LINKAGE STUDIES IN VARIETY 1 OF TETRAHYMENA PYRIFORMIS: A FIRST CASE OF LINKAGE IN THE CILIATED PROTOZOA[1]

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THE ciliated protozoa are not, in general, favorable material for cytogenetic work. The large number of chromosomes, their small size, and their tendency to form chromosomal aggregates (SONNENBORN 1949; NANNEY and RUDZINSKA 1960) make such studies exceedingly difficult. The discovery by RAY (1954) that *Tetrahymena pyriformis* possesses a haploid complement of five chromosomes opened up the possibility of such studies in this organism. A very important contribution was RAY's (1956) detailed analysis of meiosis in variety 1 of *T. pyriformis*.

Genetic analysis of this variety was initiated by NANNEY in his studies of the inheritance of the mating types (NANNEY, CAUGHEY, and TEFANKJIAN 1955). A mating type locus (*mt*) with multiple alleles was identified. NANNEY has also characterized the *H* locus as a multiple-allelic series that determines the H serotypes (NANNEY and DUBERT 1960). *mt* and *H* segregate independently (NANNEY 1960a). ORIAS (1960) discovered two lethals, fat and tiny, in certain derivatives of IL-12. These are under the control of *F* and *T*, and *F* and *T* are not linked. Three other loci have been identified; these are responsible for certain enzymes that are resolved by starch gel electrophoresis. *E-1* and *E-2* control different esterases and segregate independently (ALLEN 1961a). *P-1* controls an acid phosphatase (ALLEN, MISCH, and MORRISON 1963b).

In this report, evidence will be presented for the independent assortment of *H*, *E-1*, *E-2*, and *P-1*. In setting up these crosses, particular steps were taken to avoid pairs in which genomic exclusion occurred. Genomic exclusion, an aberrant form of conjugation (ALLEN 1963) in which meiotic products are contributed from only one mate, would confuse linkage studies. Analysis of crosses segregating for *mt*, *E-1* and *E-2* suggest that *mt* and *E-1* are linked. A preliminary note appeared previously (ALLEN 1961b).

MATERIALS AND METHODS

Inbred strains: Inbred strains A, B and C were used in the crosses. The origins of these strains have been previously described (ALLEN 1960; NANNEY 1959). They are now in their 13th or 14th generation of inbreeding. The genotypes of strains A, B and C are:

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A	mt^A/mt^A	H^A/H^A	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^A/P\text{-}1^A$
B	mt^B/mt^B	H^D/H^D	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^B/P\text{-}1^B$
C	mt^C/mt^C	H^E/H^E	$E\text{-}1^C/E\text{-}1^C$	$E\text{-}2^C/E\text{-}2^C$	$P\text{-}1^B/P\text{-}1^B$

Crosses: Crosses were made in bacterized medium (Cerophyl rye grass inoculated with *Aerobacter aerogenes*) at 23°C or at 30°C, as previously described (ALLEN, MISCH, and MORRISON 1963b).

Mating type tests: Mature cultures were tubed up in bacterized medium. After appropriate conditions of feeding were satisfied, samples of these unknown cultures were mixed with samples of each of the seven mating types. The pattern of reaction served to identify the mating type of the unknown culture. With certain exceptions, mating type genotype cannot be assigned to the exconjugants of a pair until appropriate test crosses are made. The frequency and quality of types observed among the progeny characterize the mating type genotypes of the parents.

Serotype tests: Samples of immature cultures were mixed with diluted antisera. The concentration of antiserum was adjusted so that control cultures were completely immobilized in 30 to 45 minutes. The antisera were prepared in rabbits by injection of 50 mg of protein obtained from the concentrated brei of approximately 5×10^7 cells in Freund's complete adjuvant. Maximum titers of the order 1/40 to 1/80 are reached against homologous cells at around three weeks.

