

GENETICS OF THE ST SEROTYPE SYSTEM IN *TETRAHYMENA* *PYRIFORMIS*, SYNGEN 1*

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ABSTRACT

Genetic analyses using lines of *Tetrahymena pyriformis* manifesting different serotypes indicate that the St serotypes are governed by alleles at a single genetic locus. These alleles are termed St^A and St^C . The *St* locus is not closely linked to any of the other well-studied loci examined. Differentiation in St^A/St^C heterozygotes follows a pattern very similar to that observed with lines heterozygous at the other loci. Initially both alleles are expressed, but as the synclone divides, lines develop that manifest one allele or the other but not both. The time of differentiation is very early in the clonal life cycle, and the output ratio is eccentric. The pattern of development of the *St* locus places it in a category with the mating type and H serotype loci.

A strain of *Tetrahymena pyriformis*, syngen 1, ordinarily manifests a single immobilization antigen, but has the ability to produce any one of several such antigens. The particular serotype expressed depends in part on the prevailing environment, in part on the previous history, and in part on the strain. Strains differ in the specificities of the antigens produced. The four different H serotypes—Ha, Hc, Hd, and He—found among the fourteen inbred strains (families) of *T. pyriformis*, syngen 1, are phenotypic manifestations of genetic information of four allelic variants, H^A , H^C , H^D , and H^E at the *H* locus (NANNEY and DUBERT 1960). A similar situation occurs with respect to the *T* serotypes, *Ta*, *Tb*, and *Tc* (PHILLIPS 1967a). Strain differences for the I serotypes involve the expression of different initial and secondary I types depending on the family but the genetic basis for the differences has not been determined (JUERGENSMEYER 1969). The L serotype, expressed at 15°C, exhibits no strain differences in the antigens themselves, but the rate of transformation from H to L does vary among the families of syngen 1 (JUERGENSMEYER 1969).

The *H* and the *T* loci are unlinked to each other (PHILLIPS 1967a), and are unlinked to any of the other well-studied loci (NANNEY 1960; ALLEN 1964; PHILLIPS 1967a). The only case of linkage reported in *Tetrahymena* is between the mating-type locus and the esterase-1 locus (ALLEN 1964).

Heterozygotes for serotype loci, as do most heterozygotes in *Tetrahymena*, undergo a peculiar kind of phenotypic assortment the basis of which is not well understood. Like other ciliates, *Tetrahymena* has both micro- and macronuclei.

* This report is part of the research conducted in partial fulfillment of the requirements for the Ph.D. degree at the University of Illinois, Urbana. The work was supported by USPHS grant GM-07779 and NSF grant GB-023910 to Dr. D. L. NANNEY.

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The macronucleus, a polygenomic structure, constitutes the somatic apparatus of the cell while the diploid micronucleus makes up the germ line. During conjugation (ELLIOTT and HAYES 1953; NANNEY 1953; RAY 1955) the micronucleus undergoes meiosis to give four haploid products, three of which disintegrate. The remaining haploid product undergoes a mitotic division giving two haploid products, one of which remains stationary while the other becomes migratory. The migratory pronucleus from each member of a conjugating pair moves into the other cell and fertilizes the stationary pronucleus. Each cell then contains a diploid, zygotic nucleus and the old macronucleus. As the old macronucleus is resorbed, the zygotic nucleus gives rise to the new micronucleus and two macronuclei. At the first cell division after conjugation, the micronucleus divides mitotically, while one macronucleus is distributed to each daughter cell or caryonide. Thus two members of a conjugating pair give rise to four "caryonides", genotypically identical subclones derived from different primordial macronuclei.

Although genetically alike, sister caryonides may be hereditarily different for some traits, both in *Tetrahymena* (NANNEY 1956) and in other ciliates (SONNEBORN 1937); for other traits the caryonides may not be different, but sublines of distinctive character arise later in the life cycle. Thus "vegetative assortment" occurs for most heterozygotes in syngen 1 of *T. pyriformis*, and remarkably enough, the kinetics of the assortment—once it begins—is identical for all loci. The chief variable appears to be the time at which assortment begins, and this seems to be a locus-specific characteristic.

Recent studies (GRASS, in preparation) established a new set of specificities within the serotype system of *T. pyriformis*, revealed under conditions of high-salt stress, for which strain differences have been documented. The purpose of the present study was to determine the genetic basis for the strain differences in these "St" serotypes and to assess the properties of heterozygotes for loci which might be observed.

MATERIALS AND METHODS

The families of *T. pyriformis*, syngen 1, used in this study were A, B, C1, C2, and C3. Families A and B display the Sta serotype, while C1, C2, and C3 display the Stc type. The allelic constitutions for all of the previously studied loci of these families are given in Table 1. For the derivation of these inbred families see ALLEN (1967); NANNEY, CAUGHEY and TEFANKJIAN (1955), and NANNEY (1959).

