T H E U N I V E R S I T Y O F M I C H I G A N

COLLEGE OF LITERATURE, SCIENCE, AND THE ARTS
Department of Zoology

Progress Report

GENETICS OF TETRAHYMENA

(October 1, 1958 - December 31, 1962)

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SUMMARY

Screening of the inbred strains of variety 1 of Tetrahymena pyriformis for two families of enzymes by starch gel electrophoresis revealed alternative forms for three different enzymes. Breeding analyses showed that each of these enzymes is controlled by alleles at single loci. E-1 and E-2 control different esterases, P-1 an acid phosphatase. None of these loci are linked, but E-1 is linked to mt.

Each E-1 allele produces isozymes. Since each set of isozymes behaves as a unit during breeding and subclonal analysis, the isozymes probably arise by modifications of a single protein produced by each allele. This idea receives support from experimental manipulation of the isozymes. The P-1 alleles make either one or three electrophoretically distinct acid phosphatases. The heterozygote is potentially capable of making five types, although variations in pattern occur during phenotypic drift.

This report covers several areas of our work. These areas are organized into the following sections:

a. General comments
b. Esterase isozymes in Tetrahymena
c. Variations in the electrophoretically separated acid phosphatases of Tetrahymena.
d. Genetic control of an acid phosphatase in Tetrahymena: formation of a hybrid enzyme
e. Lysosomes in Tetrahymena
f. A first case of linkage in Tetrahymena pyriformis
g. Regulation of phenotypic drift in Tetrahymena
h. Genomic exclusion in Tetrahymena: genetic basis
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I. RESEARCH ACCOMPLISHMENTS TO DATE

A. GENERAL COMMENTS

1. Inbred Strains

A number of inbred strains of variety 1 of Tetrahymena pyriformis have been derived during the course of breeding analysis. Most of these strains have been developed by D. L. Nanney (Department of Zoology, University of Illinois); four recombinant strains have been contributed from my laboratory. This brings the number of inbred strains to 13. A joint effort between our laboratories is being made in the maintenance of these strains.

These strains provide a rich source of genetic variation. Screening of these strains for only two families of enzymes by starch gel electrophoresis has so far revealed alternative forms of three different enzymes. Once differences are detected, appropriate breeding analyses are initiated.

The distribution of alleles at the mt (mating type), H (serotype), E-1 and E-2 (esterases) and P-1 (acid phosphatase) loci is shown in Table 1. Also listed are two additional potential markers, about which only partial information concerning distribution and inheritance has been obtained.

TABLE 1

DISTRIBUTION OF ALLELES AT FIVE LOCI IN THIRTEEN INBRED STRAINS

Strain	Distribution of Alleles at					Additional Markers	
	mt	H	E-1	E-2	P-1	E-3	P-2
A	A	A	B	B	A	a	a
A1	A	D	B	B	A		
A2	A?	E	B	B	B	a	
A3	A?	E	B	C	B	b	
B	B	D	B	B	B	a	a
B1	B	C	B	B	A		
C	C	E	C	C	B	a	a,b
C1	C?	A	C	B	B	a	
C2	C?	A	C	C	B	b	
D	D	D	B	B	A		
D1	D	C	B	B	A		
E	E	D	B	B	B		
F	F	D	B	B	B		

2. Methods

Tetrahymena are grown either on bacterized medium (Cerophyl rye grass inoculated with Aerobacter aerogenes) or axenic medium (1% proteose-peptone). Crosses of cells grown in either medium have been made, with similar genetic results being obtained. Since manipulations are easier and viability better, crosses of cells grown on bacterized medium are preferred. To prevent selection of subclones, large populations of cells from an exconjugant culture are transferred to the proteose-peptone medium after exposure to antibiotics.

Mating type and serotype tests are performed on cells grown in bacterized medium. For analysis of enzymes, extracts of peptone-grown cells are customarily employed.

Crude extracts are made by concentrating cells by centrifugation, washing them in glass-distilled water and breaking up the concentrate by repeated rounds of freeze-thawing. A sample of an extract is inserted into a starch gel and electrophoresis carried out in the horizontal position using 8 v/cm for 5 hours at room temperature (23-26°C). A tris-boric acid buffer system is employed in the starch (0.03M) and end trays (0.3M).

Visualization of the enzymes in starch is accomplished by incubating starch strips in the appropriate substrates in pans or in test tubes. If the product of the enzymatic reaction selected for visualization is a naphthol, it is coupled to a diazonium salt. If a phosphate, it is trapped as lead phosphate and visualized as lead sulfide. After incubation in substrate, the starch strips are washed in successive changes of distilled water and stored in the refrigerator until photographed. Photography of starch strips is best accomplished by transillumination.

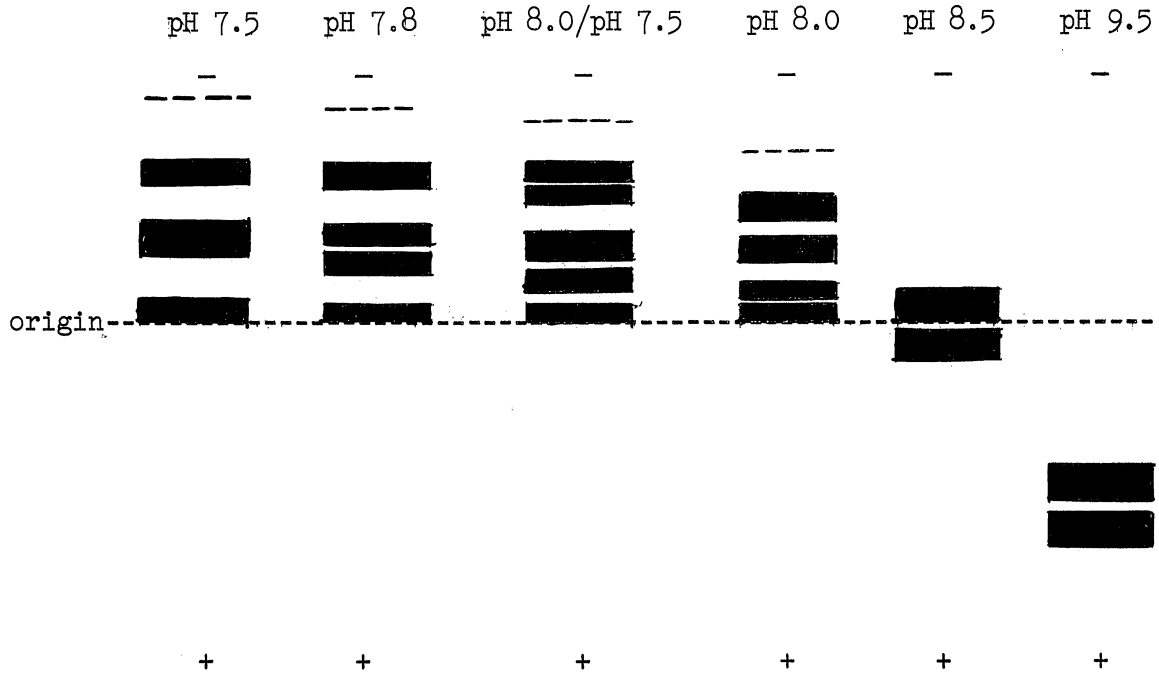
B. ESTERASE ISOZYMES IN TETRAHYMENA

Cells homozygous for each of the alleles at the E-1 locus produce four to six esterases with identical substrate, activator and inhibitor requirements. Heterozygotes produce both sets of esterases similar in pattern to that of mixed extracts of the two homozygotes. Each set of esterases behaves as a unit during breeding and subclonal analysis (Allen, S. L., 1961); hence, these multiple forms—or isozymes—probably arise by modification of a single protein produced by each allele.(Figure 1).

Although each set of isozymes is transmitted as a unit, variations in activity between isozymes within a set is observed under various growth conditions (Allen, S. L. 1960, 1961). This suggests that these modifications are sensitive to changes in cell metabolism.

Electrophoretic separation of the E-1 isozymes is pH sensitive and the isozymes do not act in parallel with increasing or decreasing pH. For example, at

pH 7.5 (both starch and end tray buffers) three major isozymes are resolved, at pH 8.0 (starch only; end tray buffer, pH 7.5) four to five major isozymes are resolved, while at pH 8.5 and pH 9.5 (both starch and end tray buffers) two isozymes are separated. The effect of pH on separation is shown diagramatically for the E-1B isozymes:



The isoelectric point of E-1B appears to be around pH 8.5; that of E-1C is known only to be lower than pH 6.5. The pH conditions for resolving a particular number of isozymes are relatively similar, although not identical, for E-1B and E-1C. In the analysis that follows most of our work was done on the E-1B isozymes at their maximum resolution (pH 8.0 starch, pH 7.5 end tray buffer).

In order to gain insight into the nature of the isozymes two approaches are being explored. First, experiments are being directed towards understanding the structure of the E-1 proteins. Second, the distribution of isozymes in cell fractions is being investigated.