Identification of E-1, E-2, and P-1: Samples of immature cultures were transferred to 1 percent proteose-peptone, as previously described (ALLEN, MISCH, and MORRISON 1963b). Flask cultures (100 ml in 250 ml Erlenmeyer flasks) were harvested after five days of growth at 30°C, the cells were concentrated by light centrifugation and whole-cell extracts were prepared by freeze-thawing. Extracts were inserted in starch gels and electrophoresis was carried out for 5 hours at room temperature (23 to 26°C), as previously described (ALLEN 1960; ALLEN, MISCH, and MORRISON 1963a). Identification of P-1 was carried out in starch gels of pH 7.5, of E-1 and E-2 in starch gels of pH 8.0. The end tray buffer was in all cases pH 7.5.

The E-1 esterases were identified by incubating the starch slices for 2 hours at room temperature in pans in a reaction mixture containing 0.01 percent alpha-naphthyl propionate (synthesized by DR. ROBERT L. HUNTER, Department of Anatomy, Medical School, Stanford University), 0.1 percent Fast Blue RR (4′-amino-2′, 5-dimethylbenzanilide; Carbic-Hoechst) and 0.01 M sodium taurocholate (Nutritional Biochemicals) in 0.1M Sörenson's phosphate buffer at pH 7.4.

The E-2 esterases were identified by incubating the starch slices for 2 hours at 30°C in pans in a reaction mixture containing 0.02 percent alpha-naphthyl butyrate (synthesized by DR. ROBERT L. HUNTER) and 0.1 percent Fast Blue RR in 0.1M Sörensen's phosphate buffer at pH 6.5.

The P-1 phosphatases were identified by incubating the starch strips for 1 hour at room temperature in test tubes (see ALLEN, MISCH, and MORRISON 1963a) in a reaction mixture containing 4mM sodium alpha-naphthyl acid phosphate (Dajac Laboratories), 0.1 percent Fast Garnet GBC (4-amino-3:1′-dimethyl azobenzene; Dupont DeNemours) in 50mM acetate buffer at pH 5.0.

After incubation, the starch slices or strips were rinsed several times in distilled water and stored in water in the refrigerator until photographed.

RESULTS

Tests of linkage of H, E-1, E-2, *and* P-1*:* In this analysis a triple cross was used so that the progeny arising from conjugation could be distinguished from those arising as a result of genomic exclusion. A distinctive *H* allele is present in strains A (H^A/H^A), B (H^D/H^D) and C (H^E/H^E). Strains A and C differ in genotype at all four of the above loci; thus, as a first step, these two strains were crossed. The resulting F_1 (AC) was then crossed to strain B.

Next, the resulting ACB progeny were screened for their serotypes. If normal conjugation had occurred, then half of the progeny should be Had and half should be Hde. Pairs formed as a result of genomic exclusion would be spotted if they possessed any of the following phenotypes: Ha, He, Hae, or Hd.

Pairs defined as Had or Hde were then characterized as to their E-1, E-2 and P-1 phenotypes. For each marker gene half the pairs would be expected to be heterozygous (E-1BC, E-2BC or P-1AB) and half should be homozygous (E-1B, E-2B, or P-1B). These distinctions are clear-cut, as illustrated in Figure 1. In the case of all four loci, the genotypes may be directly ascertained from the phenotypes, since the classification is performed on populations of pair cultures immediately following conjugation. By sampling early and by sampling a population of cells, the effects of phenotypic drift (ALLEN, MISCH, and MORRISON 1963b) can be avoided.