TABLE 1

*Allelic constitutions of five families of syngen 1, T. pyriformis**

Family	<i>mt</i>	<i>H</i>	<i>T</i>	<i>E-1</i>	<i>E-2</i>	<i>P-1</i>
A	<i>mt^A</i>	<i>H^A</i>	<i>T^A</i>	<i>E-1^B</i>	<i>E-2^B</i>	<i>P-1^A</i>
B	<i>mt^B</i>	<i>H^D</i>	<i>T^B</i>	<i>E-1^B</i>	<i>E-2^B</i>	<i>P-1^B</i>
C1	<i>mt^C</i>	<i>H^A</i>	<i>T^A</i>	<i>E-1^C</i>	<i>E-2^B</i>	<i>P-1^B</i>
C2	<i>mt^C</i>	<i>H^E</i>	<i>T^B</i>	<i>E-1^C</i>	<i>E-2^C</i>	<i>P-1^B</i>
C3	<i>mt^C</i>	<i>H^A</i>	<i>T^B</i>	<i>E-1^C</i>	<i>E-2^B</i>	<i>P-1^A</i>

* From PHILLIPS 1967a; S. L. ALLEN, personal communication.

All immobilization tests were run with cells grown in axenic peptone prepared with 200 mM NaCl. The procedures for the preparation of the antisera were identical to those of PHILLIPS (1967a). The immobilization tests were described by LOEFER, OWEN and CHRISTENSEN (1958). The breeding procedures and tests for nonconjugation and maturity were the same as those used by NANNY and CAUGHEY (1955). After maturation, the F_1 lines were transferred from bacterized peptone into 100 mM and 200 mM NaCl-peptone (axenic) for examination of the St serotype. All serotype tests were performed under axenic conditions. F_2 and backcross pairs were isolated directly into 100 mM NaCl-peptone and allowed to form clones. After two days these lines were mass transferred to 200 mM NaCl-peptone and were serotyped after growth in this medium for 24 hr.

Procedures for starch gel electrophoresis used in the study of linkage between the esterase-1 and *St* loci have been described previously (ALLEN 1961).

Determination of the kinetics of stabilization during vegetative assortment employed methods similar to those described by NANNY and DUBERT (1960).

RESULTS AND ANALYSIS

Parental and F_1 phenotypes: When grown in axenic salt medium two antigenic classes may be distinguished with available antisera among the families of syngen 1. Serotype Sta is observed in families A, A1, A3, B, D, D1, E, and F. Stc occurs in C, C1, C2, C3, and possibly B2. As demonstrated elsewhere Sta and Stc are serologically related but may be discriminated. Antisera against Stc immobilize Sta cells equally well, but anti-Sta sera—when absorbed with Stc cells—immobilize only Sta cells. Unabsorbed anti-Sa sera have a higher titre against Sta cells than against Stc cells, and at limiting dilutions can be diagnostic. Unabsorbed sera were used in many of the experiments. A possible explanation for these observations is that the Sta serotype is composed of two antigenic component, i.e. component a and component c, while the Stc serotype possesses only the c component.

The phenotypes of the F_1 will be treated in greater detail later. For the moment it is sufficient to note that "vegetative assortment" does in fact occur for the St serotype. In making crosses for the F_2 or backcross generations, matings can be arranged between sublines with various phenotypes, but the F_1 phenotypes are not consequential for their progeny's serotypes.

To understand some of the tests performed in this study, the distinction between the F_1 and parental phenotypes must be understood. In early F_1 pair cultures (synclones) most cells are immobilized by unabsorbed anti-Sta sera. These cultures are easily distinguished from parental Stc cultures which are not immobilized by antisera at the diagnostic dilutions. Distinction between F_1 synclonal cultures and parental Sta cultures is more difficult, since both contain cells with the Sta antigen. However, the distinction can be made with anti-Sta sera absorbed with Stc cells. Sta parental cultures are completely immobilized by such antisera, while F_1 cultures a few days old respond with an intermediate reaction with many of the cells immobilized and a large number unaffected by the antisera. The controls run with all tests included parental Sta and Stc types tested against both the antiserum used and normal sera or demineralized water. In summary, under the specified conditions, three phenotypes can be distinguished—parental Sta, parental Stc, and F_1 .

TABLE 2

Results of crosses between F₁ heterozygotes manifesting various phenotypes

Parental genotypes	Parental phenotypes	Serotypes of progeny			χ^2*	P
		Stc	Stac+Sta	Total		
<i>St^A/St^C × St^A/St^C</i>						
F ₁ (A × C2)	Sta × Sta	16	44	60	0.1	0.7
F ₁ (B × C3)	Stc × Stc	18	41	59	0.9	0.3
F ₁ (B × C2)	Sta × Stc	13	42	55	0.1	0.7
F ₁ (A × C2) × F ₁ (B × C2)	Sta × Stc	12	43	55	0.3	0.6
	Total	59	170	229	0.1	0.7

* χ^2 values calculated on basis of 3:1 ratio. Homogeneity χ^2 value = 1.3; P = 0.8.