1. Structure of the Esterase Isozymes

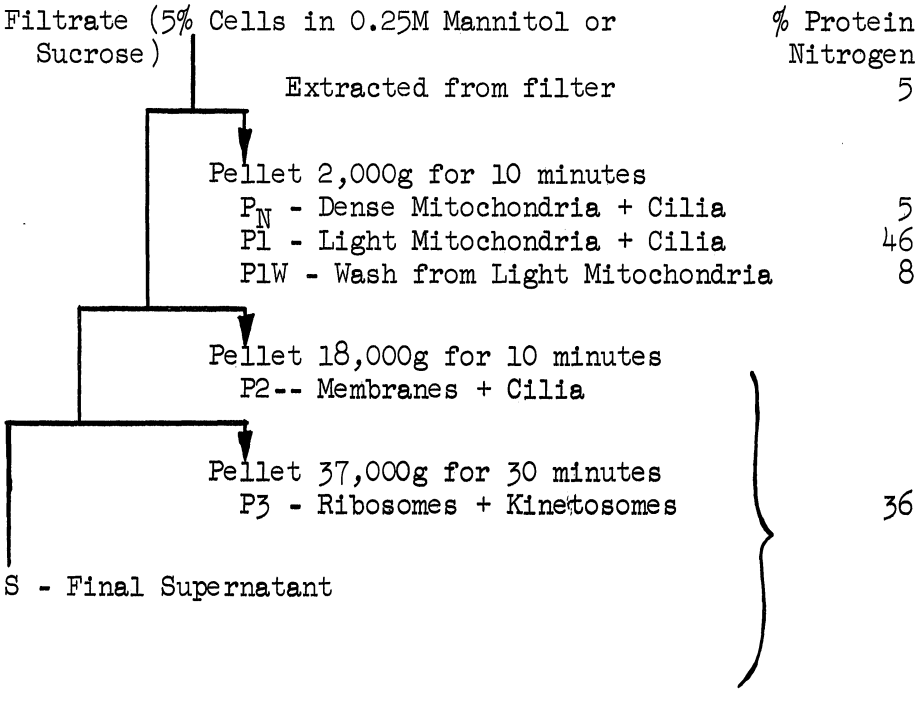
Preliminary experiments have been performed on crude extracts in an attempt to characterize the E-1 proteins. E-1B is thermostable up to 60°C for 30 minutes (a more detailed analysis of higher temperatures is in progress). If extracts are made in buffers of different pH (3.0-10.0) the E-1 esterases are inactivated by lowering the pH below 5.0. 8 M urea has no effect upon E-1B or E-1C even with as much exposure as 18 hours at 4°C. Adding thioglycollate or mercaptoethanol along with 8 M urea results in only slight inactivation of E-1B, provided these reagents are used in high concentration (2 M) or for long exposure (2 hours, 23°C).

The most interesting effect was observed with the addition of iodoacetamide and 8 M urea: additional isozymes, more basic in charge were produced (Figure 2). This effect was observed for both E-1B and E-1C extracts, and a "conversion" of the native isozymes into the new forms was noted as the concentration or time of exposure was increased. These results suggest that mere folding of the E-1 protein into various configurations may not be important in producing the isozymes. Rather, additions (or subtractions) of small groups—possibly by attachment to -SH groups at some distance from the active center of the enzyme—may bring about changes in net charge. These results suggest that manipulation of the isozymes will be feasible.

Another approach towards understanding the structure of the E-1 isozymes is by means of immunological techniques. Antisera to crude extracts of cells of different E-1 genotype were prepared and tested against extracts of various cell types in Ouchterlony double diffusion tests and by immunoelectrophoresis (with the guidance of G. W. Nace, Department of Zoology, University of Michigan). It is clear from our results that it will be necessary to purify—or at least enrich—our enzyme preparations before we can achieve any degree of specificity in our tests, since we see many precipitin lines and diffuse "staining" for esterases. Antisera to a specific cell fraction enriched in one of the isozymes are currently being made; these have not, as yet, been tested.

2. Intracellular Localization of the Esterase Isozymes

A variety of techniques have been used to attempt to localize the various esterase isozymes to specific cell particulates. Perhaps the best method used is a schedule of differential centrifugation modified after one developed by J. F. Hogg and H. L. Kornberg (J.B.C., in press). With this schedule the mitochondria are removed cleanly. The supernatant is then cleared in two subsequent steps:



One of the four major isozymes is enriched in small particles, the other three sediment in the lowest speed fractions (Figure 3).

Several runs were made with the joint efforts of J. F. Hogg (Department of Biochemistry, University of Michigan) who was able to demonstrate that almost all activity of the mitochondrial enzymes occurred in the 2,000 x g particles. Samples of the various pellets were prepared by A. M. Elliott (Department of Zoology, University of Michigan) for electron microscopy. The 2,000 x g pellets did, indeed, contain mitochondria (as well as cilia and membranes of various size). The 37,000 x g pellet contained "ribosomes" almost exclusively, while a pellet obtained at 18,000 x g for 10 minutes was made up largely of membranes (and cilia). Some of these membranes are rounded up to form vesicles usually smaller in diameter than the mitochondria.

Such cell fractions have been prepared from inbred strains differing in E-1 genotype. In general, the isozymic distributions are similar for $E\text{-}1^B$ and $E\text{-}1^C$ homozygotes, although the fractions varied in their sharpness of separation. Unfortunately, the different inbred strains behave somewhat differently in the separations achieved, the C strain (homozygous for $E\text{-}1^C$) giving the poorest separations.

Early attempts (Allen, S. L. 1961a) to achieve clean separations were met with much frustration. One of the chief disturbances was the appearance of cilia in esterase rich fractions. Only one method for preparing cilia in Tetrahymena (cold glycerol) appears to work well (Child, F. M. 1959 Exp. Cell Research 18:258 and personal communication). When clean preparations of cilia are achieved, no esterase activity is found in them.

Interpretation of these data is not easy. The most attractive hypothesis at the moment is the following: a small particle containing a single molecular species of E-1 esterase exists. Either by attachment to larger membrane surfaces or perhaps by coalescence of several small particles to form larger ones, macromolecules of esterases of larger size are formed. The differences in charge that distinguish the isozymes may emerge during the attachment or coalescence of membranes by addition or loss of side groups. This process cannot be random since only a limited array of isozymes are formed. The small particle isozyme possesses a charge intermediate in the series. It also appears almost "pure" during logarithmic growth. Thus, the differences in isozymic pattern observed in cells at different stages in the growth cycle (Allen, S. L. 1960) could reflect shifts in metabolism between enzyme synthesis and "morphogenesis."

C. VARIATIONS IN THE ELECTROPHORETICALLY SEPARATED ACID PHOSPHATASES OF TETRAHYMENA

Several zones with acid phosphatase activity can be separated electrophoretically in starch gel of pH 7.5 from extracts of different genotypes of Tetrahymena (Figure 4). Starch slices are incubated in substrate at pH 5.0

for 60 minutes in test tubes, unless otherwise specified. The activities of these acid phosphatases are optimal at pH 5-6, although the different phosphatases vary in their range of pH.

Seven substrates have so far been employed: four using the coupling technique with 4-amino-3: 1'-dimethyl azobenzene (Fast Garnet GBC) as dye coupler, and three using the Gomori-lead method. Variations occur in the splitting of sodium alpha naphthyl acid phosphate, naphthol AS phosphate, naphthol AS-Bl phosphate, and naphthol AS-MX phosphate, although for the latter three substrates, 8 hours of incubation are required (Figure 5). Some of the faint bands are relatively more intense with these three substrates, while the bands identified by the arrows in Figure 4 are much less intense. The most anodally migrating band shows the greatest variability and is much less intense with naphthol AS phosphate and naphthol AS-MX phosphate. An additional faint band, even more negative in charge, appears with naphthol AS-MX phosphate.

With one difference, comparable results are obtained using sodium alpha naphthyl acid phosphate using the coupling technique and sodium beta glycerophosphate using the Gomori-lead method, i.e. the most anodally migrating band does not appear with sodium beta glycerophosphate (Figure 5). D. B. McCormick and M. Russell (1962 Comp. Biochem and Physiol. 5:113) reported that Tetrahymena (unknown species) has two peaks of acid phosphatase activity, one at pH 5.0 and another at pH 3-4. The latter activity is particularly high when riboflavin-5-phosphate is used as substrate and is much less when sodium beta glycerophosphate or glucose-6 phosphate are used as substrates. This activity is inhibited by a number of cations. When we used glucose-6 phosphate as substrate, the pattern of activities in starch gels was similar to that found for sodium beta glycerophosphate. Riboflavin-5-phosphate was not split at all. This, however, may be due to inhibition by Pb^{++}, necessarily included in the reaction mixture as a trap for PO_4. So far, we have not designed a way around this technical problem.

A number of "adjuvants" were screened, usually at 0.016M. Complete inhibition occurred in the presence of NaF, partial inhibition with D-tartaric acid. No effect of Mn^{++} or sodium taurocholate was observed. A more extensive series of adjuvants, at various concentrations, needs to be screened.

Cultures of different age were examined (1,3,5,7 days of growth at 30°C). With one or two possible exceptions, the relative activities of the electrophoretically separated acid phosphatases were similar. All activities, however, increased with the age of the culture.

Centrifugation studies revealed that the acid phosphatases are confined to particulate materials: little activity is found in the final supernatant (provided gentle handling is employed). Most of the acid phosphatases appear to be associated with particles spun down at 2,000 x g for 10 minutes.

In addition to minor variations between inbred strains and one variation (P-2X) observed to differ between different pairs of the inbred C strain, a

major variation divides the strains into two groups. Alternative forms of this variation, indicated by arrows in Figure 4, are under control of alleles at the P-1 locus.

The observations on the electrophoretically separated acid phosphatases, taken as a composite, suggest that these variations represent a family of different enzymes with overlapping specificities. In the future it is hoped that by use of a battery of natural substrates better definition of specific enzymatic activities can be achieved.

