GENIC MUTANTS WITH ALTERED SYSTEM OF EXCITATION IN *PARAMECIUM AURELIA*. II. MUTAGENESIS, SCREENING AND GENETIC ANALYSIS OF THE MUTANTS[1]

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THE development of behavioral genetics in *Paramecium aurelia* reported here is part of an attempt to dissect the system of behavioral control centering on the excitable membrane.

Molecular lesions can be produced in cells by mutagenesis. Mutants can then be screened for behavioral deviations which indicate lesions in the excitable system. By studying the phenotypic expression behaviorally and electrophysiologically, one can try to understand the relation between the mutated molecular entities and the normal function of the system. It may also be possible to identify the mutated molecules using available immunological and biochemical techniques.

While the rationale behind this work is the same as BENZER's (1967), the level of study is quite different from the genetic dissection of the nervous system. The pellicular fibrils in ciliates have apparently no function in coordinating ciliary beats (OKAJIMA and KINOSITA 1966; NAITOH and ECKERT 1969b) as had been previously suggested (TAYLOR 1920), and are thus a poor analog of the central nervous system of multicellular forms. On the other hand, these eukaryotes can be viewed as excitable cells capable of generating receptor potentials and action potentials (KINOSITA, DRYL and NAITOH 1964a, 1964b; NAITOH and ECKERT 1968a, 1968b, 1969a) upon proper electrical, mechanical and chemical stimulations. Protozoa are thus comparable to excitable cells such as neurons, muscle fibers and hair cells in metazoa.

Paramecium as an experimental material for such study offers the following advantages:

1. Paramecium behaves relatively stereotypically (JENNINGS 1906). The locomotor behavior of the organism reflects the electrical behavior of the membrane. Ciliary reversal (hence, backward swimming) is correlated with membrane depolarization, whereas augmented ciliary beat in the normal direction (rapid forward swimming) is correlated with hyperpolarization (KINOSITA, DRYL and NAITOH 1964a, 1964b). Changes in membrane (and other structural components involved in movement) by mutation will appear as changes in behavior. Thus,

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screening for mutants with altered behavior is an effective means of finding membrane mutants.

2. It is often difficult to ascertain that certain cells are primarily responsible for the mutant behavior in multicellular animals, although it can sometimes be accomplished by ingenious techniques (HOTTA and BENZER 1970). This problem does not arise when working with unicells such as paramecia.

3. Paramecium is large enough to allow insertion of microelectrodes (KAMADA 1934). This is very difficult if not impossible to do in bacteria. Intracellular recording is important to the understanding of the excitable membrane in conventional electrophysiological terms (KINOSITA, DRYL and NAITOH 1964a, 1964b; NAITOH and ECKERT 1968a, 1968b, 1969a).

4. *P. aurelia* can undergo an almost unique nuclear reorganization, i.e., autogamy, in addition to conjugation (DILLER 1934; SONNEBORN 1937). The union of identical haploid nuclei during autogamy makes the postautogamous lines completely homozygous. This facilitates genetic analysis and makes the study of mutagenesis almost as simple as with haploid organisms.

5. Besides allowing gene transfer, conjugation is a process in which the plasma membranes of the mates are temporarily fused. Membrane fusion can be made permanent through many life cycles by cortical genetic techniques such as doublet formation and cortical grafting (BEISSON and SONNEBORN 1965; SONNEBORN 1970). Fusion of membrane together with microsurgery of the cortex (CHEN-SHAN 1969) is another way of manipulating the membrane.

6. Biochemical analysis of the neuronal membrane from nervous tissue is handicapped by the presence of other cell types besides neurons in the sample. Because pure clones of paramecia can be grown rapidly in monoxenic and axenic media (VAN WAGTENDONK, COLDMAN and SMITH 1970; SONNEBORN 1970), they are potentially good material for membrane biochemistry. The ciliary membrane is continuous with the plasma membrane and covers the major part of the total surface. In ciliates, this portion of the membrane has been fractionated and purified (GIBBONS 1963).

7. Paramecium has a short and controllable life cycle and can be handled easily. The tremendous amount of literature in all branches of biological research on this genus provides a solid basis for further research.

Wild type paramecia swim forward in a spiral course. Mechanical stimulation of the posterior part as well as certain chemical stimulations, such as transfer to $CaCl_2$ solution, induce augmentation of the ciliary beat in the normal direction. This results in accelerated forward movement, a state correlated with membrane hyperpolarization (KINOSITA, DRYL and NAITOH 1964a, 1964b; NAITOH and ECKERT 1969a). On the other hand, impact at the anterior end and many other stimuli induce the avoiding reaction (JENNINGS 1906). This reaction, in part, consists of a period of backward swimming which is accomplished by reversing the direction of the ciliary beat. Ciliary reversal is correlated with membrane depolarization. Avoiding reactions induced by transferring the cells to solutions of high concentrations of Na^+ and K^+ (with relatively low Ca^{++} concentration) are of special interest in our study.

TABLE 1

Diagnostic characteristics of different strains of P. aurelia*

Strain	Behavior in culture fluid	Behavior on transfer to Na-Ca solution†	Behavior on transfer on K-Ca solution†
Wild type	normal	repeated avoiding reaction	repeated avoiding reaction
Fast-1	rapid forward swimming	repeated avoiding reaction	repeated avoiding reaction
Fast-2	rapid forward swimming	rapid forward swimming	repeated avoiding reaction
Pawn	complete lack of avoidance	rapid forward swimming	rapid forward swimming
Paranoiac	spontaneous long distance backward swimming	rapid backward swimming	repeated avoiding reaction

* For a complete description of the mutant phenotypes see KUNG (1971a).

