gamete nucleus of one conjugant, but no $mtA^{0}mtC^{0}pwA^{+}$ nucleus of the other conjugant migrated into the mate, the results would be a haploid (pawn) exconjugant and a triploid (wild-type) exconjugant if gamete union and synkaryon development proceeded in spite of haploidy and triploidy. Like the exceptional synclone, such cells would not survive autogamy because of the aneuploidy that would result from meiosis. If this analysis is correct, the selfing caryonides (the triploids) in this exceptional synclone were genotypically $mtA^{0}/mtA^{0}/mtA^{+}$ $mtC^{0}/mtC^{0}/mtC^{+}$ $pwA^{+}/pwA^{+}/pwA$, and therefore had a larger dose of mutant *mt* alleles than the generally non-selfing diploids mtA^{0}/mtA^{+} mtC^{0}/mtC^{+} pwA/pwA^{+} in the same experiment.

Further observations on mtB^{S}: Two features of the mtB^{S}-determined selfing character stood out. First, selfing was most frequent and abundant in young cultures. Subclones developing from successive daily isolations—axial (BLEYMAN 1967a) or discard (TAUB 1966a) cultures—showed progressively less selfing. This decrease was paralleled by a decrease in reactivity when stable wild-type discard cultures were tested over a period of days against reactive testers and probably reflected the onset of autogamy which ends mating type reactivity. Second, if an mtB^{S} culture was tested for mating type, usually more pairs formed with *E* than with *O* testers. The proportions of the two mating types in these selfing cultures was typically $O > E$.

Double heterozygotes for *O* and *S* genes were constructed in the allelism tests reported above. As was the case with double heterozygotes for *O*-restricted genes, results varied from pair to pair. In the mtA^{0}/mtA^{+} mtB^{S}/mtB^{+} combination 20/56 and 17/20 caryonides selfed as $E > O$ in two different experiments. Nonselfing caryonides were *E*. Selfing as $O > E$ was observed in 7/20 mtB^{S}/mtB^{0} caryonides. The remaining caryonides were *O*. All mtB^{S}/mtB^{+} mtC^{0}/mtC^{+} caryonides examined thus far have been *E*, but this examination was not a fully critical one.

Two other simple crosses were done in the analysis of mtB^{S}. Crosses between two mtB^{S}/mtB^{S} individuals from one selfing clone yield selfing enconjugants caryonides. NANNEY (1957) reported that wild-type selfing pairs gave rise to *O* caryonides more frequently than any other class, including *S*. Earlier, the cross of the homozygous selfer (mtB^{S}/mtB^{S}) expressing *O* to wild-type *E* (mtB^{+}/mtB^{+}) was described. A reciprocal cross of the homozygous selfer (mtB^{S}/mtB^{S}) expressing *E* to wild- type *O* (mtB^{+}/mtB^{+}) gave further insight into the action of mtB^{S}. *O-E* non-sister caryonides from co-conjugant clones arose from 21/22 synclones; *S-E*, from 1/22. An autogamous F_2 taken from *E* caryonides gave a segregation of 0:53:27(*O*:*S*:*E*), a poor fit to a 1:1 ($\chi^2 = 8.45$; $P = 0.0025$) in contrast to the good fit achieved in the reciprocal cross earlier described. Clearly something other than chance enlarged the *S* class. This *S* class may have had some wild-type members. If this were so, and the result continues to be repeated, a new, relatively complex, nucleo-cytoplasmic interaction may be demonstrable. From the *O* F_1 parents, a 49:0:0: (*O*:*S*:*E*) autogamous F_2 developed. The marker gene

segregated well in both F_2 groups, indicating that cytogenetic processes had proceeded normally, i.e., the *O* and *E* F_1 clones must have been genotypically identical in spite of giving very different mating type results in the F_2. The failure to self of the 50% of the 49 clones expected to be carrying mtB^S/mtB^S shows that this gene cannot promote selfing in clones possessing odd determining cytoplasm.

DISCUSSION

The number of mt loci: In syngen 1 of *P. aurelia*, three mutant stocks restricting cells to the odd-numbered mating type were described (SONNEBORN 1947; BUTZEL 1955). All three are alleles at one locus (BUTZEL 1955). This observation suggests *mt* loci are few in number or limited to one per genome. It is important to realize that the scheme used to screen for *mt* mutations is less efficient in Group A syngens (like syngen 1) than in Group B (Syngens 4 and 7) since change of mating type is common following autogamy in A syngens. More than one locus has been demonstrated in both syngens 4 and 7; there is no proof that the upper limit of loci has yet been approached in either case. The contrasts between the A and B syngens do not necessarily reflect a difference between the mating type systems in the syngens. Repeated hits at the same locus in syngen 1 may reflect some peculiarity of the locus rather than a dearth of *mt* loci in syngen 1.