D. GENETIC CONTROL OF AN ACID PHOSPHATASE IN TETRAHYMENA: FORMATION OF A HYBRID ENZYME

1. Inheritance

When the proteins of cells derived from the various inbred strains are separated electrophoretically in starch gels, one striking difference in acid phosphatase pattern divides the strains into two groups. Extracts of strains A, A1, B1, D and D1 possess an intense band that migrates about 4.5 cm toward the anode after 5 hours at 8 v/cm (Figure 4A). Extracts of the other group of strains (A2, A3, B, C, C1, C2, E and F) do not possess this band; instead, they have three bands that migrate to about 2.5, 3.0, and 3.5 cm toward the anode, in decreasing order of intensity (Figure 4B).

Crosses were made in the bacterized medium at 23°C. Pairs were separately isolated into depression slides, exconjugants being separated in some crosses. When the medium was exhausted (c. 13 fissions), half of the culture was tubed up in sufficient medium to permit approximately 2 more fissions. The culture left over in the depression slide was tested for maturity. Only immature cultures (those in which conjugation had occurred) were used. Massive transfers (c. 5000 cells) were made of the tubed culture through antibiotics and into fresh proteose-peptone in tubes and placed at 30°C. After growth, these peptone cultures were dumped into 250 ml Erlenmeyer flasks with 100 ml of proteose-peptone, and after 5 days at 30°C, whole cell extracts were prepared.

The tubed cultures left over in bacterized medium served as the source of caryonides. A sample from each culture was placed in a depression slide and three or more single cells were isolated into separate depressions. These randomly selected caryonides were then carried to maturity by serial transfers of single cells at 13 fission intervals. After approximately 95 fissions these cultures were tubed up in bacterized medium and mating type tests were performed. After approximately 120 fissions samples of some of the caryonides were transferred to proteose-peptone and whole cell extracts prepared.

When crosses are made within a strain (A x A, A1 x A1, D x D; B x B or C x C) the pattern characteristic of the strain is observed in the progeny. For a

genetic analysis strain A possessing the P-1A band was selected as representative of the first group, and strain B or strain C, possessing the P-1B bands, were selected as representative of the second group.

Mixed extracts of cells from strains A and B or strains A and C possess the P-1A band (at 4.5 cm) and the 2.5 cm P-1B band (Figure 6, mixture). An extract of a heterozygote (screened at about 15 fissions after conjugation) not only has these bands but also an intense band lying midway between at 3.5 cm. At 15 fissions heterozygotes, regardless of genetic background (F1, F2, Backcrosses ABA or ABB) have similar patterns (Figures 4 and 6, A/B): bands of equal intensity at 3.5 and 4.5 cm and a weaker band at 2.5 cm. The new pattern found in the heterozygote will be referred to as the hybrid or P-1AB pattern.

The results of crosses between strains A and B or between strains A and C and their hybrid generations are shown in Table 2. Exconjugants from several F1 and F2 pairs were separated, and in all cases examined exconjugants from the same pair were alike in phenotype. Each entry in Table 2 represents an observation on a pair culture. In the F1 all pair cultures were P-1AB in phenotype. In the F2 three types of pair cultures were observed: P-1A, P-1AB and P-1B. In each backcross only two types of pair cultures were observed: P-1AB and P-1A, or P-1AB and P-1B. The distribution of pair cultures observed in the F2 generation fits a 1:2:1 ratio closer ($p = .95$) than a 1:3:1 ratio ($p = .05$-$.1$), although the latter is obviously not excluded. The distribution of pair cultures observed in the backcross generations is compatible with a 1:1 ratio but not with a 1P-1AB: 3P-1A or a 1P-1AB: 3P-1B ratio.

TABLE 2

INHERITANCE OF THE P-1 ACID PHOSPHATASES

Crosses	Distribution of Pairs P-1A	P-1AB	P-1B	Total	Exp. Ratio 1 Locus	X^2 p=	Exp. Ratio 2 Loci	X^2 p=
A x A	20	0	0	20	1:0:0	>.99	1:0:0	>.99
B x B	0	0	20	20				
C x C	0	0	18	18				
	0	0	38	38	0:0:1	>.99	0:0:1	>.99
A x B	0	18	0	18				
A x C	0	10	0	10				
	0	28	0	28	0:1:0	>.99	0:1:0	>.99
AB x AB	11	19	10	40				
AC x AC	13	25	12	50				
	24	44	22	90	1:2:1	.95	1:3:1	.05-.1
AB x A	10	10	0	20	1:1:0	>.99	1:1:0	>.99
AB x B	0	12	8	20	0:1:1	.5	0:1:1	.5

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AB x A	10	10	0	20	1:1:0	>.99	1:1:0	>.99
AB x B	0	12	8	20	0:1:1	.5	0:1:1	.5

Two genetic explanations for these data can be entertained. The P-1 phosphatases could be under the control of alleles at a single locus or they could be under the control of genes at two loci. If a single locus is involved, the P-1A phenotype would arise in cells homozygous for a $\underline{P\text{-}1}^{A}$ allele, the P-1B phenotype in cells homozygous for a $\underline{P\text{-}1}^{B}$ allele, and the P-1AB phenotype in heterozygotes. Thus, a 1:2:1 ratio would be expected in the F2 and 1:1 ratios in the backcrosses. The data fit this assumption well.

On the other hand, if two unlinked loci are involved, the P-1A genotype might arise in cells of genotype A-bb and the P-1B phenotype in cells of genotype aaB-. Without epistasis (A over B or B over A) the P-1AB phenotype would arise in cells of genotype A-B-. The doubly recessive homozygote (aabb) might be lethal; and, if so, 6.3% of the F2 pairs should die from this cause alone. The observed lethality in the F2 was even greater—16.8% in the F2(A x B) and 52.4% in the F2 (A x C). For the remaining three classes a 1:3:1 ratio would be expected in the F2: 3 A-bb, 9 A-B-, and 3 aaB-. In the backcross generations 1:1 ratios would be expected: 2 A-B- and 2 A-bb in the backcross to the A strain, and 2 A-B- and 2 aaB- in the backcross to the B strain. The F2 data are less compatible with the expected ratios for two loci, but they do not exclude this assumption.

In order to distinguish a 1:2:1 ratio from a 1:3:1 ratio a minimum of 150 F2 pairs should have been tested. Actually, a more critical test of these two assumptions would come out of genotypic testing of the backcross heterozygotes. If two loci are involved, two genotypes would be representative among these heterozygotes. For example, ABA heterozygotes would be either AABb or AaBb in genotype. These could be distinguished in a cross to the B strain (aaBB) where AABb would give rise to only P-1AB pairs and AaBb would give rise to equal numbers of P-1AB and P-1B pairs. If a single locus is involved, all ABA heterozygotes would be $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ in genotype and, when crossed to the B strain ($\underline{P\text{-}1}^{B}/\underline{P\text{-}1}^{B}$) all heterozygotes would give rise to equal numbers of P-1AB and P-1B pairs.

The critical crosses have not been made for the reason that data obtained from another type of cross (and made for a different purpose) clearly suggest that one locus is involved (see section h, Table 18). In this cross a 3 P-1A : 2 P-1AB : 3 P-1B distribution of F2 pairs is expected if the phosphatases are controlled by alleles at a single locus. If two unlinked loci were involved, a 7 P-1A : 29 P-1AB : 7 P-1B distribution would be expected. The observed distribution was 20 P-1A : 13 P-1AB : 17 P-1B, clearly more compatible with the frequencies expected for the single locus hypothesis.

Two P-1A and four P-1B F2 pairs have been selfed. All bred true for their particular type of acid phosphatase. In the future, additional genotypic tests will be made.

The data from all sources suggest that a single locus is involved in the control of the P-1 phosphatases, although two closely linked loci are not ruled out. For the present, we will assign the genotypes $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{A}$ and $\underline{P\text{-}1}^{B}/\underline{P\text{-}1}^{B}$ to the two homozygotes, responsible for the P-1A and P-1B phenotypes, respectively,

and $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ to the heterozygote with the P-1AB hybrid phenotype.

2. Phenotypic Drift in the Heterozygote

At 15 fissions the phenotype of the heterozygote shows very little variability, regardless of genetic background. All extracts possess the P-1A band at 4.5 cm, equal in intensity to a band at 3.5 cm, and the P-1B band at 2.5 cm, usually less intense than the other two bands. Strong extracts also possess bands that are very weak in intensity and intermediate in mobility—at 3.0 and 4.0 cm. These intermediate bands are relatively much stronger in extracts of some caryonides. This gives a total of five electrophoretic positions in starch for the P-1 acid phosphatases of heterozygotes. These positions relative to the positions of the parental enzymes are, as follows:

Band No.	$\underline{P\text{-}1}^{B}/\underline{P\text{-}1}^{B}$	$\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$	$\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{A}$	Distance in cm from Origin Toward Anode
1	■	▒		2.5
2	▒	------		3.0
3	-----	■		3.5
4		-----		4.0
5		■	■	4.5

Caryonides from a heterozygote possess a spectrum of phenotypes—with ordered variations in intensity of all five bands being observed (Figure 6, $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ at 120 or more fissions). Some caryonides are parental in phenotype (P-1A or P-1B); others are P-1AB and others are intermediate, P-1AB or P-1AB. The distribution of $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ caryonides obtained from three types of crosses (F1 or AB, and the two backcrosses, ABA and ABB) is shown in Table 3. The distributions of the two backcrosses are similar, suggesting that other genetic factors do not exert a significant effect in determining these phenotypes.