The genetic basis for strain differences in St serotypes: Crosses between inbred families of syngen 1 displaying different St serotypes were carried out and F₂ and backcross data were collected to determine if these serotypic differences were manifestations of two different alleles at a single locus. The F₂ data are summarized in Table 2. The first three crosses show the results of matings between the F₁ products of the same crosses. The fourth mating was between the F₁ products of two different crosses as indicated. In all cases the observed data fit the 3:1 ratio expected of segregating alleles. A 3:1 ratio was expected because the antisera used in these experiments were unabsorbed anti-Sta sera. This procedure avoided the necessity of absorbing the antisera with Stc cells so that heterozygotes could be distinguished from homozygotes. All lines possessing Sta factors are immobilized by unabsorbed anti-Sta sera. This would include most cells in heterozygous synclones and *St^A/St^A* homozygous synclones which together would constitute 75% of the progeny if segregating *St^A* and *St^C* alleles are involved. Totally negative reactions indicated that the Sta factor was absent and that the cells were of the *St^C/St^C* genotype. Each F₂ and backcross synclone was tested at least four times, usually with two different anti-Sta sera. At least one of these tests always employed anti-Sta serum absorbed with Stc cells.

Table 3 shows the results of backcrosses between mature F₁ lines and parental homozygous of the Sta serotype. Phenotypes of backcross progeny were distinguished in the same manner as F₁ and parental Sta phenotypes. Absorbed anti-Sta sera were used here. If a strong positive reaction was observed, the synclone

TABLE 3

Results of crosses between Sta parental homozygotes and F₁ heterozygotes of various phenotypes

Parental genotypes	Parental phenotypes	Serotypes of progeny			χ^2*	P
		Sta	Stac	Total		
<i>St^A/St^C × St^A/St^A</i>	Sta × Sta	29	24	53	0.5	0.5
F ₁ (A × C3) × A	Stc × Sta	28	26	54	0.1	0.8
	Total	57	50	107	0.4	0.5

* χ^2 values calculated on basis of 1:1 ratio. Homogeneity χ^2 = 0.1; 0.8.

TABLE 4

Results of crosses between Stc parental homozygotes and F₁ heterozygotes of various phenotypes

Parental genotypes	Parental phenotypes	Serotypes of progeny			χ^2 *	P
		Stc	Stac	Total		
<i>St^A/St^c × St^c/st^c</i>						
F ₁ (A × C3) × C3						
F ₁ (B × C3) × C2	Stc × Stc	100	102	202	.02	.9
F ₁ (A × C2) × C2	Sta × Stc	76	87	163	.7	.4
F ₁ (B × C2) × C2	Stac × Stc	25	23	48	.1	.7
	Total	201	212	413	.3	.5

* χ^2 values calculated on basis of 1:1 ratio. Homogeneity $\chi^2 = 0.5$; P = 0.8.

was classified as homozygous *St^A/St^A*. If an intermediate reaction was observed, the synclone was classified as heterozygous, *St^A/St^c*. These tests were run after each exconjugant had undergone six to twelve fissions. The results of the vegetative assortment experiment indicated that *St^A/St^c* heterozygotes yield some pure Stc lines as early as the fourth fission. Thus the classification of heterozygous and homozygous synclones in this manner, at this early stage is necessary.

Table 4 summarizes the results of backcrosses between mature F₁ lines and parental homozygotes of the Stc serotype. Determination of progeny phenotypes in this experiment was less difficult for the reasons cited previously for the parents, and unabsorbed anti-Sta sera were used after tests with absorbed antisera indicated the absence of pure Sta synclones. Lines showing any positive immobilization reaction were classified as heterozygous, since these would possess an *St^A* allele. Lines showing a completely negative reaction were classified as homozygous, *St^c/St^c*.

Neither of these sets of backcross data was significantly different from the 1:1, heterozygote to homozygote ratio expected of segregating alleles. The phenotypic differences observed in cells from different families grown in high salt concentrations are due to two different alleles, *St^A* and *St^c*, at a single genetic locus, the *St* locus.

In mature heterozygotes, one allele is "silent" but in the F₂ or backcross generation these "silent" genes are expressed. This aspect of the F₁ heterozygotes will be discussed further.

Genetic relationship between the St locus and other loci: The only case of linkage reported in Tetrahymena is that between the mating-type (*mt*) locus and the class 1 esterase (*E-1*) locus (ALLEN 1964). The small number of chromosome pairs (RAY 1955) should, however, render linkages relatively easy to find now that the number of markers surpasses the number of chromosome pairs. Examination of the distribution of the Sta and Stc serotypes among the inbred families of syngen 1 reveals a correlation between families homozygous for certain mating-type alleles and St serotype alleles. The implications of this correspondence are strengthened by a similar correlation between the families homozygous for various *E-1* alleles and *St* alleles. Families that carry the *St^c* allele in the homozygous

TABLE 5

Linkage relationship between the St and the E-1 loci in dihybrid backcrosses, St^c/St^c, E-1^c/E-1^c × St^A/St^c, E-1^B/E-1^c [C3 × F₁ (A × C3)]

Progeny phenotypes	Observed frequencies	Expected frequencies*		
		A	B	C
Stac, E-1bc	14	10	15	20
Stac, E-1c	6	10	5	0
Stc, E-1bc	12	10	5	0
Stc, E-1c	8	10	15	20
Total	40	40	40	40

Maximum likelihood estimate of linkage: 45 ± 15.5
 χ^2 calculated on basis of independent assortment = 4.0; $P = 0.3$.
 χ^2 calculated on basis of 25% recombination = 13.23; $P = 0.005$.