One of the mutant lines in syngen 1 arose after a series of heat shocks during three successive meioses (BUTZEL 1955). Heat shock is known to interfere with macronuclear division in Paramecium (SONNEBORN 1947, p. 294) and in some circumstances may cause abnormalities in developing micronuclei (SONNEBORN 1950). As a group, paramecia are karyotypically complex (DIPPELL 1954; KOŚCIUSZKO 1965). Haploid chromosome numbers range from 33 to 63 in different stocks. In some stocks duplications might lead to chromosomes or chromosome regions with few unique sequences. If an *mt* locus were such a unique region among otherwise duplicated genes, loss of that region could be relatively undamaging to the organism and result in a genically-restricted *O* phenotype. The sort of chromosomal aberration resulting in such a deletion may be more frequent than any particular point mutation under the conditions employed, resulting in a supramolecular hotspot. This interpretation could be tested by standard methods distinguishing point mutations from deletions. Reversion or recombination tests could be applied efficiently using the selection technique for mutants or recombinants that has worked so well in Group B.

The E F_1 phenotype: When the corresponding wild-type allele was introduced into any of the mutant lines, usually *E* clones resulted. Either the heterozygous genotypes dictated the *E* phenotype, or the mutant lines carried *E*-determining cytoplasm despite their *O* or *S* phenotype. An *E* cytoplasmic state in the mutants could be a result of cyclic determination of new nuclei for their initial, premutant, cytoplasmic state (*E*) independent of expressed mating type. Alternatively, *E* cytoplasm could be a second effect of the mutant genes themselves. TAUB (1963) found both situations in syngen 7, in the only previously-described mating-type mutations in the Group B syngens. His mutant gene *n* has no effect on the cytoplasmic state while mt^{XIII} acts as a dominant gene for *E*-determining

cytoplasm in this O-restricted stock. In syngen 4, crosses of some selected mtA^O and mtC^O clones to wild type gave rise to O and E co-conjugant clones. Heterozygosity was confirmed by the marker gene and segregation of the mating type character in the autogamous F_2 from the F_1 E clones. J. DILTS (personal communication) reports O-E co-conjugant clones resulting from a cross of mtB^O to wild type where each parent carried several marker genes. No F_2's were followed. Evidently, mtA^O, mtB^O, and mtC^O can be and sometimes are accompanied by O-determining cytoplasm. Heterozygotes for these genes need not be E.

In relation to these observations, it is important to realize that in wild type a small percentage of stable O clones is known to arise after fertilization (conjugation or autogamy) from E clones (SONNEBORN 1947; NANNEY 1951) in Group B. This indicates that the cyclic redetermination of nuclei to produce E-determining cytoplasm is sometimes broken. Hence a continued mass culture derived from a clone carrying E-determining cytoplasm will come to include clones with O-determining cytoplasm. In agreement, some mtA^O, mtB^O, and mtC^O individuals gave rise to O heterozygous progeny. It is therefore likely that these mutations resemble TAUB's n mutation in having no effect on the cytoplasmic state. In both his and the present examples, the determination of the expressed mating type and the determination of the cytoplasmic state have been uncoupled.

Dihybrid F_1 variability as to selfing: Single heterozygotes selfed no more frequently than wild-type clones; double heterozygotes selfed more frequently, if unpredictably. The cause of selfing among the caryonides doubly heterozygous at two *mt* loci remains undecided, as does the reason some do not self. The lack of selfing cannot be attributed to infrequent observation or gross cultural differences. However, F_1 selfing was curiously more prevalent in crosses made soon after the initial construction of the double-mutant parental stocks than crosses of the same stocks twenty months later.

Phenotypic lag cannot explain the observed pattern of selfing, as it did for the cytoplasmic progeny of E parents in another syngen (KIMBALL 1939). Selfing was found in the present study among the cytoplasmic progeny of both the O and E parents. If E were to be the final stable F_1 phenotype of double heterozygotes, selfing owing to phenotypic lag would be expected only in the descendants of the O parent.

The exceptional synclones described in RESULTS and the tendency of double heterozygotes for *mt* loci to self suggests that one kind of genically-mediated selfing is curiously related to the number of mutant genes present. Whether allelic or non-allelic, increasing dosages of mt^S seem to increase the probability of selfing. The effect is not simply additive, however. Two mutant genes at the same locus give a decidedly different phenotype (O) than two mutant genes at different loci (S or E).

Models of mating-type systems: BUTZEL (1955) suggested that the *mt* loci in syngens 1 and 7 control a terminal reaction in the development of E mating type. The mt^O-like alleles are incapable of completing this reaction and the mutant cells are O by default. BUTZEL had earlier (1953) described his model in more detail. Based on the absence of E-restricted mutations (still a void in spite of the

unpublished efforts of many), *O* mating-type substance was assumed to be a precursor of *E* mating-type substance. Mutations which prevent the production of *O* substance might also restrict the production of *E* substance on this hypothesis. It is this model, $O \rightarrow E$, which has been most widely discussed. TAUB (1966a, 1966b) believed his results on wild-type selfers in syngen 7 supported the $O \rightarrow E$ hypothesis. Cells that changed mating type over a short period of time did so in the direction of $O \rightarrow E$. KIMBALL's (1939) finding that phenomic lag is much shorter when $O \rightarrow E$ than when $E \rightarrow O$ following fertilization also fits the hypothesis, as do BLEYMAN's (1967b) results with syngen 5, *P. aurelia.*