The control of these phenotypes appears to be exerted at the level of the caryonide. From a single P-1AB caryonide, subclones can be initiated and, if these are separately maintained for 120 fissions, these give rise to P-1AB cell lines, to P-1A and to P-1B cell lines as well as to a host of intermediate cell lines. If single cells are isolated from selected representatives of these lines and the subclones are immediately assayed, the subclones show a much narrower distribution of phenotypes. Thus, a P-1A cell line gives rise to only

TABLE 3

DISTRIBUTION OF $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ CARYONIDES AT 120 FISSIONS

Cross	No. of Caryonides with P-1 Phenotypes					Total	% Parental Phenotypes
	A	$\underline{AB}$	AB	$A\underline{B}$	B		
AB	1	5^{1*}	10^{2*}	3	2	21	14.3
ABA	4	4^{1*}	8^{1*}	1	3	20	35.0
ABB	3	1	15^{1*}	2	7	28	35.7
	8	10	33	6	12	69	

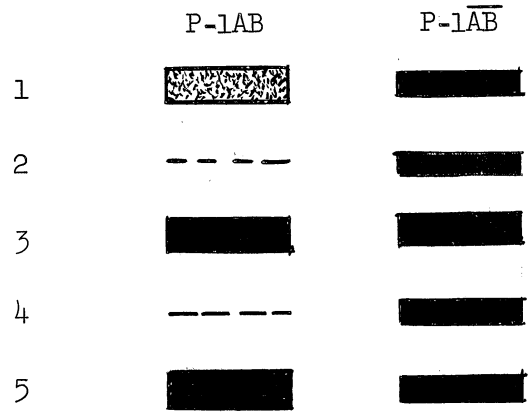
*$\overline{AB}$ in phenotype

P-1A subclones and a P-1B cell line to only P-1B subclones. Cell lines that are P-1AB in phenotype give rise to P-1AB, P-1$\underline{AB}$ or to P-1$A\underline{B}$ subclones. P-1$\underline{AB}$ cell lines give rise to P-1AB, P-1$\underline{AB}$ and to P-1A subclones and P-1$A\underline{B}$ cell lines give rise to P-1AB, P-1$\underline{AB}$ and to P-1B subclones.

These observations suggest that the $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ heterozygote undergoes phenotypic drift, the extent of drift being a function of the number of fissions that have transpired since conjugation. Phenotypic drift in heterozygotes in variety 1 of T. pyriformis is not a new observation. It has been observed for three other loci—H (Nanney, D. L. and J. M. Dubert, 1960, Genetics 45: 1335), E-1 and E-2 (Allen, S. L. 1961). It arises from changes taking place in the composition of the somatic nucleus. This macronucleus is hypothesized to contain 45 diploid subnuclei (Allen, S. L. and D. L. Nanney, 1958, Am. Nat. 92: 139). In heterozygotes interallelic interactions occurring in separate subnuclei give rise to a heterogeneous macronucleus. With random assortment of these differentiated subnuclei to daughter macronuclei during division, cells with variable phenotype appear. Depending upon the time of interaction and the proportion of subnuclei expressing a given allele, a certain number of cell lines appear that are "pure" for one or the other parental phenotype. These changes that take place in the macronucleus are removed once conjugation takes place and the old macronucleus disappears.

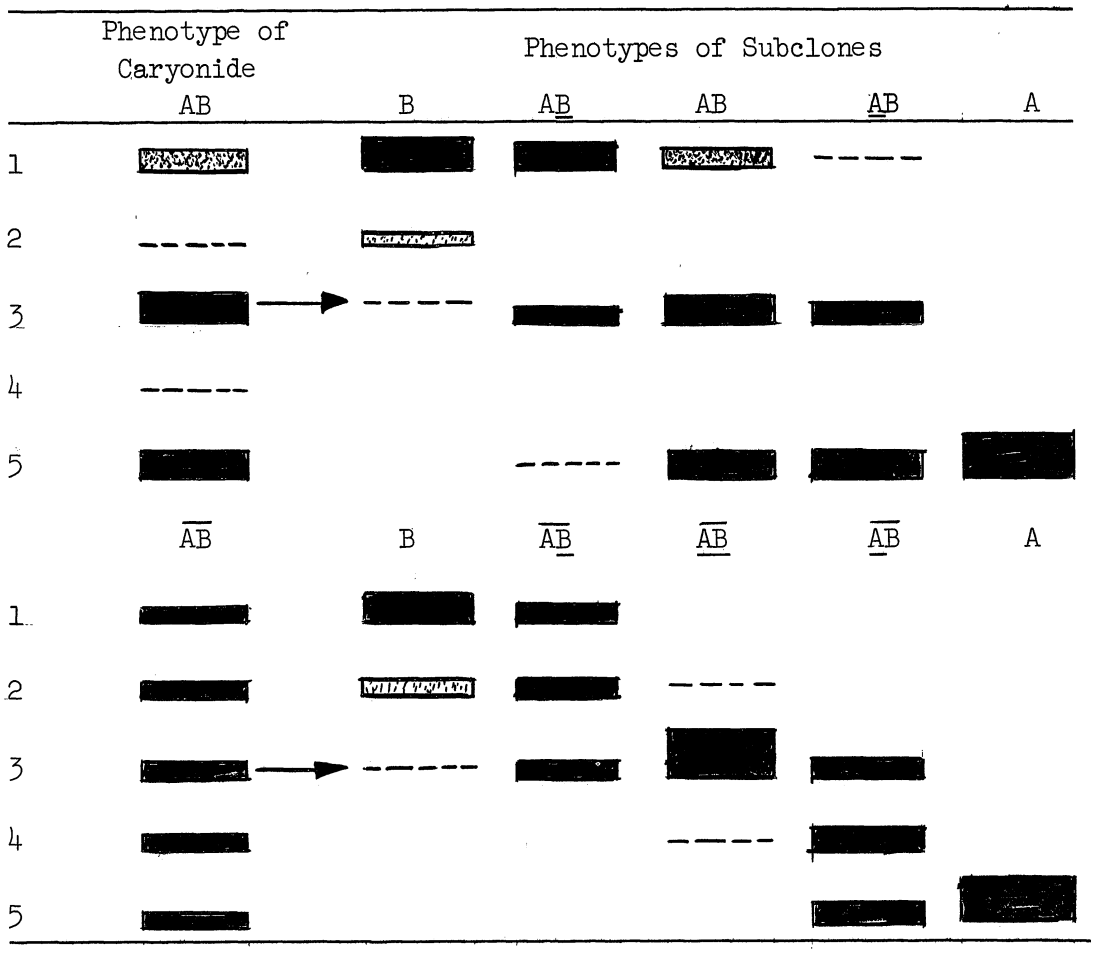
Some features of phenotypic drift are peculiar to the phosphatases. These will be taken up in a later section devoted to an understanding of phenotypic drift. Here, the phenomenon of phenotypic drift will be used as a means of understanding the conformations of the "hybrid" enzymes. By this I mean that the phenotypic sequences observed in clonal analysis may provide clues as to possible structural arrangements and modes of synthesis. An extensive clonal analysis will be necessary before a detailed map of the sequence of patterns can be made. However, the results of a preliminary analysis are sufficiently provocative to warrant special attention.

In addition to variations among the caryonides suggestive of a different "dosage" of the P-1^A or P-1^B alleles, differences were noted in the extent to which the intermediate bands, 2 and 4, were present. Some of the P-1AB caryonides seemed to possess five bands of equal intensity, with band 3 being relatively more intense. These will be referred to as P-1$\overline{AB}$ in phenotype. A comparison of the P-1AB and P-1$\overline{AB}$ phenotypes is illustrated below:



For a clonal analysis three P-1AB caryonides and three P-1$\overline{AB}$ caryonides were selected. Thirty subclones were initiated from each of these caryonides when they had completed approximately 175 fissions (ABA or ABB caryonides) or 200 fissions (AB caryonides). The subclones were separately maintained for 120 fissions by serial transfers of single cells at 13 fission intervals (9 transfers) Then, 20 of the subclones were tubed up and samples transferred to proteose-peptone and whole cell extracts made.

The P-1A and P-1B subclones obtained from the AB and $\overline{AB}$ types of caryonides did not appear to differ in phenotype. But, to our surprise, we observed that the intermediate and hybrid subclones from the two types of caryonides were, on the whole, distinctly different in phenotype. A diagram of the phenotypes observed will illustrate this point. Moreover, from the $\overline{AB}$ caryonides a new type of cell lineage appeared: the $\overline{AB}$ type. In extracts of such a cell line band 3 is present almost exclusively, with trace amounts of bands 2 and 4. This cell type appears to be quite stable, since subclones initiated from a P-1$\overline{AB}$ line are all P-1$\overline{AB}$. Whether it is completely stable, as are the P-1A and P-1B cell lines, has not been determined.



A complete breakdown of the subclonal analysis of the six caryonides is shown in Table 4.

The difference in the types of intermediate subclones obtained from the two types of caryonides is not absolute; thus, one P-1$\underline{\overline{AB}}$ and one P-1$\overline{\underline{AB}}$ subclone were derived from AB caryonides. Other differences were observed between the two groups of subclones. In general, the frequency of parental types was higher in subclones obtained from AB caryonides (61.8%) than from $\overline{AB}$ caryonides (22.1%). Moreover, the two parental types were produced in equal frequency from AB caryonides, while many more P-1B than P-1A subclones were produced from $\overline{AB}$ caryonides.

These data suggest that the direction of phenotypic drift may be tied up in some manner with the ability to make the intermediate acid phosphatases (bands 2 and 4). Of importance may be the fact that the P-1B phenotype is associated with isozymes while the P-1A phenotype is not; i.e., only one electrophoretically distinct phosphatase is formed. Yet, paradoxically, the P-1B phenotype also seems to be associated with the ability to make the phosphatase that appears almost exclusively as band 3. This phosphatase (band 3) is present in trace amounts in P-1B extracts but is exceedingly active in P-1AB extracts.