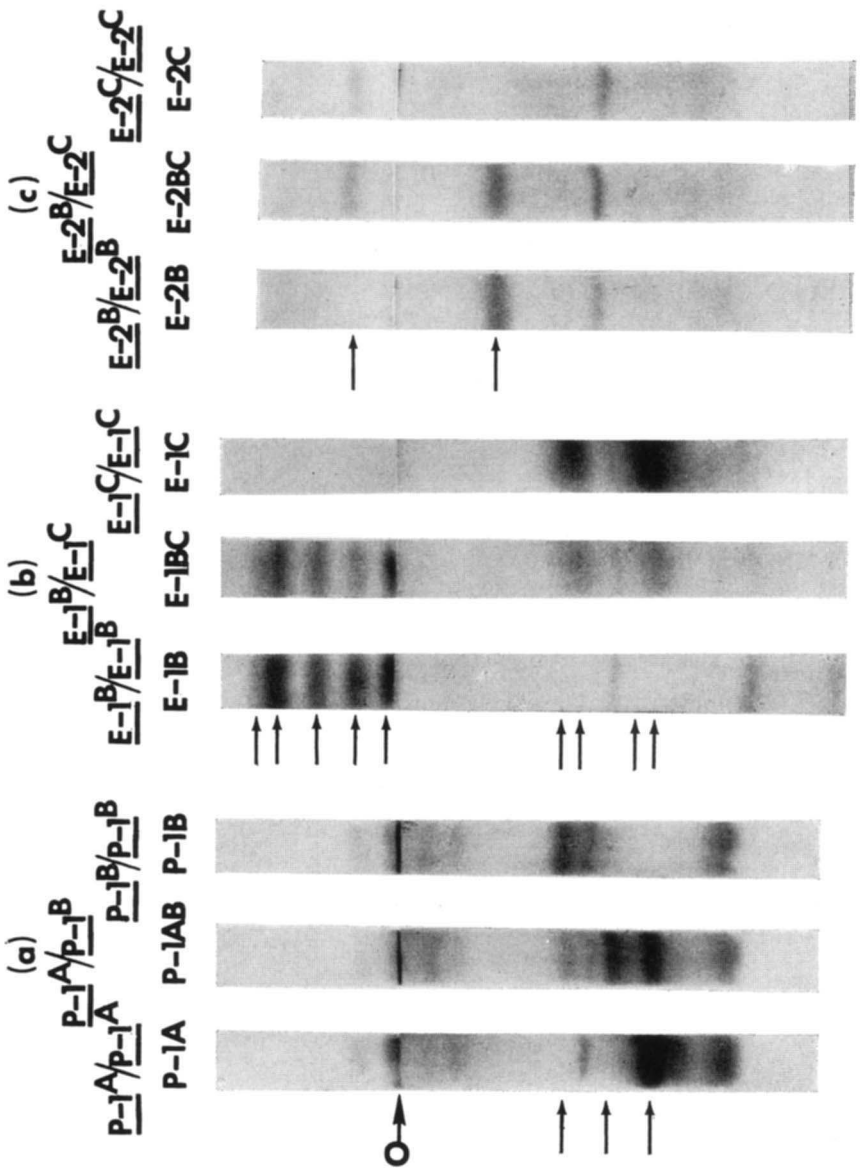

FIGURE 1.—Zymograms of extracts of different genotypes. The corresponding phenotype is indicated below each genotype. The cathode is at the top, the anode is at the bottom, and the origin is indicated by a "O". The sites of each enzyme type are marked by arrows. The zymograms were incubated in (a) sodium alpha-naphthyl acid phosphate at pH 5.0, (b) alpha-naphthyl propionate at pH 7.4, and (c) alpha-naphthyl butyrate at pH 6.5.

For the analysis, two AC stocks were selected that differed in their viability when selfed. 70 percent of the progeny of AC-1 × AC-1 were viable, while 55 percent of the progeny of AC-2 × AC-2 were viable. The B stock, when selfed, gave rise to 100 percent viable progeny. NANNEY (1963) has found an association between viability and genomic exclusion. Stocks that are less than 50 percent viable when selfed tend to give rise to a higher frequency of pairs formed as a result of genomic exclusion when they are outcrossed.