† Na-Ca test solution is that of DRYL (1959) and the K-Ca test solution is 8 mM KCl, 0.3 mM $CaCl_2$ and 1 mM Tris, pH 7.2. These diagnostic tests were made by transferring small populations by micropipette gently from the cerophyl culture medium to the test solutions.

Mutants in *P. aurelia* have been studied in which the system of behavioral control is affected but apparently not the ability to move nor even the physical ability to reverse the ciliary beat (KUNG 1969, 1971a, 1971b). Mutants "Fast of the 1st type" (abbreviated Fast-1) have as their outstanding characteristic an accelerated forward movement. "Fast of the 2nd type" (Fast-2) are much less sensitive to Na-stimulation whereas their reaction to K^+ is apparently normal. "Pawn", being insensitive to Na^+, K^+, Ba^{++} or mechanical stimulation, show a virtually complete lack of avoiding reaction. Mutants "Paranoiac" retain the normal threshold for Na-induced reactions but the reactions are much exaggerated into continuous ciliary reversals of long duration. The diagnostic features of the mutants are given in Table 1. A detailed description and analysis of the phenotypes of these mutants is given in the first paper of this series (KUNG 1971a). The present paper, which is the second of the series, describes the induction, selection and genetic analysis of these behavioral mutants.

All mutants except the type Fast-1 were recently discovered and have no previously known parallels. A strain of fast swimmers (now classified as Fast-1), apparently appearing spontaneously, was studied by COOPER (1965). They swam at accelerated speeds but could not mate. No tests of their reactions to stimulation were made. SONNEBORN and SCHNELLER (unpublished) also found a fast swimmer (designated as "thin") which could be crossed only with great difficulty. Upon crossing, however, the trait was found to be controlled by a recessive gene.

MATERIALS AND METHODS

All strains used were of syngen 4 of *P. aurelia*, including the wild type stock 51s (non-kappa bearing) and d4-85 carrying the temperature sensitivity (T.S.) gene ts_{111} (BEISSON and ROSSIGNOL 1969).

The technique of culturing paramecia was basically that of SONNEBORN (1950, 1970). The culture medium used was cerophyl medium buffered with Na_2HPO_4 and inoculated with *Aerobacter aerogenes* 12 to 48 hr before use. The methods of obtaining sexually reactive animals and mating pairs, induction of autogamy, replications of depression cultures and other general techniques of handling paramecia are given by SONNEBORN (1970). Cytological examinations of the states of the macronucleus were made by using DIPPELL's (1955) stain. Temperature sensitivity was tested at 37°C overnight in rapidly growing cultures.

In each mutagenesis experiment a population of paramecia of the order of 10^6 was treated with the mutagen. These cells, over 20 fissions from the last autogamy, were growing rapidly and they were never starved. Cytological examinations were made to be sure that no cell was undergoing autogamy before the treatment. N-methyl-N'-nitro-N-nitrosoguanidine was dissolved in Dryl's solution (DRYL 1959) to 150 μg/ml. The cells were then concentrated, first with the oil testing centrifuge (PREER and PREER 1959) and then with the clinical centrifuge. The pellet was resuspended in a small volume (10–20 ml) of Dryl's solution. An equal volume of the nitrosoguanidine solution was added, giving a final concentration of the mutagen of 75 μg/ml. The animals were left undisturbed in the solution at room temperature for one hour. The animals were then washed three times with Dryl's solution by centrifugation and resuspension. Finally, the cells were returned to fresh culture fluid, allowing roughly 2 fissions of growth before they were starved. In most experiments, the treated cells were separated into 8 or 16 different flasks. When the cultures cleared, the starved animals were examined cytologically to be sure that they were in autogamy. Over 100 cells were taken at random from the culture in single cell isolations to estimate the proportion of exautogamous deaths. Desirable amounts were taken from different flasks and were fed with fresh culture fluid to permit a given number of exautogamous fissions (usually 5 to 10). The cultures were then ready to be screened for mutants.

In all the mutagenesis experiments carried out in the course of this investigation, the exautogamous deaths amounted to 40–60%. While only a few kinds of behavioral mutants are of special interest here many different kinds of mutants were isolated. These included sluggish swimmers, clear-bodied mutants, chain formers, trichocyst mutants and gross morphological mutants. A few of these were examined and proved to be genic. One of them, with body deformation (abbreviated B.D.), was used to provide a gene marker, beside ts_{111}, for some of the analyses below. This mutant carries a pair of recessive alleles (*bd bd*) with high penetrance which produce a phenotype ranging from a slightly bent anterior end to extreme, monstrous distortion.

RESULTS

Devices and techniques for screening mutants less reactive to Na^+ stimulation: Paramecia show negative geotaxis and tend to gather near the surface. Avoiding reactions make the cells unable to swim in any definite direction and thus interfere with the spatial orientation necessary for geotaxis. For instance, when a wild-type population was introduced into the bottom of a column filled with a solution with a high concentration of Na^+ and relatively low Ca^{++} concentration, the cells went into repeated avoiding reactions moving randomly near the bottom of the column. On the other hand, when the same treatment was given to a mutant population which was insensitive to Na^+, the cells would swim actively upward since the lack of chemotaxis to Na^+ allowed the exhibition of geotaxis. For instance, there was a drastic difference in the distribution of wild-type and Na-insensitive mutant populations in a vertical column 5 min after the animals were transferred from their cerophyl medium to a medium containing NaCl (20 mM), $CaCl_2$ (0.3 mM) and Tris (pH 7.2) (1 mM). The Na-insensitive mutants (Fast-2, in this case) moved up and aggregated at the top while the wild types stayed almost exclusively at the bottom. Less than 0.01% of the wild type