TABLE 4

DISTRIBUTION OF SUBCLONES AFTER 120 FISSIONS INITIATED FROM SELECTED $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ CARYONIDES AT 175—200 FISSIONS

AB Caryonides	No. of Subclones with P-1 Phenotypes					Total	% Parental Phenotypes
	A	$\underline{AB}$	AB	$\underline{AB}$	B		
ABB-5	3	3^{1*}	8	1	5	20	40.0
ABA-3	8	0	3	1*	8	20	80.0
ABB-4	6	2	4	1	7	20	65.0
	17	5	15	3	20	60	61.8
$\overline{AB}$ Caryonides	$\underline{A}$	$\underline{\overline{AB}}$	$\overline{AB}$	$\overline{\underline{AB}}$	B	Total	% Parental Phenotypes
ABA-6c	1	1	3	6	9	20	50.0
AB-5	0	0	17	0	2	19	10.5
AB-7a	0	0	19	0	1	20	5.0
	1	1	39	6	12	59	22.1
TOTALS	18	6	54	9	32	119	42.0

Whether these phosphatases with similar mobilities are qualitatively similar is not known.

These data suggest that two types of processes may be involved in the formation of the five P-1 phosphatases in heterozygotes. A hybrid molecule may arise by recombination of subunits derived from the action of $\underline{P\text{-}1}^{A}$ and $\underline{P\text{-}1}^{B}$. Further recombinations may give rise to the intermediate molecules. These latter recombinations may be associated with a genetic predisposition to make isozymes. This ability, usually associated with the $\underline{P\text{-}1}^{B}$ allele, may perhaps become dissociated from its influence and also work in cells in which the $\underline{P\text{-}1}^{A}$ allele is predominant. In arriving at a working hypothesis for the structural organization and synthetic pathways of these phosphatases, some relationships are suggested by the sequences in pattern observed during phenotypic drift. The initial phenotype of $\underline{P\text{-}1}^{A}/\underline{P\text{-}1}^{B}$ heterozygotes is P-1AB. Subsequently, phenotypic drift may follow along two pathways: either cells remain P-1AB or drift to P-1$\overline{AB}$. From either type of cell line parental phenotypes may emerge with further drift, although the P-1B phenotype may be associated more closely with the P-1$\overline{AB}$ lineage.

Several different molecular models could be considered, but only one will be discussed. The fact that each of the five bands differs in a regular fashion in electrophoretic mobility (and they migrate in parallel dependent upon pH) suggests an ordered difference in charge. This situation is reminiscent of the

LDH's (Appella, E. and C. L. Markert, 1961, Biochem. and Biophys. Res. Comm. 6: 171; Cahn, R. D. et al., 1962, Science 136: 962). Here, it has been postulated, and confirmed by physical and chemical analysis, that the five electrophoretically distinct forms represent tetramers that arise by recombination of two subunits. If such a model is applied to the phosphatases and the P-1^B allele makes a "B" protein and the P-1^A allele an "A" protein, the following sets of subunits might appear in the three genotypes:

P-1^B/P-1^B	P-1^A/P-1^B	P-1^A/P-1^A
BBBB	BBBB	
BB		
B	ABAB	
	AAAA	AAAA

If recombination of subunits took place in two steps (a) formation of a dimer (b) formation of a tetramer, the intermediate phosphatases of heterozygotes might arise if the dimers associated randomly during step (b). Initially, we would suggest that non-randomness is the rule. The various cell types would arise depending upon the amount of A or B synthesized before step (a) and the degree of randomness occurring in step (b). Schematically, the following temporal relationships might exist:

P-1^A and P-1^B active:

(1)		(2)		(3)
BBBB		BBBB		
		BBAB		BBAB
ABAB	⟶	ABAB	⟶	ABAB
		AAAB		AAAB
AAAA		AAAA		

	P-1^B active	P-1^B > P-1^A	P-1^A > P-1^B	P-1^A active
(1)	(4)	(5)	(6)	(7)
BBBB	BBBB	BBBB	BBBB	
	BB			
ABAB ⟶	B	ABAB	ABAB	
AAAA		AAAA	AAAA	AAAA
(2)	(4)	(5a)	(6a)	(7)
BBBB	BBBB	BBBB		
BBAB	BB	BBAB		
ABAB ⟶	B	ABAB	ABAB	
AAAB			AAAB	
AAAA			AAAA	AAAA

ABAB, cell type (3), poses the greatest difficulty in interpretation. In order to make it fit into the above scheme, it is necessary to invoke non-randomness in the formation of the dimer in step (a). This is particularly paradoxical since this cell type seems to be derived from type (2) where randomness has been invoked for step (b). An alternative, though less likely hypothesis is that this phenotype represents a modified P-1B. This seems less likely because trace activities of bands 2 and 4 are usually seen in such extracts, and the presence of band 4 would imply that the P-1^A allele is also participating in the synthesis of the phosphatases. Additional subclonal analyses of the P-1$\overline{AB}$ and P-1$\underline{AB}$ lineages at more closely spaced fission intervals will help to solve this question.

In conclusion, we believe that the P-1 acid phosphatases are under the control of alleles at a single locus. Initially, heterozygotes possess a distinctive phenotype, not found in mixed extracts of the homozygote. With subsequent fissions, heterozygotes undergo phenotypic drift and several different cell types are formed that vary in activity at five electrophoretic positions. The temporal sequence in pattern observed during phenotypic drift may permit us to speculate about the structure and synthesis of these phosphatases in hybrids. A molecular model is proposed, based on the LDH model, in which the five electrophoretically distinct types are viewed as five tetramers arising through recombinations of two subunits. Variations in the pattern of phosphatase activity at these five electrophoretic positions may reflect variations at three steps during the synthesis of these acid phosphatases: first, in the amount of the two subunits synthesized; secondly, in the degree of randomness in the formation of the dimer; and, thirdly, in the degree of randomness in the formation of the tetramer.

E. LYSOSOMES IN TETRAHYMENA

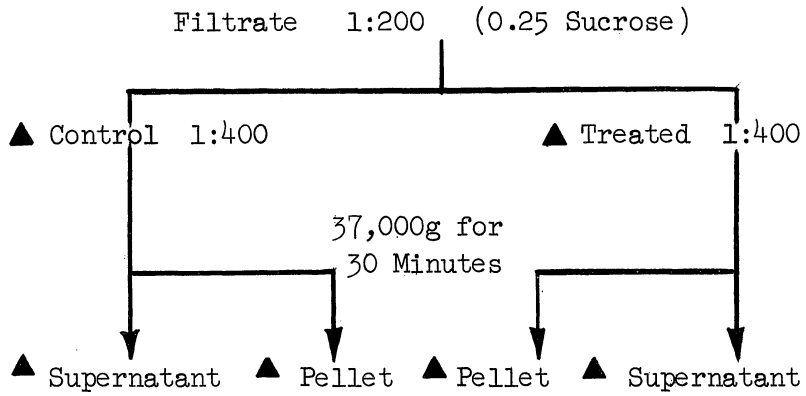
Acid phosphatase activity has been associated with lysosomes (deDuve, C., 1959, In Subcellular Particles, ed. T. Hayashi, p. 128; Novikoff, A., 1961, In The Cell II., ed. J. Brachet and A. E. Mirsky, p. 423). In Tetrahymena localizations under the light microscope are confined to small vesicles in short incubations with substrate; these usually merge to form larger vesicles with longer periods of incubation (Allen, S. L., 1958). G. R. Seaman (1961, J. Biophys. and Biochem. Cytol. 9: 243) and M. Müller and I. Toro (1962, J. Protozool. 9: 98) have noted the association of acid phosphatase localizations and vacuole formation. Electron microphotographs of sections of T. pyriformis stained for acid phosphatase show that the activity is confined within bodies remembling lysosomes (Elliott, A. M. and I. Bak, personal communication).

We have been interested in seeing if the customary treatments used to demonstrate lysosomal activity will work on the acid phosphatases of variety 1 of T. pyriformis. And, if so, whether the electrophoretically separated acid phosphatases show differences in behavior after the various treatments.

In good separations achieved by differential centrifugation, most of the

phosphatase activities are confined to particulate materials. All activity that is sedimentable comes down at 37,000 x g for 30 minutes in the Servall Refrigerated Centrifuge. Thus, a supernatant obtained at 105,000 x g for 60 minutes in the Spinco Ultracentrifuge shows no decrease in activity over one obtained at the lower speed. The activity found in the final supernatant does vary depending upon the age, the type, and the handling of the culture. A localization of some of the electrophoretically separated acid phosphatases to different sized particles was suggested in certain preparations (Allen, S. L. and M. S. Misch, 1962). With a couple of possible exceptions variations between the electrophoretically separated acid phosphatases were not observed in cultures of different age.

For quantitative determination of total acid phosphatase activity in various cell fractions the following protocol was adopted. Cells were concentrated from peptone grown cultures, washed once with cold (4°C) glass-distilled water, then with three changes of cold 0.25 M sucrose. The washed cells were suspended in a certain volume of cold 0.25 M sucrose and a brei prepared by passing the cells through a scintered glass filter ("M" pore size, Corning Glassware). All operations were carried out at 0-4°C unless otherwise specified. The following schedule was adopted for comparing control and treated cell fractions:



Total acid phosphatase activity was aasayed on duplicate samples of the various fractions (indicated by ▲) by a modification of the method of C. H. Fiske and Y. J. Subbarow (1925, J.B.C. 66: 375) which measures the amount of phosphorus released by acid phosphatase activity. In Table 5 acid phosphatase activity is represented as µM P released per L of culture (1.0-1.5 ml packed cells) in 10 minutes. Currently, we are working up a method for determining protein N that will permit us to convert these activities to specific activities. In Table 5 the activities of all fractions are represented as percentages of the activity of the control filtrate. In the last column the percentage change with respect to control filtrate, pellet, and supernatant is tabulated for each treated fraction.