AC-1 crossed to B (Cross 1) resulted in 82 percent viable progeny, while AC-2 crossed to B (Cross 2) resulted in 100 percent viable progeny. A difference in the viability of the outcrosses was not necessarily expected. The inviable progeny in the first cross were probably H^D/H^E since there is a significant deficiency of pairs of this class (Table 1). Out of 120 pairs, 62 were Had, 36 were Hde, and 22 were dead. The ratio 62:36 is significantly different from a 1:1 ratio ($P < .01$). If the 22 dead pairs were H^D/H^E the ratio would be that expected for a 1:1 segregation of the H alleles, i.e. 62:58 ($P = .8$).

Neither cross gave rise to progeny that were formed as a result of genomic exclusion. This was true for all four markers. From the first cross, the 36 Hde pairs and 36 of the Had pairs were screened for their *E-1*, *E-2*, and *P-1* genotypes. The segregation of heterozygous and homozygous pairs was very close to 1:1 for all three markers. From the second cross, 20 Had pairs and 20 Hde pairs were screened. Here, the segregation of the three markers did not depart significantly from a 1:1 ratio, but an excess of $E\text{-}2^B/E\text{-}2^C$ and $P\text{-}1^B/P\text{-}1^B$ pairs was observed.

The distribution of the *H*, *E-1*, *E-2*, and *P-1* genotypes among 112 progeny from the two crosses was random (Table 2). All 16 classes were observed among the progeny derived from Cross 1, and 13 of the 16 classes were observed among the progeny derived from Cross 2. In 112 progeny, the expected number is seven for each of the 16 classes. The observed numbers fit this expectation very closely ($P = .9$).

An analysis of all six combinations of two loci was carried out for each cross separately as well as for the combined data. In no case was there evidence for linkage of any two of the loci. Although some of the P values were low (.1) for

TABLE 1

Segregation of H, E-1, E-2, *and* P-1 *in ACB crosses*

Genotypes	Cross 1 AC-1 × B	Cross 2 AC-2 × B
H^A/H^D	62	66
H^D/H^E	36	54
Dead	22	0
$E\text{-}1^B/E\text{-}1^B$	38	22
$E\text{-}1^B/E\text{-}1^C$	34	18
$E\text{-}2^B/E\text{-}2^B$	35	15
$E\text{-}2^B/E\text{-}2^C$	37	25
$P\text{-}1^A/P\text{-}1^B$	38	15
$P\text{-}1^B/P\text{-}1^B$	34	25

TABLE 2

The distribution of H, E-1, E-2, *and* P-1 *genotypes among the progeny of* $AC \times B$

Genotypes				Observed			Expected
				Cross 1	Cross 2	Total	
H^A/H^D	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^A/P\text{-}1^B$	6	4	10	7
			$P\text{-}1^B/P\text{-}1^B$	4	0	4	7
		$E\text{-}2^B/E\text{-}2^C$	$P\text{-}1^A/P\text{-}1^B$	5	2	7	7
			$P\text{-}1^B/P\text{-}1^B$	5	4	9	7
	$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^A/P\text{-}1^B$	5	0	5	7
			$P\text{-}1^B/P\text{-}1^B$	4	1	5	7
		$E\text{-}2^B/E\text{-}2^C$	$P\text{-}1^A/P\text{-}1^B$	4	4	8	7
			$P\text{-}1^B/P\text{-}1^B$	3	5	8	7
H^D/H^E	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^A/P\text{-}1^B$	4	0	4	7
			$P\text{-}1^B/P\text{-}1^B$	5	4	9	7
		$E\text{-}2^B/E\text{-}2^C$	$P\text{-}1^A/P\text{-}1^B$	5	1	6	7
			$P\text{-}1^B/P\text{-}1^B$	4	7	11	7
	$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^B/E\text{-}2^B$	$P\text{-}1^A/P\text{-}1^B$	3	3	6	7
			$P\text{-}1^B/P\text{-}1^B$	3	3	6	7
		$E\text{-}2^B/E\text{-}2^C$	$P\text{-}1^A/P\text{-}1^B$	5	1	6	7
			$P\text{-}1^B/P\text{-}1^B$	6	1	7	7
			Totals	72	40	112	112

Cross 2, the P values were all high for Cross 1 and for the combined data. Detection of linkages up to those with approximately 40 percent crossing over should be possible in 112 progeny resulting from this type of cross. Some of the four loci might still turn out to be very loosely linked, but it is also possible that *H*, *E-1*, *E-2*, and *P-1* each marked a different chromosome.