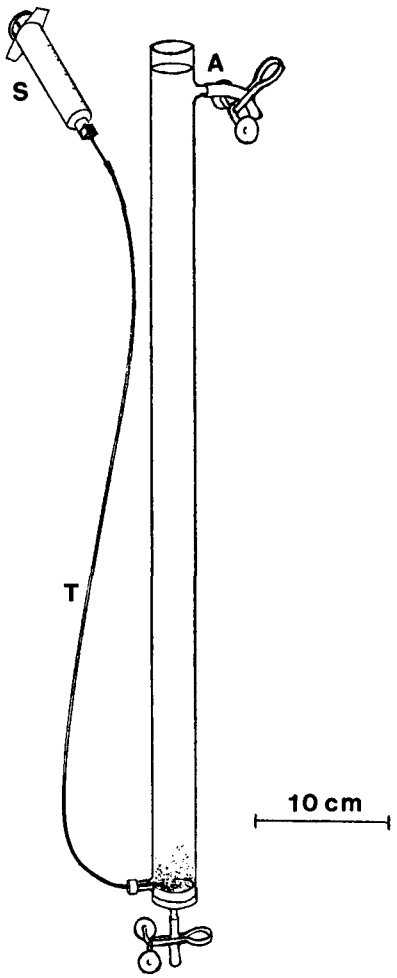

FIGURE 1.—Apparatus for the screening of mutants less sensitive to cationic stimulation based on the principle of chemotactic interference with geotaxis. See text for description and operation.

managed to appear near the top (KUNG 1971a.) This difference allowed us to screen for the variants which are insensitive to Na^+.

A liquid column (Figure 1) was used for the screening. Animals were injected with a syringe (S in Figure 1) (gauge 16 B-D hypodermic needle) through polyethylene tubing (T) (PE 240, i.d. 1.68 mm) to the bottom of the column of Na-Ca solution filled to 3 cm above the upper side arm (A). From this outlet, a fraction of fluid was withdrawn at a certain time after the injection. The content of this fraction was carefully examined. With reflected light the movement of the animals in the column was observable.

In early experiments, mutagen-treated populations (of the order of 10^5 cells) in 2–4 ml of cleared cerophyl were injected into the bottom of the column filled with a solution of 20 mM NaCl, 0.05 mM $CaCl_2$ and 1 mM Tris (pH 7.2) and the

TABLE 2

*Results of screening using columns filled with 20 mM NaCl, 0.05 mM $CaCl_2$ and 1 mM Tris, pH 7.2**

Experiment	Number of runs with the column	Number of lines from cells in the top fraction				Total
		Wild type	Slow grower	Dead	Fast or Pawn	
I	5	26	13	17	5	61
II	6	21	9	15	0	45
III	5	40	8	3	1	52
IV	6	15	12	7	5	39
Total	22	102	42	42	11	197
†Control of III		140	23	5	0	168
IV		123	20	4	0	147
Total		263	43	9	0	315

* Top fractions were collected within 8 min. See text for method of screening.
† Control lines were derived from cells of the same mutagenized, exautogamous population in numbered experiments. These cells, however, were taken at random from the population without screening.

top fractions were collected within 8 min. Single cell isolations were made of all cells in these fractions. The results are summarized in Table 2.

Many of the lines derived from isolations of single cells in these top fractions were dead or slow growing but apparently had no behavioral abnormalities. Many more of the lines were wild type. However, 11 of a total of 197 lines isolated were Fast-1, Fast-2 or Pawns. Most of these variant lines and others obtained from similar experiments were examined genetically and are described in this paper.

In Table 2, each experiment (I to IV) used populations descended from different nitrosoguanidine-treated cells. Controls for experiment III and IV were made by isolating lines directly from the populations of exautogamous animals without screening. None out of 315 lines isolated was the variant type sought. Many fewer deaths (not to be confused with the exautogamous deaths) were observed as compared with the experimentals. It was noticed that the solution used was toxic if the cells stayed in it for a long period of time. Also, the injection was found to be crucial. Disturbance caused by the injection could cause passive upward displacement of wild-type populations (KUNG 1971a).

Using a solution with a higher concentration of Ca^{++} (20 mM NaCl, 0.3 mM $CaCl_2$ and 1 mM Tris, pH 7.2) and exercising extreme care in injecting, a new set of experiments was carried out. Collection was made 6.5 min after the injections. The experiment was abandoned if unnecessary disturbance occurred. The results are summarized in Table 3. While 11 Fast or Pawns were recovered out of a total of 78 lines of experimentals, none out of 600 was found in the controls obtained as in previous experiments.

It is concluded that the column used in the above manner is effective in obtaining fractions much enriched with Fast and Pawn mutants. While it is clear from the previous study (KUNG 1971a) why the Fast-2 and Pawn can be selected

TABLE 3

*Results of screening using columns filled with 20 mM NaCl, 0.3 mM $CaCl_2$ and 1 mM Tris, pH 7.2**

Experiment	Number of runs with the column	Number of lines from cells in the top fraction				Total
		Wild type	Slow grower	Dead	Fast or Pawn	
V	3	14	6	1	7	28
VI	3	23	7	16	4	50
Total	6	37	13	17	11	78
†Control of V		287	10	3	0	300
VI		282	15	3	0	300
Total		569	25	6	0	600

* Top fractions were collected at 6.5 min after injections. See text for method of screening.
† See note on Table 2.

in our column, it is not yet clearly understood why Fast-1 was also selected. At present, there is no reliable method of screening for Paranoiacs.