* Columns under "expected frequencies" indicate expectations based on (A) independent assortment, (B) 25% recombination, and (C) complete linkage.

state also carry the *E-1^c* and *mt^c* alleles in the homozygous state. Families homozygous for the *St^A* allele carry one of the other five *mt* alleles in the homozygous state along with the *E-1^B* allele. The *St* serotype of family B2 has not been determined precisely, and there is some indication that it may be an exception to this pattern (GRASS, in preparation). Such distribution analyses are however, at best only suggestive and breeding studies are required to establish linkage. The procedures were straightforward. Doubly heterozygous lines of known parentage were backcrossed and their progeny were examined for deviations from expected phenotypic ratios.

1. Relationship between the *St* locus and the *E-1* locus: Forty backcross lines from as many individual pairs that had been examined for the *St* serotype were transferred individually to 250 ml flasks containing 100 ml of standard axenic peptone, grown, harvested and homogenized. The homogenate of each line was then tested for *E-1* specificity. The results of these tests are given in Table 5. The observed distribution of the *St* and *E-1* phenotypes in the backcross generation did not differ significantly from the 1:1:1:1 ratio expected of randomly assorting genes. *E-1^B/E-1^c* and *E-1^c/E-1^c* lines were distributed randomly throughout the *St^A/St^c* and the *St^c/St^c* lines. A few more lines were found (22:18) in the parental than the recombinant classes, but *St* and *E-1* are obviously not closely linked. A maximum likelihood estimate (MATHER 1951) yields linkage values of 45 ± 15.48 indicates that the two loci could be no closer than 30 map units.

2. Relationship between the *St* locus and the *mt* locus: A preliminary experiment was performed to determine if there was any indication of linkage between the mating-type locus and the *St* locus. The *mt* locus was more difficult to examine than the *E-1* locus or any of the serotype loci. Mating-type alleles specify an array of mating types and the frequencies with which specific mating types occur (NANNEY 1959). Cells of the genotype *mt^c/mt^c* may be any mating type of the array I, II, III, V, or VI. Cells of the genotype *mt^B/mt^B* may be any mating type of the array II, III, IV, V, VI, and VII. The critical types are I, IV, and VII. Mating-type frequencies are dosage dependent, and mating types I, IV, and VII occur twice as frequently in homozygotes as in heterozygotes (NANNEY 1959).

Mating-type specificities are under macronuclear control, and all cells with macronuclei derived from a given macronucleus tend to have the same mating type (NANNEY and CAUGHEY 1953). Therefore, all lines derived from a single caryonide tend to be the same. Lines derived from different caryonides of a single pair do not tend to be alike. Though these mating-type specificities are determined during macronuclear development prior to the first macronuclear division they are not manifested until sexual maturity, approximately 65 fissions after conjugation (BLEYMAN and SIMON 1968).

All of these factors make determination of the mating-type genotype a laborious process of progeny testing a large number of caryonides from a cross (NANNEY 1960). A simpler procedure was adopted to determine if there was any indication of linkage between the *mt* locus and the St serotype locus. A backcross involving an *mt^B/mt^C* heterozygote and an *mt^C/mt^C* homozygote was employed. Synclones derived from fifteen backcross pairs observed to be homozygotes of the *St^C/St^C* genotype were selected and expanded by single-cell isolations to twenty-one lines each. This process gave a total of 315 lines. Some lines died, and the remaining 287 were transferred to maturity. At maturity each line was tested for mating type to determine if the critical types were produced and to get an idea of the relative frequencies at which other types were produced. These results are summarized in Table 6.

The appearance of mating type I in the progeny indicated that the *mt^C* allele was present. Since this was a backcross situation, this allele was present in all cells. The observation of mating types IV or VII indicated the presence of the *mt^B* allele.

TABLE 6

Relationship between the mating type and the St serotype loci in dihybrid backcrosses, St^C/St^C, mt^C/mt^C × St^A/St^C, mt^B/mt^C [C3 × F₁ (B × C3)]

Line	I	II	III	Mating type IV	V	VI	VII	Probable genotype
a	.30	.35	—	.35	—	—	—	<i>mt^B/mt^C</i>
b	.60	.38	—	.35	—	—	—	<i>mt^B/mt^C</i>
c	.09	.48	—	.24	—	.19	—	<i>mt^B/mt^C</i>
d	—	.25	.30	—	—	—	—	<i>mt^B/mt^C</i>
e	.40	—	—	.60	—	—	—	<i>mt^B/mt^C</i>
f	.47	.26	—	.21	—	.06	—	<i>mt^B/mt^C</i>
g	.33	.37	—	—	—	.30	—	<i>mt^C/mt^C</i>
h	.80	—	—	.20	—	—	—	<i>mt^B/mt^C</i>
i	.11	.11	.05	—	—	.73	—	<i>mt^C/mt^C</i>
j	.44	—	.17	—	—	.39	—	<i>mt^C/mt^C</i>
k	—	.47	.35	—	—	.18	—	<i>mt^B/mt^C</i>
l	.85	—	—	—	—	.15	—	<i>mt^C/mt^C</i>
m	.82	.18	—	—	—	—	—	<i>mt^C/mt^C</i>
n	.61	.17	—	.22	—	—	—	<i>mt^B/mt^C</i>
o	.35	—	.06	.06	—	.53	—	<i>mt^B/mt^C</i>