TABLE 5

EFFECT OF VARIOUS TREATMENTS ON TOTAL ACID PHOSPHATASE ACTIVITY

	μ M P/L/10 Min	% Control Filtrate	% Change c.f. Control
Control Filtrate	778	100	-----
Pellet	605	78	-----
Supernatant	103	13	-----
Violently Resuspended			
Pellet	514	66	- 15
Supernatant	214	31	+ 139
0°C, 24 Hrs, Filtrate	780	100	0
Pellet	562	72	- 8
Supernatant	210	27	+ 108
Frozen-Thawed Filtrate	918	118	+ 18
Pellet	762	98	+ 26
Supernatant	220	28	+ 115
37°C* Filtrate	810	104	+ 4
Pellet	497	64	- 18
Supernatant	297	38	+ 192
Triton* Filtrate	1130	145	+ 45
Pellet	146	19	- 76
Supernatant	775	100	+ 670
PVP* Filtrate	762	98	- 2
Pellet	654	84	+ 8
Supernatant	97	13	0

*30 minute treatments

Several treatments resulted in a doubling of soluble acid phosphatase: mechanical treatment (resuspsnsion of pellet and supernatant), storage (0°C for 24 hours), and freeze-thawing. Treatment of a sample of filtrate at 37°C for 30 minutes resulted in a tripling of soluble acid phosphatase. Some of these treatments effected very slight increases in the activity of the filtrate. PVP (polyvinylpyrrolidinione) had no effect at all. Triton X-100 (method of R. Wattiaux and C. de Duve, 1956, Biochem. J. 63: 606) resulted in a noticeable increase in the activity of the filtrate (45%) which was detected as a 670% increase in soluble acid phosphatase. In other runs this increase has varied between 400 and 1200%.

These results suggest that the acid phosphatases in Tetrahymena are bound

to particles since solubilization can be brought about with various treatments. Since activation can also be effected by some treatments, particularly by Triton X-100, at least some of the acid phosphatases may be localized within lysosomes.

A preliminary examination of some of these treatments upon the electrophoretically separated acid phosphatases has been made. Freeze-thawing, Triton X-100 and PVP have been applied to filtrates containing a higher concentration of cells (1:10 initial concentration). After treatment of the filtrate, a pellet and supernatant were obtained as above, and a sample of each fraction inserted into a starch gel. Qualitative and quantitative comparison of the patterns of treated with control fractions were made after electrophoresis. Although the differences in pattern are not striking, four categories of acid phosphatases emerge. Three of these are mobile: some are already soluble, some are easily solubilized, and others are solubilized with greater difficulty. One category is only slightly mobile: those that are solubilized by Triton X-100. In the filtrate and supernatant after Triton treatment an intense band appears that extends from the origin to about 1 cm toward the anode.

Before we can draw final conclusions, this type of analysis obviously needs to be extended. The preliminary observations suggest that not all acid phosphatases behave in the same manner after various treatments. This may mean that they are localized to different regions of a cell structure. Perhaps some are freely soluble within lysosomes and others are more intimately bound to the lysosomal membrane.

The acid phosphatases are presumed to function in hydrolytic processes in association with "digestive" vacuoles. G. R. Seaman (1961, J. Protozool. 8: 204) has found that "water" vacuoles form in the peptone medium but not in synthetic medium. In the future, use of the synthetic medium may provide some interesting answers to the functional significance of the family of acid phosphatases. Are all phosphatases synthesized in a cell not forming vacuoles? Perhaps some are and some are not. By correlating information obtained by several techniques, we may achieve some understanding of the relationship of function and specific localizations within cell structures of the different acid phosphatases.

F. A FIRST CASE OF LINKAGE IN TETRAHYMENA PYRIFORMIS

The haploid chromosome number of variety 1 of Tetrahymena pyriformis is five; thus, this ciliate should be good material in which to look for linkage between various markers. To date, the following seven loci have been identified in this organism: mt (mating type), H (serotype), E-1 and E-2 (esterases) and P-1 (acid phosphatases) as well as two lethals, F and T. No linkage was found in tests between mt and H (Nanney, D. L., 1960, Genetics 45: 1351), E-1 and E-2 (Allen, S. L. 1961) and F and T (Orias, E., 1960, J. Protozool. 7: 64).

Other combinations have also been examined: H and P-1, H and E-1, H and E-2; P-1 and E-1 and P-1 and E-2 (see appended Tables 8 and 9). In none of these

combinations was linkage detected, although the data are by no means good enough to exclude loose linkage.

Only one case of linkage has been detected. This involves the E-1 and mt loci. The crosses giving rise to the heterozygotes used in these tests are shown below in Table 6. Two of the heterozygotes were F1 hybrids obtained in crosses of the B and C strains. They were $(\underline{E\text{-}1}^{B}\ \underline{mt}^{B})/(\underline{E\text{-}1}^{C}\ \underline{mt}^{C})$ in genotype. When backcrossed to strain B, segregation of the alleles at these two loci was normal since 1:1 ratios of heterozygotes to homozygotes were observed. This was not true for the backcrosses to the C strain. Abnormal segregation of the alleles occurred, signalled by the appearance of unexpected homozygotes ($\underline{E\text{-}1}^{B}/\underline{E\text{-}1}^{B}$; $\underline{mt}^{B}/\underline{mt}^{B}$). Abnormal segregation is a phenomenon often associated with members of the C strain, due to abnormal nuclear events (to be discussed in section H). For the present purpose, the backcross data showing abnormal segregation were discarded.

A third heterozygote was used in these tests, an F2 crossover between E-1 and mt:

$$\frac{\underline{E\text{-}1}^{B}\ \underline{mt}^{C}}{\underline{E\text{-}1}^{C}\ \underline{mt}^{B}} .$$

Backcrosses to both strains B and C showed normal segregation; hence, data from both crosses are included.

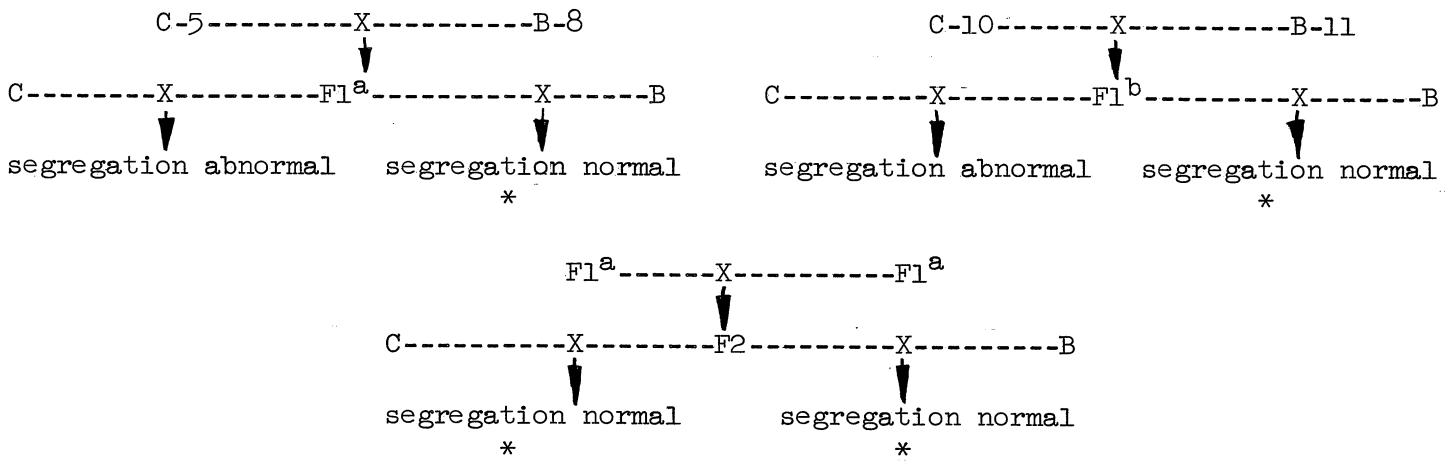
The E-1 phenotype of each backcross pair culture was screened at about 15 fissions. Genotypic tests of the esterases of 16 of the backcross pairs were made, and in each test phenotype and genotype corresponded; i.e., E-1BC = $\underline{E\text{-}1}^{B}/\underline{E\text{-}1}^{C}$; E-1B = $\underline{E\text{-}1}^{B}/\underline{E\text{-}1}^{B}$; E-1C = $\underline{E\text{-}1}^{C}/\underline{E\text{-}1}^{C}$. In these tests a minimum of 10 pairs was screened if the progeny resulted from selfing. Twenty pairs were screened if the progeny were obtained from a second backcross.

Three or more caryonides were selected at random from each pair, and these were carried to maturity by serial transfers of single cells. The mating type of each mature culture was then ascertained. Except for certain pairs, mating type genotype was assigned to a pair only after progeny tests. These exceptions were pairs producing caryonides that were mating type I in the B backcross or IV and VII in the C backcross. In the respective crosses only a heterozygote can produce these particular mating types. For all other pairs testcrosses were necessary, preferably between the caryonides derived from the same pair (selfing) and at least 30 progeny examined for mating type. Where only a single mating type appeared among the caryonides from a single pair, it was necessary to perform a second backcross, and a minimum of 60 progeny were screened for mating type.