Genetic analysis. Fast-1: Over 15 lines of variants of this type were selected, of which 8 lines were genetically examined. To avoid the cumbersome numbers originally assigned to these lines in the mutagenesis experiments, these lines are coded with letters Q to X in Table 4. This table gives the crosses of Fast-1 with the behaviorally wild-type strain, d4-85, carrying the genetic marker ts_{111}. F_1 showed normal behavior in clones from both parents and the autogamous F_2 gave segregation of Fast-1 to wild type consistent with a 1:1 ratio. Two lines (W and X) were unable to cross with either mating type of other stocks in repeated trials.

Thus the Fast-1 can be grouped under 2 classes; those which can be crossed easily and have been shown to have the recessive alleles controlling the pheno-

TABLE 4

Crosses between mutant lines of Fast-1 and d4–85 giving F_1 phenotype and F_2 segregation ratios

Line	Code	F_1	F_2	
			Fast-1 : wild type	T.S. : wild type
F 2–3–6	Q	wild type	41 : 40	39 : 42
F 5–5–9	R	wild type	43 : 41	43 : 41
F 11–2–34	S	wild type	36 : 45	40 : 41
F 11–3–35	T	wild type	35 : 46	44 : 37
F 11–3–38	U	wild type	72 : 81	82 : 71
F 11–4–25	V	wild type	34 : 37*	33 : 38
F 8–1–20	W		does not mate	
F 6–2–35	X		does not mate	

* This is one of the only 3 crosses reported in this paper in which a large number of non-viable clones (13 in this case) appeared in the autogamous F_2. All the other crosses gave no or only very few deaths.

TABLE 5

Crosses between Fast-1 mutants giving F_1 phenotype and F_2 segregation ratios

Cross	F_1	F_2	
			T.S. : wild type*
S × U‡	Fast-1	all Fast-1	14 : 17
S × T	Fast-1	all Fast-1	10 : 21†
R × T	Fast-1	all Fast-1	16 : 17
R × U	Fast-1	all Fast-1	12 : 20
R × S	Fast-1	all Fast-1	———
		Fast-1 : wild type	T.S. : wild type
R × V	wild type	23 : 13	17 : 19
S × V	wild type	46 : 26†	32 : 40
U × V	wild type	21 : 7	———
T × V	wild type	30 : 6	———

* Spaces in this column where no number is given mean the marker was not available for the cross.

† These are ratios inconsistent with the expectation at the 95% confidence level in χ^2-test. Macronuclear regeneration, which would result in persistence of the F_1 phenotype, seems to be a reasonable explanation in some of these cases. The Fast-1 : wild type segregations of the lower 4 crosses involving line V can be pooled to give a ratio of 120 : 52 which is not significantly different from a 3 : 1 expectation.

‡ Code letters refer to lines on Table 4.

type, and those which have not yet been shown to cross with ease under normal conditions, including 51.8 fast (COOPER 1965) and "thin" (SONNEBORN and SCHNELLER, unpublished).

Crosses were made between descendant lines of the above crosses bearing the Fast-1 phenotype to determine the relations of the genes responsible for their common characteristic—rapidity in forward swimming. Table 5 summaries the results. They suggest that lines R, S, T and U carry the same allele (*fA fA*) for fastness since both the F_1 and F_2 were fast. Line V apparently carries another gene pair (*fB fB*) for rapidity unlinked to *fA* since the F_1 and some F_2 were wild type. Re-examination of line V showed that the speed was not as high as in the other lines. All *fA fA* bearers were also slightly smaller in size than normal.

No linkage between *fA* or *fB* and ts_{111} was observed.

Fast-2: Ten lines of variants of this type were isolated in different mutagenesis experiments. Five lines from one mutagenesis experiment (coded K to O in Table 6) were genetically analyzed. The results are given in Table 6. When these lines were crossed with d4-85, the Fast-2 phenotype did not appear in F_1 but segregated out in roughly half of the autogamous F_2. This indicates that the Fast-2 trait is controlled by a recessive allele (*fna*). No linkage is observed between *fna* and ts_{111}.

In order to study the genetic relations of these lines, crosses K × L, K × M and K × O were made. All F_1 and F_2 were Fast-2 while the markers segregated normally. Thus the same gene pair (*fna fna*) seems to reside in lines K, L, M and O. An earlier mutagenesis experiment in which these lines were taken was ambiguous in revealing the origin of these lines since they came from the only

TABLE 6

Crosses between mutant lines of Fast-2 and d4–85 giving F_1 phenotype and F_2 segregation ratios

Line	Code	F_1	F_2					
			Fast-2	:	wild type	T.S.	:	wild type
Fna 1–24	K	wild type	(a) 20	:	18	17	:	21
			(b) 19	:	22	19	:	22
Fna 1–35	L	wild type	12	:	10	12	:	10
Fna 1–14	M	wild type	14	:	26*	16	:	24
Fna 1–21	N	wild type	22	:	18	16	:	24
Fna 1–42	O	wild type	19	:	21	6	:	34†

* Twenty exautogamous dead clones were found in this cross.
† See note to Table 5.

flask of descendants of the mutagenized population. It is therefore very likely that the lines (or some of them) were descendants of the same original mutant. Fortunately, variants of this type—Fast-2—were again isolated in subsequent mutagenesis experiments. The genetic analysis of these variants and their relation to the above lines will be studied in the future.