New observations presented here fit within the bounds of the $O \rightarrow E$ hypothesis. The mt^o-like alleles have been considered null alleles or amorphs (BUTZEL 1953). One could consider mtB^s a hypomorph or a low-level producer of an element in the $O \rightarrow E$ pathway. On the $O \rightarrow E$ hypothesis the existence of an amorphic allele of mtB^s could have been and was correctly predicted. The epistatic relationship between mtB^s-mtA^o and mtB^s-mtC^o also fits the hypothesis. If *mtA*, *mtB*, and *mtC* are all loci directing different steps in the pathway $O \rightarrow E$, then the effect of a partial block at *B* would be obscured by a complete block at *A* or *C*. The absence of mtB^s-induced selfing in *O*-determining cytoplasm is also compatible with the hypothesis. If some *mt* locus (or loci) were the site of the determinative switch(es), regulating *O* versus *E* expression in wild type (BUTZEL 1953, 1955; TAUB 1963), *O*-determining cytoplasm would inactivate (or fail to activate) the switch(es), thus preventing *E* substance formation and preempting the activity of mtB^s.

The suggestion that *O*-determining cytoplasm prevents selfing is not consistent, it would seem, with observations on wild type. Genic (mtB^s) selfers have *E*-determining cytoplasm and self as $O > E$. Wild-type selfers usually have *O*-determining cytoplasm and usually self as $E > O$ (NANNEY 1957). If *O*-determining cytoplasm inhibits selfing in the mutant mtB^s, why does it not also do so in wild type? The cytoplasmic state of a clone or cell is defined by the mating type developed by its offspring after fertilization. NANNEY's wild-type selfers thus could have had non-*O*-determining cytoplasm at the time of their origin, but later, under the influence of the new macronucleus determined for selfing, could have produced *O*-determining cytoplasm. This interpretation means that *O*-determining cytoplasm is restrictive (or *E*, permissive) only *vis à vis* developing, not vegetative, nuclei.

Conventional genetic analyses have now demonstrated several relationships between cytoplasmic determining factors and genes governing the expressed mating type. The spectrum of mating type mutations found to date and the observations on wild type are suggestive, but not proof, of the $O \rightarrow E$ hypothesis. As has been pointed out, speculations on models of differentiation are only useful if they promote new experimental avenues, and molecular approaches are, at present, lacking (PREER 1968). Mutant stocks could certainly play a key role in the promotion of biochemical analyses.

Evolutionary considerations: In all, there are three major modes of mating type determination in ciliates: genic differences controlling mating type at one

or more loci, nuclear differentiation overlying genic identity, and incorrigible (by genetic standards) selfing. The largest class, by number of taxa, is probably the last (RAIKOV 1972). Many of the most carefully studied examples exhibit features of more than one mode. In some paramecia, only one stable mating type can be isolated under non-selective conditions. Selfing is the rule among some clones of many stocks in syngen 2, *P. aurelia* (PREER, RUDMAN and SONNEBORN personal communication) and among clones that are not exclusively *O* (IX) in syngen 5, *P. aurelia* (BLEYMAN 1967a). In Syngen 13, *P. aurelia*, the two alternative mating types are directly determined by two alleles of one locus. Heterozygotes may self (SONNEBORN 1966). HIWATASHI (1968) demonstrated the genic basis for mating type in syngen 3, *P. caudatum*. Here some clones having either one or two dominant alleles at the *mt* locus self. HIWATASHI found that certain crosses between stocks encouraged the appearance of selfers, possibly under the influence of genes at other loci interacting with the dominant allele at the *mt* locus. In *Tetrahymena pyriformis*, syngen 1, NANNEY, CAUGHEY and TEFANKJIAN (1955) discovered that genes determine the possible spectrum of mating types, and nuclear differentiation is responsible for limiting expression to one type.

The genes and gene action described in this paper provide several examples of possible evolutionary steps—simple steps—that could have led to the diversity of modes of mating type inheritance noted above. This is not to suggest that the Group B system is the primeval mode, but only that it is not far removed from other modes. Constitutive production of a permissive cytoplasmic state and the introduction of an *O*-restricted gene (mt^{XIII}) could transform the pattern of mating type inheritance. If only two stocks were known in syngen 7—one with mt^{XIII} and the other with a gene whose action is like mt^{XIII} with respect to cytoplasmic determination but was not *O*-restricted—the mode of mating type inheritance would be like syngen 13. Alleles like mtB^{S} introduced into such a hypothetical system might simulate the situation for *P. caudatum*, syngen 3. A population where mtB^{S}-like genes were prevalent (and the cytoplasmic milieu permissive) would self.

The imposition of genic lesions on genomes otherwise capable of expression of either mating type has not provided the definitive answers to problems of mating type development or evolution. But with accumulation of more data, particularly on the biochemical level, the mutational analysis approach could become a touchstone to these problems.

This paper is dedicated to T. M. SONNEBORN on the thirty-fifth anniversary of his discovery of mating types. I am greatly indebted to him for his help and criticism throughout the course of this work.

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