mt^B/mt^C : mt^C/mt^C = 10:5; χ^2 value based on 1:1 ratio expected for random assortment = 1.4; P = 0.2 with classes d and k included. ($\chi^2 = 0.8$; P = 0.4 with classes d and k excluded).

Maximum likelihood estimate of linkage: 60 ± 25.0.

Likely genotypes were assigned to each line on the basis of the absence of mating types IV or VII. If one of these mating types occurred, the mt^B allele had to be present, and the lines must have been heterozygous. The absence of one of these mating types does not totally rule out the possibility of mt^B/mt^c heterozygosity, although a sample size this large renders fairly likely that all four caryonides would be represented, since the expected frequency is 0.26 for mating-type IV in heterozygous caryonides. The inference that the mt^B allele was absent is thus fairly strong. To establish the absence of an allele with certainty would require progeny tests.

In two cases, lines d and k in Table 6, none of the critical mating types appeared in the expansion lines. These were considered more likely to be mt^B/mt^c heterozygotes because of the slightly greater probability of obtaining no mating types I and IV with expected frequencies of 0.21 and 0.26 respectively in the mt^B/mt^c state than of getting no mating type I with an expected caryonidal frequency of 0.60 in the mt^c/mt^c state.

If the mt and St loci were linked, one would expect to observe a tendency for the mt^c and the St^c alleles to remain together. Since the lines selected were known St^c/St^c homozygotes, linkage would be implied by a preponderance of mt^c/mt^c homozygotes. If these loci were not linked, one would expect the mt^B/mt^c heterozygotes and the mt^c/mt^c homozygotes to be randomly distributed within this group. The results indicate that the ratio of likely mt^B/mt^c to likely mt^c/mt^c genotypes was 10:5, not significantly different from the 1:1 ratio expected if alleles at the mt locus and the St locus had assorted independently. Even if the two somewhat ambiguous cases are not considered, the ratio of likely heterozygotes to likely homozygotes was 8:5, again not significantly different from the expected 1:1 ratio based on independent assortment. The total number of synclones examined was small, but the bias is the opposite of that expected if the mt and St loci were linked. A maximum likelihood estimate yields a recombination value of 60 ± 25 and constitutes sufficiently strong evidence against any close linkage between these loci that a further detailed analysis of the relationship is unwarranted.

3. Relationship between the St serotype and the H and T serotypes: Although no obvious correlations exist among inbred families with various H and T serotypes, experiments were performed to determine if the St locus might be linked to either of these other serotype loci.

Table 7 summarizes the experiments to determine if St and H are linked. These tests were performed with backcross progeny that had been grown in 200 mM NaCl-peptone so that the immature lines could be tested for the St serotype. The synclonal lines were transferred by loop inoculation into bacterized peptone so that the H serotype could be examined. Once the H serotype was expressed, the lines were tested with anti-Ha or anti-Hd sera and anti-He sera. The observed frequencies of the various dihybrid backcross serotypes were not significantly different from the frequencies expected on the basis of independent assortment of alleles at the St locus and the H locus.

A summary of the results of the tests for linkage between St and T is given in

TABLE 7

Linkage relationship between the H and the St loci in dihybrid backcrosses

Progeny phenotypes	Observed frequencies	Expected frequencies*		
		A	B	C
a) $St^C/St^C, H^E/H^E \times St^A/St^C, H^A/H^E$ ($C2 \times F_1 (A \times C2)$)				
Stac, Hae	9	13.25	19.87	26.50
Stac, He	17	13.25	6.63	0
Stc, Hae	11	13.25	6.63	0
Stc, He	16	13.25	19.87	26.50
Total	53	53.00	53.00	53.00
χ^2 based on independent assortment: $\chi^2 = 3.1$; $P = 0.3$				
χ^2 based on 25% recombination: $\chi^2 = 28.7$; $P < 0.0001$				
Maximum likelihood estimate of linkage: 53 ± 13.4				
b) $St^C/St^C, H^E/H^E \times St^A/St^C, H^D/H^E$ ($C3 \times F_1 (B \times C3)$)				
Stac, Hde	11	14.75	22.13	29.50
Stac, He	17	14.75	7.37	0
Stc, Hde	18	14.75	7.37	0
Stc, He	13	14.75	22.13	29.50
Total	59	59.00	59.00	59.00
χ^2 based on independent assortment: $\chi^2 = 2.2$; $P = 0.5$				
χ^2 based on 25% recombination: $\chi^2 = 39.2$; $P < 0.0001$				
Maximum likelihood estimate of linkage: 59 ± 12.5				

* Columns under "expected frequencies" indicate expectations based on (A) independent assortment, (B) 25% recombination, and (C) complete linkage.