Pawns: Over 25 lines of Pawns were isolated through the course of this study. Some grew poorly and a few lines died before genetic analysis could be made on them. Six lines (coded A to F in Table 7) from one mutagenesis experiment were examined by crossing with d4-85. While basically Pawn in nature, members of line Pw 10-4-7 (code E) showed transient slowing in the diagnostic tests with Na-Ca or K-Ca solutions before they darted out into the test solutions. This "leaky" phenotype (E-type) can be distinguished from that of the other Pawns. The trait Pawn was found to be controlled by recessive genes unlinked to ts_{111}. Table 7 summarizes the data.

Interline crosses making use of the descendants of the above crosses were made and the results are given in Table 8. Lines B, C and D apparently carried the same allele (*pwB pwB*) since the crosses among them gave only Pawn in the

TABLE 7

Crosses between mutant lines of Pawns and 51s giving F_1 phenotype and F_2 segregation ratios

Line	Code	F_1	F_2					
			Pawn	:	wild type	T.S.	:	wild type
Pw 5–5–28	A	wild type	39	:	42	45	:	36
Pw 10–2–8	B	wild type	49	:	32	41	:	40
Pw 2–2–26	C	wild type	41	:	43	34	:	50
Pw 1–2–34	D	wild type	46	:	35	38	:	43
Pw 10–4–7	E	wild type	81	:	80	91	:	70
Pw 2–2–25	F	wild type	32	:	35	40	:	27

TABLE 8

Crosses between Pawn mutants giving F_1 phenotype and F_2 segregation ratios

Cross	F_1	F_2		
				T.S. : wild type
B × C*	Pawn	all Pawn		16 : 20
B × D	Pawn	all Pawn		15 : 21
		E-type : B-type		T.S. : wild type
D × E	Pawn	(all Pawn)	14 : 18	18 : 14
B × E	Pawn	(all Pawn)	14 : 18	15 : 17
		Pawn : wild type		T.S. : wild type
A × E	wild type	54 : 18		41 : 31
A × B	wild type	46 : 19		32 : 33

* Code letters refers to lines on Table 7.

F_1 and F_2. Although all F_1 and F_2 were Pawns, in crosses B × E and D × E there was a 1:1 autogamous F_2 segregation of the "leaky" E-type and the B-type (relatively non-leaky, same as the above *pwB pwB* bearers). A different allele (*pwB'*) at the same locus seems to be present in line E. A different allele at an unlinked locus (*pwA*) is assigned to line A to account for the 3:1 ratio in the autogamous F_2 when A was crossed to B or E. Since 1/3 of the F_2 pawns in this cross were expected to be double mutants, two lines of temperature resistant F_2 Pawns (line 7a-13 and line 7a-7) from cross A × B were chosen at random for a backcross with the behaviorally wild-type parent (d4-85). While the F_1 of the cross line 7a-7 × d4-85 was wild type in behavior, the autogamous F_2 segregated as: 30 pawn : 47 wild type, with the marker segregating: 34 T.S. : 43 wild type. χ^2-tests indicated $P<0.001$ for a 3:1 (Pawn : wild type) expectation and $0.05<P<0.1$ for a 1:1 expectation.

The cross 7a-13 × d4-85 also produced normal F_1, and the F_2 segregated as: 55 pawn : 26 wild type, with the marker segregating: 34 T.S. : 47 wild type. χ^2-tests showed $0.1<P<0.2$ for a 3:1 (Pawn : wild type) expectation and $P<0.001$ for a 1:1 expectation. Thus, double mutants (*pwA/pwA*; *pwB/pwB*) such as line 7a-13 did exist among the F_2 Pawns of the A × B cross.

Paranoiacs: Nine lines with this remarkable phenotype were isolated in one mutagenesis experiment. Five (coded G, H, I, Y and Z as in Table 9) were subjected to genetic analysis. Crosses involving the mutant trait and markers (ts_{111} and/or *bd*) were made repeatedly. In these crosses the result was consistent with a 1:1 expectation in the autogamous F_2 segregation. The F_1 from both parents showed a weak Paranoiac trait, i.e., a much smaller proportion of the population exhibited continuous ciliary reversal at a given time and the duration of this reversal was shorter than that of the parental Paranoiacs. The allele which controlled the Paranoiac trait was thus considered to be partially dominant over (or co-dominant with) its wild-type allele (Table 9).

Line Pa 2-211 (code Z) gave slow growing or non-viable F_2 in 3 crosses. This could have been accidental because one cross with a derived line from B_{21} (a

TABLE 9

Crosses between mutant lines of Paranoiacs and others showing F_1 phenotype and F_2 segregation ratios

Line	Code	F_1	F_2 Paranoiac : wild type	F_2 T.S. : wild type*	F_2 B.D. : wild type*
Pa 4–30	G	slight Paranoiac	(a)† 47 : 36	42 : 41	42 : 41
			(b) 79 : 72	72 : 79	———
			(c) 76 : 75	75 : 76	73 : 78
Pa 1–50	H	slight Paranoiac	(a)† 32 : 36	36 : 32	34 : 34
			(b) 88 : 55*	68 : 75	———
			(c) 73 : 71	76 : 68	———
Pa 7–40	I	slight Paranoiac	(a)† 55 : 28*	46 : 37	43 : 40
			(b)† 46 : 35	49 : 32	44 : 37
			(c) 76 : 70	———	61 : 85
Pa 5–68	Y	slight Paranoiac	(a) 18 : 20	14 : 24	19 : 19
			(b) 43 : 40	———	36 : 47
Pa 2–211	Z				

* See notes to Table 5.