Additional data were obtained in other crosses, and none of these provided any evidence of linkage of any of these four loci. Backcross data obtained from crosses involving the A and B strains gave no evidence for linkage of *H* and *P-1*. An observed distribution of 18:22:20:26 was not significantly different from a 1:1:1:1 ratio. Fifty F_2 (A × C) pairs that were *H* homozygotes were classified as to their *E-1*, *E-2* and *P-1* genotypes. This sample was small, but in no case was there any evidence of linkage of any two of the four loci.

Tests of linkage of mt, E-1 *and* E-2*:* In this analysis crosses were made between the B and C strains and the resulting hybrids were backcrossed to either strain B or strain C. These strains differ in genotype at each of the above loci. The B strain possesses the B alleles at *mt*, *E-1* and *E-2*; the C strain possesses the C alleles. These strains also differ in *H* genotype, but this useful marker was not included in this study, since the investigations of the *H* serotypes were in their infancy at the time these crosses were initiated.

The backcross progeny were screened, first for their E-1 and E-2 phenotypes. Half the progeny would be expected to exhibit the heterozygous phenotypes for each marker (E-1BC, E-2BC) and half would be expected to exhibit the homozygeous phenotypes, E-1B and E-2B in the B backcross and E-1C and E-2C in the C backcross. These distinctions are easily made, as illustrated in Figure 1b and c.

Since the classification of progeny was based on populations of pair cultures immediately following conjugation, genotypic assignments may be made directly. Confirmation of these assignments was also obtained by testcrosses.

In order to test the mating types, it is necessary to obtain from each pair culture mature lines that are representative of at least two of the caryonides. This is performed by selecting three cells at random from each immature pair culture and carrying each line to maturity by repeated serial transfers of single cells until each lineage has undergone at least 80 fissions. Then, each line is typed for mating type. In at least half the pairs, two of the lines from a pair will differ in mating type. These probably represent different caryonides or different subcaryonides. A few lines will exhibit mating types that permit direct identification of the mating type genotype of a pair, but in most cases it is necessary to cross the lines derived from a pair and to raise 30 progeny to maturity before the mating type genotype can be identified. Where all lines from the same pair are identical in mating type, it is necessary to perform a second backcross. Here, at least 60 progeny must be raised to maturity in order to ascertain the mating type genotype.

Each mating type genotype gives rise to an array of mating types. Some types are unique to a genotype; others are produced by several genotypes, but the frequencies in which they are produced are different. The frequencies are also temperature sensitive (NANNEY 1960b). At 23°C the following array of mating types is observed in the two homozygous genotypes:

	I	II	III	IV	V	VI	VII
mt^B/mt^B	0	.12	.04	.58	.06	.06	.14
mt^C/mt^C	.42	.10	.09	0	.01	.39	0

The unique mating types are I for mt^C/mt^C and IV and VII for mt^B/mt^B. Heterozygotes produce all seven mating types, and the frequencies of I, IV and VII are about half those of the homozygotes.

A pair was assigned the genotype mt^B/mt^C without further testing if one of the lines was I in the B backcross or if one of the lines was IV or VII in the C backcross. If the lines were II, III, V, or VI (or I in the backcross to C, or IV or VII in the backcross to B), it was necessary to make a testcross. Since the frequency of I is optimal at 23°C, testcrosses of B backcross pairs were conducted at this temperature. However, the frequency of IV is higher at 30°C; thus, testcrosses of C backcross pairs were performed at the higher temperature.