Table 8. The immobilization tests for the T serotype were also performed with cells that had been tested for the St serotype and transferred to bacterized peptone. Transformation to the T serotype was achieved by growing the cells in test tubes with liver peptone at 40°C (PHILLIPS 1967a). The observed phenotypic

TABLE 8

Linkage relationship between the St and the T loci in dihybrid backcrosses
 $St^C/St^C, T^B/T^B \times St^A/St^C, T^A/T^B$ [$C3 \times F_1 (A \times C3)$] and [$C3 \times F_1 (B \times C3)$]

Progeny phenotypes	Observed frequencies	Expected frequencies*		
		A	B	C
Stac, Tab	12	17.50	26.25	35.00
Stac, Tb	17	17.50	8.75	0
Stc, Tab	22	17.50	8.75	0
Stc, Tb	19	17.50	26.25	35.00
Total	70	70.00	70.00	70.00
χ^2 value calculated on basis of independent assortment = 3.1; $P = 0.4$				
χ^2 value calculated on basis of 25% recombination = 37.6; $P < 0.0001$				
Maximum likelihood estimate of linkage: 56 ± 11.8				

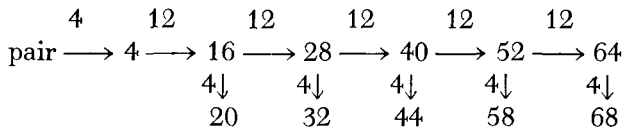
* Columns under "expected frequencies" indicate expectations based on (A) independent assortment, (B) 25% recombination, (C) complete linkage.

frequencies were not significantly different from the 1:1:1:1 ratio expected with independently assorting loci.

Although some difficulty was encountered in obtaining unambiguous results with the anti-T sera, reasonably discriminatory results were obtained using absorbed anti-Tb sera and one specific anti-Ta serum (serum #62). The cross reactivity of the anti-Tb sera has been discussed (PHILLIPS 1967a). One problem that made the tests for the T serotype difficult was that several of the sera had titres much lower than had been recorded at the time bleedings were performed. Similar observations have been made with some of the older anti-H sera. Possibly these antisera have deteriorated over a period of several years.

The linkage tests with the loci examined indicated that all assorted independently of the *St* locus. Although maximum likelihood estimates rule out close linkage to any of the markers, these results do not eliminate the possibility of distant linkage which could be detected only by the examination of a much larger number of synclones.

Vegetative assortment in Stac heterozygotes: Lines of *Tetrahymena* initiated from heterozygotes shortly after conjugation give rise to mixed cultures which contain cells expressing both parental alleles. Later the lines stabilize and clones initiated from single cells are pure, i.e. they express one allele or the other but not both. The lines tested for the vegetative assortment of pure types from mixed heterozygous synclones in the present report were initiated from ten pairs isolated into 100 mM NaCl-peptone. Five of these pairs were eliminated because of death or positive tests for non-conjugation. After four fissions for each exconjugant the remaining five pair cultures were expanded to twenty-one lines each by single-cell isolation. The procedure for reinitiating lines on alternate days along with mass transfer of "leftover" cells into 200 mM NaCl-peptone can be summarized as follows:



The horizontal lines represent the single-cell transfers in 100 mM NaCl-peptone. The vertical lines represent the mass transfers of leftover cultures from 100 mM to 200 mM NaCl-peptone. The figures with the arrows indicate the approximate number of fissions that occurred between transfers, while the figures at the end of the arrows represent the age of the sublines at each transfer and test point expressed in fissions. The number of fissions that occurred at each stage of this experiment was estimated with cell counts by the dilution method described by PHILLIPS (1967a). The lines were serotyped after they had grown in 200 mM NaCl-peptone for twenty-four hours. Thus lines initiated by single-cell isolations from pair cultures at 4 fissions were serotyped after they had undergone 16 fissions. This method enabled the events that occurred prior to a given single-cell isolation to be examined after phenotypic magnification.

Table 9 shows the cumulative number of pure and intermediate lines observed at various times in the development of the synclone. Three facts should be noted

TABLE 9

The distribution of serotype phenotypes in subclones derived from single cells at various times since conjugation

St phenotypes	Fissions since conjugation				
	4	16	28	40	52
c	4	6	20	43	46
ca	93	85	67	36	27
a	0	3	6	11	17
Total	97	94	93	90	90

about these data. As pure lines accumulated, the number of lines of mixed phenotype declined. Pure Stc lines appeared early and accumulated rapidly until the fortieth fission. Pure Sta lines appeared later and developed at a slower rate. The five pair cultures appeared to be homogeneous and behaved in about the same way, although the sample size may have been too small to detect any inter-pair heterogeneity.