† All these crosses were done at the same time and showed hastened autogamy, sometimes before 10 fissions after conjugation. The reason is not understood. It could be due to cultural conditions since other crosses of the same or similar nature did not have early autogamy.

strain with a morphological marker) did produce viable F_2 with a segregation of 30 Paranoiac : 12 wild-type (see note, Table 5). In the study below (Table 10), line Z was successfully crossed with another Paranoiac line (I).

Although most of the data on F_2 segregation for the Paranoiac trait in Table 9 are consistent with the 1:1 expectation, most of them have a slight preponderance on the Paranoiac side in these crosses. Macronuclear regeneration may have occurred in some cases. This preponderance of Paranoiac phenotypes did not always occur in other crosses involving segregation of this trait.

The five lines, though from the same mutagenesis experiment, were descendants of different nitrosoguanidine-treated cells. Interline crosses using the

TABLE 10

Crosses between Paranoiac mutants giving F_1 and F_2 phenotypes and marker segregation ratios

Cross	F_1	F_2	F_2 T.S. : wild type	F_2 B.D. : wild type
I × Z*	Paranoiac	all Paranoiac	50 : 33	45 : 38
G × I	Paranoiac	all Paranoiac	33 : 43	39 : 37
I × Y	Paranoiac	all Paranoiac	47 : 32	36 : 43
H × Y	Paranoiac	all Paranoiac	37 : 41	40 : 38
G × Y	Paranoiac	all Paranoiac	41 : 40	33 : 48

* Code letters refer to lines on Table 9.

TABLE 11

Crosses between Fast-1 and Fast-2 mutants giving F_1 phenotype and F_2 segregation ratios

Cross	Genotype of P	F_1	F_2* Fast-2 : Fast-1 : wild type
N × U‡	*fna/fna* +/+ × +/+ *fA/fA*	wild type	17 : 8 : 11
N × T	*fna/fna* +/+ × +/+ *fA/fA*	wild type	13 : 9 : 14
N × Q	*fna/fna* +/+ × +/+ *fA/fA*	wild type	18 : 7 : 9
N × S	*fna/fna* +/+ × +/+ *fA/fA*	wild type	11 : 12 : 12†
N × V	*fna/fna* +/+ × +/+ *fB/fB*	wild type	31 : 17 : 21

* Markers were not available for these crosses.
† Macronuclear regeneration might have occurred in the F_2 to give this ratio which deviates significantly from the 2 : 1 : 1 expectation. (See note to Table 5).
‡ Code letters refer to lines on Tables 4 and 6.

descendants of the above crosses showed, however, that they seemed to carry the same allele (*Pa*) for the Paranoiac trait since both F_1 and F_2 were all Paranoiac while the markers segregated in F_2 (Table 10).

Crosses between strains of different behavioral mutants: To understand the genetic relations and interactions, crosses among the above four types of behavioral mutants were carried out using descendants with different mating types and markers derived from various crosses of the original mutant lines.

Fast-1 × Fast-2: The data are summarized in Table 11. Although markers were not available for these crosses, all the F_2 segregations except the S × N cross were consistent with an expectation of 2:1:1 (Fast-2 : Fast-1 : wild-type) derived from a null hypothesis assuming that *fna* is epistatic over *fA* and *fB*. That is, the double mutants (*fA/fA;fna/fna*) among the F_2 were expected to be Fast-2 in phenotype.

In the cases of *fna/fna*;+/+ × +/+;*fA/fA* the F_2 clones that gave Fast-2 in the diagnostic tests seemed to be of two classes. One was more like the original Fast-2 phenotype and the others had a higher speed and smaller bodies than the original Fast-2. Although a good 1:1 ratio of these two classes was found in all four crosses, genotypes of the two classes could not readily be assigned because speed and body length were more variable characters than the Na-insensitivity. Three lines of Fast-2 F_2 were crossed back to d4-85 for genotypic analysis. They were line 32a-5 (from cross N × Q), which resembled the original Fast-2 in phenotype, and lines 32a-4 and 38a-16 (from crosses N × Q and N × U respectively), which were smaller and faster than the original Fast-2 type. While the F_1 of all crosses were wild type, the F_2 of line 32a-5 × d4-85 segregated as: 16 Fast-2 : 20 wild type (marker—14 T.S. : 22 wild type) that of line 32a-4 × d4-85 segregated as: 39 Fast-2 : 16 Fast-1 : 16 wild type (marker—38 T.S. : 33 wild type), and that of line 38a-16 × d4-85 segregated as: 37 Fast-2 : 17 Fast-1 : 18 wild type (marker—36 T.S. : 36 wild type).

It is thus clear that the fast-2 F_2 from Fast-1 × Fast-2 crosses does include animals with *fA fA* and these animals are likely to be those that have a smaller

TABLE 12

Crosses between Fast and Pawn mutants giving F_1 phenotype and F_2 segregation ratios

Code	Genotype of P	F_1	F_2					
			Pawn	Fast-1	wild type	T.S.	wild type*
U × A‡	*fA/fA* +/+ × +/+ *pwA/pwA*	wild type	20	7	7	19	15
R × A	*fA/fA* +/+ × +/+ *pwA/pwA*	wild type	20	5	4	———	
			Pawn	Fast-2	wild type	T.S.	wild type
N × E	*fna/fna* +/+ × +/+ *pwB¹/pwB¹*	wild type	33	19	20	32	40
N × B	*fna/fna* +/+ × +/+ *pwB/pwB*	wild type	52	28	21	43	58
N × D	*fna/fna* +/+ × +/+ *pwB/pwB*	wild type	16	4	16†	18	18
N × A	*fna/fna* +/+ × +/+ *pwA/pwA*	wild type	28	13	23	23	41*

* See notes to Table 5.
† It is not probable that the deviation from 2 : 1 : 1 expectation here, significant at the 95% confidence level, is due to macronuclear regeneration since the marker segregates perfectly. The 5% of crosses expected to deviate from the norm at 95% level may be the answer.
‡ Code letters refer to lines on Tables 4, 6 and 7.

body and higher speed in addition to the Fast-2 characteristics. In the case of *fna/fna*;+/+ × +/+;*fB/fB*, the subdivision of the Fast-2 F_2 was not practical. This is probably because of the more subtle character of line V (see above).