Three heterozygotes were used in the crosses: two were F_1 hybrids; the other was an F_2 hybrid. The backcrosses of the F_1 hybrids to the B strain resulted in normal 1:1 segregations of the alleles at all three loci. The backcrosses to the C strain, however, resulted in abnormal segregations. Some $F_1 \times C$ pairs were observed that were mt^B/mt^B, or $E\text{-}1^B/E\text{-}1^B$, or $E\text{-}2^B/E\text{-}2^B$ in genotype. Pairs of these genotypes could only have arisen by genomic exclusion. Thus, only the $F_1 \times B$ backcross data could be used in this analysis. The F_2 hybrid, on the other hand, showed normal segregations for all three markers in both the backcross to the C strain as well as the backcross to the B strain. Table 3 summarizes the information on the crosses exhibiting normal segregation. The data from the two F_1's were pooled, since there was no statistical evidence of internal inconsistencies.

TABLE 3

Segregation of E-1, E-2, *and* mt *in backcross hybrids*

Genotypes	$F_1 \times B$	$F_2 \times B$	$F_2 \times C$
$E\text{-}1^B/E\text{-}1^C$	21	12	13
$E\text{-}1^B/E\text{-}1^B$	21	8	0
$E\text{-}1^C/E\text{-}1^C$	0	0	12
$E\text{-}2^B/E\text{-}2^C$	20	9	15
$E\text{-}2^B/E\text{-}2^B$	22	11	0
$E\text{-}2^C/E\text{-}2^C$	0	0	10
mt^B/mt^C	14	3	7
mt^B/mt^B	12	9	0
mt^C/mt^C	0	0	7

Fifty-two backcross pairs were analyzed for linkage of *mt*, *E-1* or *E-2*. Twenty-six pairs were derived from the $F_1 \times B$ cross. Twelve of these were $E\text{-}1^B/E\text{-}1^C$ and 14 were $E\text{-}1^B/E\text{-}1^B$. Another 26 pairs were derived from the backcrosses of the F_2 hybrid, 12 from the $F_2 \times B$ cross and 14 from the $F_2 \times C$ cross. Among these were an equal number of $E\text{-}1^B/E\text{-}1^C$ pairs and homozygous pairs.

Eleven pairs could be assigned mating type genotype without further testcrossing. From 31 pairs that were selfed, and 10 pairs that were backcrossed, more than 1600 progeny were raised and tested for mating type, permitting the assignment of mating type genotype to these 41 pairs.

The distribution of the *mt*, *E-1* and *E-2* genotypes among the 52 backcross pairs is shown in Table 4. Cross 1 consists of the pooled data from the F_1 hybrids. Cross 2 consists of the pooled data from the F_2 hybrid. In the second cross the homozygotes are lumped under common categories, i.e., mt^X/mt^X, $E\text{-}1^X/E\text{-}1^X$, $E\text{-}2^X/E\text{-}2^X$ where X denotes the inclusion of both B/B and C/C pairs. For example, the mt^X/mt^X class consists of both mt^B/mt^B and mt^C/mt^C pairs.

All eight classes were observed among the progeny derived from Cross 2, and seven of the eight classes were observed among the progeny derived from Cross 1. However, inspection of the frequency of pairs observed among these classes reveals that the distribution is not random. Moreover, Cross 1 and Cross 2 differ in their pattern of distribution.

In these crosses *E-1* and *E-2* assort independently, an observation consistent with the results previously discussed. This statement is true for the 52 pairs included in Table 4 as well as for the total data based on 87 pairs (Table 3) in which a 20:21:23:23 distribution of pairs was observed. Thus, these data support the conclusion that *E-1* and *E-2* are not linked.

Segregation of *mt* and *E-2* also appears to be independent. In Cross 1 a 7:5:7:7 distribution of pairs was observed, while in Cross 2 a 9:7:4:6 distribution was observed. Neither of these sets of data is significantly different from a 1:1:1:1 ratio. Nor are the combined data 16:12:11:13 significantly different from a 1:1:1:1 ratio. Therefore, *mt* and *E-2* do not appear to be linked.