The final subclones represented in this table were initiated 52 fissions after conjugation. Because of bacterial contamination in many of the depression cultures, the experiment had to be terminated at this point. Later tables will show results through 40 fissions after conjugation. Lines that were scored pure for the first time at 52 fissions could not be tested a second time, a procedure necessary to determine if the lines were truly pure or if they were misclassified, the situation found with both the H and the T systems (NANNEY and DUBERT 1960; PHILLIPS 1967a). These misclassified lines were artifacts resulting from limitations of the immobilization tests (NANNEY and DUBERT 1960). Data were collected to determine the fraction of lines which were scored intermediate at various readings after being scored pure. These results indicate that lines should have been tested at least two and preferably three time before being classified as pure. Because lines that were first scored pure in the late stages of this experiment could not be tested three times, some of these may be false pure; in this case the rate of stabilization would appear to be higher than it actually was. This would explain the inflated rates observed in lines isolated at 40 fissions.

The numbers of new pure Stc and Sta lines at each transfer are shown in Table 10. From these figures the R_t values, or rates of fixation per transfer, were determined. R_t values represent the fraction of lines derived from impure cultures that were scored pure in all subsequent tests. By dividing the mean R_t value by the mean number of fissions between single-cell isolations, in this case 12, the R_f value or rate of fixation per fission of stable pure lines from mixed cultures was found to be 0.0153 stabilizations per fission, a highly significant value as will be discussed later.

Although the experiment had to be terminated prematurely and the sample size was smaller than anticipated, the similarities between the patterns of differentiation of pure types from heterozygous synclonal cultures of mixed phenotype in the St serotype system and other systems are impressive.

One other piece of evidence obtained from these data suggests a similarity be-

TABLE 10

Rate of stabilization of pure types from St^A/St^C heterozygotes of intermediate type

Parental genotype	Transfer	Fissions	New pure types			Fraction stabilized	× Fraction stabilized (± S.E.) per:	
			Sta	Stc	Total		Transfer	Fission
<i>St^C/St^C × St^A/St^A</i>	1	4	0	4	4	.0414		
	2	16	3	2	5	.0555		
	3	28	3	14	17	.2030		
	4	40	5	23	28	.4400		
							.1850	.0153
							±.0920	±.0078

tween the St system and other systems. To determine the distribution of stable sublines, the output ratio was calculated by the formula $100 (2 Stc + Stac)/2N$; where the *Stc* is the number of sublines pure for the Stc type, *Stac* the number of sublines manifesting both serotypes and *N* is the total number of lines examined (NANNEY *et al.* 1963). For lines examined at 40 fissions after conjugation, the output ratio was determined to be 68:32, an eccentric ratio significantly different from a 1:1 ratio ($\chi^2 = 13.0$ P < .001).

DISCUSSION

Genetic and chemical relatedness of the H, T, and St systems: Different serotypes in Tetrahymena are expressed under different growth conditions. A superficially plausible interpretation of these serotypes is that they are based on slightly modified forms of a single antigen. This interpretation is, however, rendered improbable by several considerations. The first of these is the exclusive dependence of each serotype on a different genetic locus for its specificity (PHILLIPS 1967a). The present study enlarges that evidence by one locus. The St serotypes depend upon the St locus. Little immobilization cross reaction occurs with other serotypes. Immunodiffusion studies indicated little or no similarity in the banding patterns formed by the H and St antigens (GRASS, in preparation). If the chemical relationship between the H and the T antigens is any indication of the relationship between these antigens and the St antigens, they are different molecules. Isolation and partial characterization of the H and T antigens indicated that they are different molecules with different molecular weights (BRUNS 1969). Thus the three antigens appear to be unrelated molecules.

Differentiation and allelic expression: Although genetically unrelated, the St serotype system and the other systems do appear to be influenced by the same epigenetic assortment processes as the similarities in patterns of differentiation during the clonal cycle indicate. Before a detailed discussion of this pattern of development is embarked upon, two aspects of the differentiated F₁ heterozygotes should be mentioned.

The macronucleus of a mature differentiated heterozygote has one allele that is expressed, while the other is repressed or absent at any given locus. If such a heterozygote is then crossed to another heterozygote or to a homozygous parent,

the allele that had been inactive in the macronucleus of the F_1 generation reappears in the F_2 or backcross generation in an unaltered, active state after a new macronucleus is generated from the zygotic micronucleus. For example, the cross between two St^A/St^c heterozygotes, each of the St^A serotype, yielded F_2 progeny 25% of which were of the St^c serotype. The cross between an F_1 heterozygote expressing the St^c allele and parental homozygotes of the St^c/St^c genotype gave heterozygous progeny with an active St^A allele. Differentiation of heterozygotes will be discussed in greater detail later, but clearly alleles passed through a heterozygous state and non-functional in the macronucleus of mature cells remain unaffected in their ability to be transmitted by the micronucleus and to be active in the macronucleus in succeeding generations.

One other point should be stressed. The F_1 cells had to be carried to maturity before they could be crossed or backcrossed. The lines were never exposed to high salt concentrations during this time and never expressed the St serotype. After the lines matured and were ready to be mated, they were grown in 200 mM NaCl-peptone and serotyped. Some lines pure for the St^c type and some lines pure for the St^A type were obtained from a single pair. Thus differentiation of pure types had occurred even though the St locus was "silent." Similar observations were made for the T serotype (PHILLIPS 1967a).