Fast-1 or Fast-2 × Pawn: The results of crosses Fast-1 × Pawn and Fast-2 × Pawn are summarized in Table 12. Data on F_2 segregations all fit a 2:1:1 (Pawn : Fast : wild type) expectation except cross N × D (see notes to Table 12). This implies epistasis of *pwA*, *pwB* or *pwB¹* over *fA* or *fna*.

Three Pawn F_2 lines from Fast-2 × Pawn crosses (line 22b-2 and line 22b-6 from cross N × E and line 23b-1 from cross N × B) were analyzed genetically by crossing them back to a behaviorally wild-type stock (51s or d4-85). All 3 crosses gave wild-type F_1. The F_2's of cross line 22b-6 × d4-85 and line 23b-1 × 51s segregated as: 14 Pawn : 20 wild type (marker—16 T.S. : 18 wild type) and 12 Pawn : 23 wild type (marker—20 T.S. : 15 wild type), respectively, whereas the F_2 of line 22b-2 × 51s segregated into: 28 Pawn : 20 Fast-2 : 21 wild type (marker not available at the time of this cross). These results indicate that there are indeed some F_2 from the Fast-2 × Pawn crosses that contain *fna fna* although they are phenotypically Pawns. Approximately half of the F_2 from Fast-1 × Pawn and Fast-2 × Pawn (such as line 22b-2) appeared to be faster in movement than the parental Pawns and were often slow growers. These characters may be an indication of the presence of the genes for Fast-1 or Fast-2 in addition to the genes for Pawn.

Fast-1, Fast-2 or Pawn × Paranoiac: The results of crosses Fast-1, Fast-2 or Pawn × Paranoiac are given in Table 13. The F_2 segregation of Fast-1 × Paranoiac has consistent with the 1:1:1:1 expectation of no linkage and no genic

TABLE 13

Crosses of Fast or Pawns with Paranoiacs giving F_1 phenotype and F_2 segregation ratios

Code	Genotype of P	F_1	F_2					
			Paranoiac and Fast-1	Paranoiac	Fast-1	wild type	T.S.	wild type*
Q × Z§	*fA/fA* +/+ × +/+ *Pa/Pa*	slight Paranoiac	12	16	24	20	———	
V × Y	*fB/fB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	5	10	9	12	16	20
			Fast-2	Paranoiac		wild type	T.S.	wild type
K × H	*fB/fB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	49	20		15	38	40†
K × G	*fB/fB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	37	18		27	33	49
K × I	*fB/fB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	37	24		23	35	49
			Pawn	Paranoiac		wild type	T.S.	wild type
F × Z	*pwB/pwB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	23	16		13‡	20	32
D × G	*pwB/pwB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	17	9		7	18	15
B × Z	*pwB/pwB* +/+ × +/+ *Pa/Pa*	slight Paranoiac	33	22		14	———	
A × Z	*pwA/pwA* +/+ × +/+ *Pa/Pa*	slight Paranoiac	28	14		24	———	

* See note to Table 5.
† Six clones were lost in an accident which prevented testing for temperature sensitivity.
‡ Twenty clones of this cross were nonviable.
§ Code letters refer to lines on Tables 4, 6, 7 and 9.

interaction. A new group of animals was found in F_2. They swam rapidly forward when slightly disturbed and spontaneously went into continuous ciliary reversal. These were very likely the double mutants (*fA/fA;Pa/Pa* or *fB/fB; Pa/Pa*). Three such lines (30a-8, 30a-14 and 30a-34 from cross Q × Z) were crossed with d4-85. F_1 of all three crosses were partial Paranoiac and F_2 segregated as follows: in line 30a-8 × d4-85; 12 Paranoiac and Fast : 9 Paranoiac : 10 Fast-1 : 5 wild type (marker—22 T.S. : 14 wild type). In line 30a-14 × d4-85; 10 Paranoiac and Fast : 6 Paranoiac : 9 Fast-1 : 11 wild type (marker—23 T.S. : 13 wild type). In line 30a-34 × d4-85; 6 Paranoiac and Fast : 8 Paranoiac : 14 Fast-1 : 8 wild type (marker—20 T.S. : 16 wild type).

In the Fast-2 × Paranoiac crosses, one fourth of the F_2 clones were wild type, but the expected one fourth with the double mutant phenotype were not found. Instead, a 2:1:1 (Fast-2 : Paranoiac : wild type) relationship was established. This suggested that *fna* showed recessive epistasis over *Pa*. To be sure that the Fast-2 F_2 comprised both the single mutants and the double mutants, three of these lines (line 2a-6 and line 2b-23 from the K × G cross and line 1b-42 from the K × H cross, all temperature resistant) were taken to cross with d4-85. Autogamous F_2 of the cross line 1b-42 × d4-85 segregated as follows: 40 Fast-2 : 34 wild type (marker—34 T.S. : 40 wild type). No paranoiac clones were found. The line 2a-6 × d4-85 and line 2b-23 × d4-85 crosses segregated respectively:

76 Fast-2 : 46 wild type : 41 Paranoiac (marker—83 T.S. : 80 wild type); and 83 Fast-2 : 35 wild type : 29 Paranoiac (marker—63 T.S. : 84 wild type).