A cell that is $\underline{mt}^{B}/\underline{mt}^{C}$ in genotype potentiates the expression of all seven mating types. One that is $\underline{mt}^{B}/\underline{mt}^{B}$ potentiates the expression of all but mating type I, and one that is $\underline{mt}^{C}/\underline{mt}^{C}$ potentiates the expression of all but IV and VII.

TABLE 6

PEDIGREES OF HETEROZYGOTES



The frequencies of the mating types are temperature sensitive (Nanney, D. L., 1960, Physiol. Zool. 33: 146). Hence, the crosses were performed at a temperature that would facilitate the appearance of a key type. Crosses of B backcross pairs were made at 25°C where I appears in highest frequency and crosses of C backcross pairs were made at 30°C where IV appears in optimal frequency.

A pair was assigned the genotype $\underline{mt}^B/\underline{mt}^C$ if I and IV appeared among the progeny, $\underline{mt}^B/\underline{mt}^B$ if no I and a high frequency of IV appeared, and $\underline{mt}^C/\underline{mt}^C$ if a high frequency of I and no IV appeared. From 41 pairs approximately 1800 progeny were raised and tested for mating type, permitting the assignment of mating type genotype to a total of 52 backcross pairs.

A tabulation of the linkage data for E-1 and mt is found in Table 7. These data suggest that E-1 and mt are linked, with crossing over being of the order of 25 percent.

TABLE 7

LINKAGE TESTS OF E-1 AND mt

Cross	$\frac{\text{E-1}^C\ \text{mt}^C}{\text{E-1}^B\ \text{mt}^B}$	$\frac{\text{E-1}^C\ \text{mt}^B}{\text{E-1}^B\ \text{mt}^B}$	$\frac{\text{E-1}^B\ \text{mt}^C}{\text{E-1}^B\ \text{mt}^B}$	$\frac{\text{E-1}^B\ \text{mt}^B}{\text{E-1}^B\ \text{mt}^B}$	Total
$F1^a$ x B	6*	1*	2*	3*	12
$F1^b$ x B	4	1	2*	7	14
	10	2	4	10	26

	$\underline{\text{E-1}}^B/\underline{\text{E-1}}^C$		$\underline{\text{E-1}}^X/\underline{\text{E-1}}^X$		
	$\underline{mt}^X/\underline{mt}^X$	$\underline{mt}^B/\underline{mt}^C$	$\underline{mt}^X/\underline{mt}^X$	$\underline{mt}^B/\underline{mt}^C$	
F2 x B	6	0	3*	3	12
F2 x C	5	2	2	5	14
	11	2	5	8	26
Total	21	4	9	18	52

*Classification of E-1 genotypes confirmed by progeny tests; $F1^{a,b}$ x C → segregation abnormal; Fit to 1:1:1:1 ratio, $p < .01$; Fit to assumption of 25% crossing over, $p = .7$.

In the future we hope we can establish a stock of variety 1 that is genotypically $(\underline{\text{E-1}}^C\ \underline{mt}^C)/(\underline{\text{E-1}}^C\ \underline{mt}^C)$ and that is free of segregation difficulties.

Once, this is accomplished, we will be able to analyze the linkage relations of E-1 and mt in greater detail. Only one other case of linkage is known in the ciliated protozoa (in Paramecium aurelia, discovered by I. Gibson and G. H. Beale, 1961, Genet. Res. 2: 82); thus, it is important that the details of this linkage should be worked out in a system free of extraneous ambiguities.

TABLE 8

BACKCROSS DATA FOR H AND P-1

Cross	P-1AB		P-1A (or B)		Total
	Had	Ha(or d)	Had	Ha(or d)	
ABA	6	4	5	5	20
ABB	4	8	5	3	20
	10	12	10	8	40

TABLE 9

F2 (A x C) DATA

Distribution of H Pairs:	Hae	Ha	He	Total
	51	21	29	101

Tests of Ha and He Pairs:

	Total	E-1			E-2			P-1		
		B	BC	C	B	BC	C	A	AB	B
Ha	21	5	11	5	7	11	3	6	10	5
He	29	8	17	4	8	12	9	7	15	7
	50	13	28	9	15	23	12	13	25	12
P-1A	13	3	10	0	4	8	1			
P-1AB	25	7	13	5	7	8	10			
P-1B	12	3	5	4	4	7	1			
	50	13	28	9	15	23	12			
E-1B	13				2	8	3			
E-1BC	28				9	12	7			
E-1C	9				4	3	2			
	50				15	23	12			

G. REGULATION OF PHENOTYPIC DRIFT IN TETRAHYMENA

Immediately following conjugation a heterozygote typically possesses a heterozygous phenotype for traits that are under control of co-dominant genes; that is, both parental phenotypes are observable in the same cell. With subsequent fissions the phenotype changes, and only one of the parental phenotypes can be scored in some of the cells. This phenomenon, phenotypic drift, has now been observed for cells heterozygous at four loci: H, E-1, E-2 and P-1. It is based upon certain changes that take place within the somatic nucleus, or macronucleus. Segregation of cells possessing macronuclei with a varying composition takes place as a function of the number of fissions. When the macronucleus disappears during conjugation and is replaced by products of the new zygotic nucleus, these changes in macronuclear composition also disappear and new phenotypes are established dependent upon the genotype of the new nuclei.

The kinetics of phenotypic drift in the H heterozygote were found by D. L. Nanney and J. M. Dubert (1960, Genetics 45: 1335) to be similar to those previously determined for the mt locus (Allen, S. L. and D. L. Nanney, 1958, Am. Nat. 92: 139). Here it was hypothesized that there were a number of "subnuclei" in the macronucleus that assorted randomly during cell division. The number of subnuclei following cell division is estimated as 45 and is derived from the equilibrium rate of production of "pure" cell types per fission.

A cell line with a parental phenotype that has been derived from a heterozygote by phenotypic drift is stable throughout the remainder of its somatic life. It even persists after a period of non-functioning of the gene controlling it (Nanney, D. L. and J. M. Dubert, 1960, Genetics 45: 1335). So far, no means has been found for effecting phenotypic reversal of a heterozygote in Tetrahymena. Thus, phenotypic drift could be explained if the subnuclei were haploid—and somatic segregation of the chromosomes were taking place. An alternative hypothesis is that the subnuclei are diploid and that phenotypic drift is a two-stage process. As a first step, interallelic interaction would occur in each subnucleus resulting in the functioning of only one of the alleles in each subnucleus. If the interactions were somewhat independent in different subnuclei, a macronucleus would emerge that would be heterogeneous in composition. As a second step, random assortment of the differentiated subnuclei to daughter macronuclei during cell division would occasionally give rise to cells containing macronuclei with similarly differentiated subnuclei.

If somatic segregation were the explanation for phenotypic drift, a critical test of the hypothesis would involve cells doubly heterozygous for linked markers. If the phenotypes associated with linked genes tend to show correlated behavior during phenotypic drift, the hypothesis of somatic segregation would be strengthened. For such a study cells that were doubly heterozygous for the E-1 and mt loci were selected. E-1 and mt are linked, with crossing over estimated at 25%. The genotype of the heterozygotes was $(\underline{E\text{-}1^{B}\ mt^{B}})/(\underline{E\text{-}1^{C}\ mt^{C}})$. Certain mating types are associated with homozygosis at the mt locus. Thus, cells that are $\underline{mt^{B}}/\underline{mt^{B}}$ may express all but mating type I, while cells that are $\underline{mt^{C}}/\underline{mt^{C}}$ may express all but mating

types IV and VII. Heterozygotes that were mating type I, IV or VII were selected. The question was asked: will cells that are I (mt^C) more often drift to E-1C, and cells that are IV or VII (mt^B) more often drift to E-1B?

Twenty-five heterozygotes that were I, IV, or VII were examined after they had undergone a minimum of 100 fissions (100-270 fissions; see Table 10). Eleven were mating type I, 10 were IV and four were VII. Five subclones were initiated from each clone, and the E-1 phenotype of each subclone was determined by starch-gel electrophoresis.

TABLE 10

E-1 PHENOTYPES OF HETEROZYGOTES OF MATING TYPES I, IV, OR VII

Clone Letter	Mating Type	E-1 Phenotypes of 5 Subclones 1	2	3	4	5	Clonal Average	No. Fissions Since Conjugation at Time of Isol. of Subclone
A	IV	75	100	80	67	80	80	100
B	I	20	75	0	25	25	29	100
C	IV	67	50	0	20	20	31	100
D	VII	0	0	0	0	0	0	200
E	I	100	100	50	100	50	90	100
F	I	0	0	75	0	0	15	100
G	IV	100	100	100	100	100	100	100
H	I	100	100	100	67	100	93	100
I	IV	100	100	100	100	100	100	100
J	I	50	100	0	0	100	50	100
K	I	67	0	25	0	13	21	150-200
L	IV	100	100	100	100	100	100	150-200
M	VII	0	50	50	50	0	30	150-200
N	I	0	0	0	0	0	0	150-200
O	I	100	100	100	100	100	100	150-200
P	IV	100	100	75	100	100	95	150-200
Q	VII	20	0	25	40	50	27	100
R	VII	33	33	0	80	50	39	100
S	IV	75	0	33	20	33	32	100
T	I	100	100	100	100	60	92	100
U	IV	100	100	100	100	100	100	100
V	IV	0	0	0	0	0	0	270
W	I	0	0	0	0	0	0	270
X	I	100	100	100	100	100	100	270
Y	IV	0	0	0	0	0	0	270

Clones derived from the same pair are in brackets.