This leaves the segregation of *mt* and *E-1* as the source of the departure from

TABLE 4

The distribution of mt, E-1, *and* E-2 *genotypes among backcross hybrids*

		Genotypes			Observed	Expected
F_1						
	(1)	mt^B/mt^B	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	5	3
				$E\text{-}2^B/E\text{-}2^C$	5	3
	(2)		$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^B/E\text{-}2^B$	2	3
				$E\text{-}2^B/E\text{-}2^C$	0	3
	(3)	mt^B/mt^C	$E\text{-}1^B/E\text{-}1^B$	$E\text{-}2^B/E\text{-}2^B$	2	3.5
				$E\text{-}2^B/E\text{-}2^C$	2	3.5
	(4)		$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^B/E\text{-}2^B$	5	3.5
				$E\text{-}2^B/E\text{-}2^C$	5	3.5
F_2						
	(1)	mt^X/mt^X	$E\text{-}1^X/E\text{-}1^X$	$E\text{-}2^X/E\text{-}2^X$*	3	4
				$E\text{-}2^B/E\text{-}2^C$	2	4
	(2)		$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^X/E\text{-}2^X$	6	4
				$E\text{-}2^B/E\text{-}2^C$	5	4
	(3)	mt^B/mt^C	$E\text{-}1^X/E\text{-}1^X$	$E\text{-}2^X/E\text{-}2^X$	3	2.5
				$E\text{-}2^B/E\text{-}2^C$	5	2.5
	(4)		$E\text{-}1^B/E\text{-}1^C$	$E\text{-}2^X/E\text{-}2^X$	1	2.5
				$E\text{-}2^B/E\text{-}2^C$	1	2.5

* X=B or C homozygotes; i.e., mt^B/mt^B or mt^C/mt^C, etc.

randomness observed in these data. If classes (1) and (4) are lumped and classes (2) and (3) are lumped, both crosses show a significant departure from a 1:1 ratio ($P < .01$) although the crosses differ in which lumped category is in excess. If *mt* and *E-1* are linked classes (1) and (4) should be the parental classes in Cross 1. This is observed and the ratio is 20 (1)+(4):6 (2)+(3). A similar excess of classes (2) and (3) is found in Cross 2 and the ratio is 19 (2)+(3):7 (1)+(4). The difference in distribution between the two crosses is easily explained if the F_2 hybrid is postulated to be a crossover of the genetic constitution $mt^B E\text{-}1^C/mt^C E\text{-}1^B$.

These data, therefore, are compatible with the conclusion that *mt* and *E-1* are linked. An estimate of crossing over can be made if the parental classes and the crossover classes from the two crosses are lumped. The combined data result in a 21:7:6:18 distribution of pairs. Crossing over is thus estimated at 25 percent.

DISCUSSION

The linkage relationships of five loci in variety 1 of *T. pyriformis* were examined. *H*, *E-1*, *E-2*, and *P-1* segregated independently in a four-factor cross. Each of these loci may mark a different chromosome. In a three-factor cross involving *mt*, *E-1* and *E-2*, *E-1* and *E-2* and *mt* and *E-2* segregated independently, while *mt* and *E-1* did not; 25 percent crossing over was observed between *mt* and *E-1*. The linkage of *mt* and *E-1* is the first to be recorded in this organism as well as in the ciliated protozoa.

In line with these observations, NANNEY (1960) found that *mt* and *H* segre-

gated independently. Two unlinked lethals, fat (*F*) and tiny (*T*), discovered by ORIAS (1960), were not included in the present analysis.