Since Paranoiac F_2 clones segregated out of the crosses with the phenotypically Fast-2 parents in the statistically acceptable 2:1:1 (Fast-2 : wild type : Paranoiac) ratio, it was concluded that some of the phenotypically Fast-2 lines, such as line 2a-6 and line 2b-23, contained *Pa* genes, although the Paranoiac trait did not appear in the diagnostic tests.

The Pawn × Paranoiac crosses showed a genic relationship similar to the Fast-2 × Paranoiac crosses. The 2:1:1 (Pawn : wild : Paranoiac) ratio in the F_2 of these crosses indicated that *pwA* and *pwB* are also epistatic over *Pa*. Two lines (line 6a-15 from A × Z cross and line 5a-21 from B × Z cross) were chosen for crosses with d4-85. Both gave partially Paranoiac F_1 and showed F_2 segregation as follows: In line 6a-15 × d4-85: 41 Pawn : 16 wild type : 22 Paranoiac (marker —43 T.S. : 36 wild type). In line 5a-21 × d4-85: 41 Pawn : 16 wild type : 19 Paranoiac (marker—23 T.S. : 53 wild type) (see note, Table 5).

Roughly one fourth of the clones reappeared as wild type in F_2 and one fourth as Paranoiacs. This reappearance of Paranoiacs from the crosses with phenotypically Pawn parents proved that these parents (which were themselves F_2 of Pawn × Paranoiac) carried *Pa Pa* genes not expressed in the diagnostic tests.

Some F_2 Pawn clones from Pawn × Paranoiac crosses, including line 5a-21, showed the Paranoiac behavior before the expression of the Pawn phenotype. Whether this transient appearance of the Paranoiac traits indicated the presence of *Pa* genes has not yet been verified.

CONCLUSIONS AND DISCUSSION

This study leaves no doubt that the system for excitation can be altered by non-lethal gene mutations. The results open the way for genetic dissection of an apparently complicated system which controls the behavior of these protozoa.

Although several mutant lines were analyzed, most lines which gave the same phenotype seemed to carry the same mutant alleles. This raises the question of the origin of these lines. Although in most experiments the lines studied were derived from different mutagenized cells, it is possible that some of them may have arisen from spontaneous mutation before the mutagen treatment. However, the fact that this repeated appearance of the same mutation occurred in all four types of mutants studied here argues against this hypothesis since spontaneous mutation does not happen often. Unrelated repetitions of mutation at the same loci were found in trichocyst mutants (POLLACK, personal communication) and odd-restricted and selfer-mating type mutants (BYRNE, personal communication) in *P. aurelia* derived from the same stocks used in this experiment. The fact that *pwB* and *pwB*^{1} are allelic proves the occurrence of independent mutations at the same locus. Also, many of the lines studied show differences in characters such as growth rate and body clearness, indicating their different origins.

It is possible that what is called the same locus here could yield many alleles differing subtly in their effects. Since the laborious broad range stimulation-

reaction tests (KUNG 1971a) have not yet been performed on all the lines, this possibility cannot be ruled out. The classification of phenotypes here is based on the diagnostic tests of Table 1. Broad range tests are also desirable for a more penetrating study of the extent of co-dominance or epistasis.

The sequence of epistasis:

$$pw > fna > f = Pa$$

(genes for Pawn, Fast-2, Fast-1 and Paranoiac respectively) established in our analysis is interesting. That *pw*>*fna*>*f* is not surprising because Pawns are more drastically mutated than Fast-2, while Fast-1 is least affected by mutation as shown in the diagnostic tests of Table 1. This, however, does not necessarily mean that the genes unexpressed in the double mutants in these tests are inactive.

Detailed phenotypic analysis of Paranoiac shows that there is no change in the threshold in reaction to Na^+ (KUNG 1971a). The long duration of ciliary reversal is not a hypersensitivity to low concentration of Na^+ but rather an exaggeration of the weaker avoiding reactions observed in wild type upon proper Na-stimulation. Thus, the genic lesions do not affect the mechanism for initiation of ciliary reversal but the mechanism for shutting off reversal. The epistasis of *pw*>*Pa* and *fna*>*Pa*, although superficially puzzling, simply indicates that the defects in the reversal "shut-off" mechanism caused by *Pa Pa* cannot be expressed due to the coexisting defects in the reversal "turn-on" mechanism caused by *pw pw* or *fna fna*. In other words, the exaggeration of the avoiding reaction cannot appear when the reaction is not initiated in the double mutants.

Besides demonstrating the possibility of inducing nuclear genic mutation in the system of behavioral control, this study provides us with easily identifiable genetic markers, which are still needed in the genetics of *P. aurelia*. We have not exhausted the use of the screening method described here. Screening methods based on other behavioral differences will also be tested in the future.

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SUMMARY

In an attempt to dissect genetically the electrically excitable membrane in *Paramecium aurelia*, mutants over-reactive or insensitive to Na^+, K^+ or mechanical stimuli were obtained. A method based on the principle of Na-chemotactic interference with geotaxis was invented to screen for most of these mutants. Genetic analysis revealed that mutant "Paranoiac" was controlled by a pair of incompletely dominant alleles. Mutants "Fast-1", "Fast-2" and "Pawn" were governed by unlinked recessive loci. The genic interactions are also analyzed and discussed.

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