Macronuclear differentiation, vegetative assortment and intraclonal variation: The mean rate of fixation per fission of pure lines from mixed cultures was determined to be 0.0153 ± 0.0078 for the St serotype. This value was obtained with cultures that were transferred every other day, and approaches the R_f values of 0.0118 ± 0.0004 obtained for the T serotype (PHILLIPS 1967a) and 0.013 ± 0.0015 obtained for the H serotype (NANNEY and DURBERT 1960) with similar transfer schedules. The original work on unstable heterozygous systems involved an analysis of selfers in *Tetrahymena* (ALLEN and NANNEY 1958) which developed pure lines from mixed cultures at a rate of 0.0113 ± 0.0004 per fission with daily transfers. The rates obtained with daily transfers and alternate day transfers were compared by NANNEY and DUBERT (1960) for the H serotype. In each of these systems as well as in the enzyme systems (ALLEN 1965) the kinetics of differentiation appear to be identical. Initially cells isolated from heterozygous synclones produced unstable clones of mixed phenotype; later they produce clones pure for one serotype or the other. A model for the ciliate macronucleus has been proposed to explain these observations (ALLEN and NANNEY 1958; SCHENSTED 1958). This model has been reviewed several times (NANNEY and DUBERT 1960; NANNEY 1964; PHILLIPS 1967b) and will be only summarized briefly here. The model proposes that the macronucleus is a multigenomic structure composed of 45 diploid subnuclei after macronuclear division, each differentiating independently of the others by the activation or inactivation of one allele at each locus. These subnuclei replicate in the differentiated state. The timing of subnuclear differentiation certainly varies with the locus involved and possibly with the alleles. Vegetative pedigree analysis of H^A/H^B heterozygotes indicates that differentiation occurs just prior to the first macronuclear division, whereas H^c/H^B and H^A/H^B heterozygotes differentiate after the first macronuclear divi-

sion (NANNEY, NAGEL and TOUCHBERRY 1964). Later work (BLEYMAN, SIMON and BROSI 1966), however, suggests that all H differentiation may occur before the first fission. Similar analyses for the mating-type locus indicate that differentiation occurs prior to the first macronuclear division (BLEYMAN, SIMON and BROSI 1966). The T serotype locus, however, does not differentiate until approximately 30 fissions (PHILLIPS 1967b), and the esterase and phosphatase loci do not differentiate until 40–50 fissions after conjugation (ALLEN 1965).

After subnuclear differentiation, assortment occurs by random distribution of subnuclei to daughter macronuclei. If the input ratio (ratio between subnuclei expressing one allele and subnuclei expressing the other allele) is highly eccentric, there is a high probability that one daughter macronucleus will possess all subnuclei of the same type early in the period after differentiation, and all the progeny from such a line would be pure for that phenotype, the early or majority type. The daughter macronucleus composed of both types of subnuclei would give rise to further mixed subclones. Eventually daughter macronuclei would be produced which contain exclusively subnuclei in which the alternate allele is expressed. Cells with such macronuclei would give rise to pure lines of the late or minority type.

The observations of differentiation in the *St* serotype system fit this model very well. Presumably a majority of the subnuclei in the macronuclei of the F_1 heterozygotes differentiated so that the *St^c* allele was expressed. As a result the lines pure for the *St^c* serotype appeared very early. Such lines developed from cells isolated at 4 fissions after conjugation. Subnuclear differentiation of the *St* locus must occur early, at about the same time as in the H system and the mating type system. The *St* system is similar to the H and *mt* systems in another respect. All have eccentric output ratios, and all differentiate late. Thus there is some correlation between time of differentiation and eccentricity of output ratio, although this relationship is not absolute. For a given heterozygous type, the output ratio of different synclones may vary from being close to equality to being highly eccentric (NANNEY *et al.* 1963).

Thus clonal differentiation is a result of cellular differentiation which in turn is a manifestation of subnuclear differentiation and assortment. One model for subnuclear differentiation (NANNEY and DUBERT 1960) invokes a system of allelic repression. This hypothesis assumes the presence of diploid subnuclei throughout the macronuclear life span, with the activation of one allele and repression of the other allele at a locus. However recent evidence from a number of sources has suggested that if the macronucleus contains 45 subunits, these must be haploid, because the macronucleus contains 23 times as much DNA as the micronucleus rather than the 45 times as much which would be expected with diploid subunits (WOODARD, GOROVSKY and KANESHIRO 1968; NILSSON 1970). NANNEY (unpublished) has proposed a new model for subnuclear differentiation which suggests that at some time during macronuclear development chromosome diminution reduces each subnucleus to about the haploid amount of DNA, but retains 45 units diploid for the relevant loci. This hypothesis is consistent with the genetic and biochemical evidence reported to date.

The author wishes to thank Dr. DAVID L. NANNEY for his valuable suggestions and criticisms in the preparation of the manuscript.

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