Considerable difficulty has been encountered in deriving highly viable lines of the C strain by selection during inbreeding. Viability and genomic exclusion seem to be inversely correlated (NANNEY 1963), and a high frequency of genomic exclusion is often observed in outcrosses of the C strain (ALLEN 1963). It is for this reason that the "triple cross" was developed. The triple cross aids in the immediate distinction between progeny derived from conjugation and progeny derived from genomic exclusion. Ideally, this type of cross should have been used in the crosses involving *mt* and *E-1*. This would have prevented the undesirable necessity of lumping data obtained from the "good" backcrosses in order to achieve sufficient numbers for statistical analysis.

RAY (1954) discovered that the haploid complement of chromosomes in variety 1 of *T. pyriformis* was five. Five linkage groups would, therefore, be expected. The results, to date, suggest that four of the chromosomes may be marked: *mt-E-1*; *H*; *E-2*; and *P-1*. Of course, some of the markers that do not appear to be linked could be on the same chromosome if they are separated by more than 50 map units.

RAY's (1956) analysis of meiosis in this organism suggests that an unconventional linkage mechanism could operate to associate markers located on the ends of adjacent chromosomes. During the early stages of the first meiotic division individual chromosomes are not seen. Polarized chromatin threads, first single, then double, are visible during the "parachute" stage. These threads then elongate until the threads coil around the macronucleus, forming a "crescent." RAY observed that the chromatin threads are "clearly paired in the crescent" and that "the appearance of chromosomes is that of late pachytene to early diplotene." Moreover, the double nature of the chromatin threads "precludes the conclusion that all chromosomes lie side by side and favors the interpretation that they are oriented end to end." As the crescent shortens five discrete tetrads appear and they are often observed to be oriented in a linear order. If the chromosomes are oriented in a particular order in the crescent, and if there is some type of physical continuity between the ends of adjacent chromosomes, the crescent may be composed of homologous "super" chromosomes. Super chromosomes may be formed as the crescent spins out, or they may have been formed in the previous sexual generation as a terminal stage following the reconstitution of diploidy. Discrete chromosomes are observed until the first post-zygotic division but are not seen once the nuclei differentiate into macronuclei and micronuclei. If super chromosomes form during the diploid stage, the crescent may serve as a preliminary step in the reestablishment of individual chromosomes (NANNEY and RUDZINSKA 1960). Random distribution of chromosomes conceivably could occur at two stages (1) during the first meiotic division, as observed in other organisms; or (2) during the formation of the super chromosomes following conjugation. Since the significant stage (2) occurs in the previous generation, preceding the meiosis in question, the results would be equivalent if the distribution is random at either (1) or (2). Segregation should be normal, and markers on adjacent chromosomes should

not show linkage. If nonrandomness occurred during (1) and (2), then markers on the ends of two adjacent chromosomes could show linkage.

The genetic data support, but do not prove, that the distribution of chromosomes is random. Nine of the ten possible pairwise combinations of five loci have been examined, and in eight combinations no correlation in parental genotypes was observed. If nonrandomness occurred at both (1) and (2) the observed random segregation of these markers could occur if crossing over were exceptionally frequent. A final decision as to the behavior of the chromosomes during meiosis rests upon a detailed cytological analysis, although certain observations (RAY, personal communication) suggest that, following the crescent stage, meiosis is probably similar to that observed in other organisms. The linkage between *mt* and *E-1* is, therefore, most likely interpreted as a conventional linkage of markers on the same chromosome.

The peculiar features of meiosis observed in Tetrahymena and in other ciliated protozoa may have been elaborated as special packaging devices for the chromosomes. The genetic evidence suggests that the central feature of meiosis—i.e., the random distribution of parental markers—occurs as in other organisms.

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SUMMARY

The linkage relationships of five loci in variety 1 of *T. pyriformis* were explored. *H*, *E-1*, *E-2*, and *P-1* segregate independently. *mt* and *E-2* also segregate independently. *mt* and *E-1* are linked, with 25 percent crossing over. This linkage is the first to be reported in the ciliated protozoa. The genetic evidence suggests that in spite of certain peculiar features observed during meiosis in the Ciliates that the central feature of meiosis—i.e., the random distribution of parental markers—occurs as in other organisms.

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