The relative intensities of the E-1B and E-1C groups of isozymes were examined visually. A numerical scoring system was adopted as follows: 0 for E-1B; 100 for E-1C; 50 for B = C; > 50 for C > B; and < 50 for B < C. See Table 10 for the scores of all 125 subclones. The average of each set of 5 subclones gave rise to a score for each clone. Some of the starch gels were run through a Photovolt Densitometer (see Allen, S. L., 1960, for the method). The computed values were very similar to those obtained by visual inspection; hence, we feel they measure the E-1 phenotypes with a degree of accuracy sufficient for our purposes.

Table 11 summarizes the data obtained on the distribution of clones as to mating type and E-1 phenotype:

TABLE 11

DISTRIBUTION OF $\frac{\text{E-1}^B\ \text{mt}^B}{\text{E-1}^C\ \text{mt}^C}$ CLONES AS TO MATING TYPE AND E-1 PHENOTYPE

	E-1BC	E-1B	E-1C	
I	7	2	2	11
IV	4	2	4	10
VII	3	1	0	4
	14	5	6	25

Table 12 summarizes the data obtained on the subclones. If linked traits show correlated behavior during phenotypic drift, then cells that are I should more often drift to E-1C and those that are IV or VII should more often drift to E-1B. This does not seem to hold for mating types I and IV: both mating types more often drift to E-1C. It does hold for mating type VII, since drift was exclusively to E-1B. However, the VII sample is much smaller than the others.

On the whole, these observations do not offer strong support for the hypothesis of somatic segregation, unless somatic crossing over is invoked. If somatic crossing over occurred, the contribution of linkage might be slight, especially in a system where the number of chromosome replications is high.

Another line of evidence, however, tends to weaken the hypothesis of somatic segregation. If the 45 subnuclei are haploid and homologous chromosomes are distributed randomly among the subnuclei, then there is no a priori reason why the segregation of homologues of different chromosomes should not proceed at the same rate. In other words, the segregation of markers on different chromosomes should begin at the second cell division and should show identical kinetics.

TABLE 12

DISTRIBUTION OF $\frac{\text{E-1}^{B}\ \text{mt}^{B}}{\text{E-1}^{C}\ \text{mt}^{C}}$ SUBCLONES

AS TO MATING TYPE AND E-1 PHENOTYPE

Mating Type	Fissions	E-1BC	E-1B	E-1C
I	100	9	7	14
	150-270	3	12	10
		12	19	24
IV	100	12	2	16
	150-270	1	10	9
		13	12	25
VII	100	8	2	0
	150-270	3	7	0
		11	9	0

Nanney has found that the kinetics of phenotypic drift in H heterozygotes is similar to that found for the mt locus (Nanney, D. L. and J. M. Dubert, 1960, Genetics 45: 1335) but the process does not seem to be initiated before 8 fissions (Nanney, D. L. 1963 in press). Our observations on phenotypic drift in P-1 heterozygotes (see Section D) suggest a similar conclusion, except that the delay in drift may result from a third stable macronuclear condition. If drift were initiated at the first division, approximately 62% of the cell lines should be parental in phenotype by 120 fissions (calculated from values computed on MIDAC; see Schensted, I.V., 1958, AM. Nat. 92: 161). In Table 3 the observed percentages for P-1 were considerably lower—14.3, 35.0, 35.7%. The value 35.2% is the theoretical value expected for 70 fissions. Either phenotypic drift is not initiated before 50 fissions, or the system is more complex. The lower values probably arise from the additional complexities of the system. Certain lineages (P-1AB) give rise to an observed frequency of parental types, 61.8% (Table 4), very close to the expected theoretical frequency (62%) for 120 fissions. Other lineages (P-1$\overline{\text{AB}}$) give rise to a much lower frequency of parental types, 22.1%, and they produce a new cell type (P-1$\overline{\text{AB}}$) that may also be stable.

The accumulated observations are less compatible with the hypothesis that the subnuclei are haploid than with the hypothesis that they are diploid. Naturally, convincing proof of their diploid condition would arise with the demonstration of reversibility of a parental phenotype derived from a heterozygote. In Paramecium aurelia such reversibility has been demonstrated (Sonneborn, T. N., M. V. Schneller, and M. Craig, 1956, J. Protozool. 3, Suppl.: 8). A search for ways of promoting reversibility in Tetrahymena may, therefore, eventually be rewarded.

If phenotypic drift is a two-stage process involving diploid subnuclei, our attention needs to be focussed on the first step. What information do the various observations supply about the site of interallelic interaction? If linked genes behave independently and if the alleles at each locus interact at a different time and in complex but specific ways, then the site of the interaction appears to be highly localized—perhaps to the vicinity of the locus itself. Nanney (1963, in press) refers to the interaction between alleles as "interallelic repression." He has preliminary evidence that some form of soluble RNA may be involved in the control of repression.

Interallelic repression may be an evolutionary mechanism of utmost significance for Tetrahymena and perhaps for all Ciliates. It increases the genotypic potential enormously by permitting the full expression of all possible phenotypes. The full phenotypic potential is rather staggering, if phenotypic drift occurs at all loci, when heterozygous. Thus, a cell heterozygous for alleles at five of the known loci (mt, H, E-1, E-2, P-1) could display one of 7 x 2 x 2 x 2 x 2 (3) or 112-224 different stable phenotypes. In Tetrahymena the macronucleus seems to have evolved as a mechanism for endowing a cell with all of the advantages usually associated with haploidy. And yet, Tetrahymena also possesses a micronucleus, which endows a cell with all of the advantages of diploidy. Depending upon which of the nuclei is functioning, Tetrahymena alternates between a life of genotypic diploidy and phenotypic haploidy. This division of function between the nuclei may have arisen in Tetrahymena—and perhaps in all Ciliates—as a very special solution to fundamental evolutionary problems.

H. GENOMIC EXCLUSION IN TETRAHYMENA: GENETIC BASIS

Four years ago a most unexpected observation was made. In an outcross of a member of the inbred C strain to the B strain, most of the progeny did not appear to be B/C heterozygotes; instead, they seemed to behave as if they were B/B homozygotes. The phenomenon was initially observed in crosses involving a new marker, E-1, and it was at first confused with properties ascribed to E-1 (Allen, S. L. 1960). Later, it was found that segregation is also abnormal at other loci: at mt (Allen, S. L., 1960b), E-2, H, and P-1. Since all genes seem to be involved in this phenomenon, a failure in the participation of nuclei derived from the C parent during conjugation is suspected. The term, genomic exclusion (suggested by D. L. Nanney), will be adapted for this phenomenon, since it seems to be an apt descriptive title.

The frequencies of pairs that arise as a result of genomic exclusion varies when different members of the C strain are used as the C parent. Some C stocks behave completely normally in crosses. Some give rise to only a few pairs that have resulted from genomic exclusion. Others result in more abnormal segregations. C*, inbred five generations (C-5573), is an example of the latter type. In outcrosses of C* almost all pairs arise as a result of genomic exclusion.

The phenomenon of genomic exclusion is demonstrated for three of the loci

in Table 13. 1, 2, and 3 are crosses of normal stocks; 4 and 5 are crosses of C*. Crosses 2 and 4 result in distributions that are comparable. But in the outcrosses, differences in segregation are observed: cross 3 results in the expected observation, while cross 5 shows distorted segregation. Thus, in cross a3 E-1BC pairs are observed, while in cross a5 most of the pairs are E-1B, and only 1/26 pairs shows the expected phenotype. In cross c3 Hae pairs are observed, while in cross c5 most of the pairs are Ha, and only 2/86 of the pairs show the expected phenotype. The mating types are also similarly distorted in their segregation. The examples given in Table 13b were obtained from crosses made at 30°C. The same pattern was found in crosses carried out at 23°C. All seven mating types are produced, if a cell is heterozygous for mt^B/mt^C. Moreover, they are produced in certain characteristic frequencies depending upon temperature. Cross 3 resulted in an array of types typical for a heterozygote. However, cross 5 resulted in an array of types characteristic of mt^B/mt^B. In only 1/112 pairs could mating type I be extracted. This one pair was the same unique pair that showed the phenotype E-1BC, expected of a heterozygote.

A number of different crosses have been made in which the exconjugants were separated. Both exconjugants give rise to viable cultures. When these are tested for a number of markers (mt, E-1, E-2, H and P-1), the phenotypes of the two exconjugants from the same pair were found to be alike. Table 14 shows that in very few pairs (7/97) did only one of the exconjugants of a pair die. It also shows that genomic exclusion affects both exconjugants of a pair. In 62 of the pairs in which both exconjugants were recovered, both were Ha. One normal pair was produced, and both exconjugants were He in phenotype (though genetically H^A/H^E).

Genomic exclusion does not result from mere changes in expression of the alleles in a heterozygote. Abnormal pairs were genetically homozygous for the alleles of the normal parent. Testcrosses of five F1 pairs and five F2 pairs showed that their mating type genotype was mt^B/mt^B (Table 15). Several of these pairs were also tested for their E-1 genotype; all were $E\text{-}1^B/E\text{-}1^B$. The viability of these crosses was good, and it was not less than the viability of crosses of normal pairs. Crosses of haploid clones are highly inviable (Elliott, A. M. and G. M. Clark, 1956, J. Protozool. 3: 181). Therefore, the abnormal pairs resulting from genomic exclusion are not only homozygous but they are also diploid.

Testcrosses of pairs that are heterozygous in phenotype show that they are genetically heterozygous. For example, 20 F2's descended from the E-1BC pair described in Table 13 (cross a5) were tested for their mt and E-1 genotype. Thirteen were mt^B/mt^C, six were mt^B/mt^B and one was mt^C/mt^C. Eleven were $E\text{-}1^B/E\text{-}1^C$, two were $E\text{-}1^B/E\text{-}1^B$ and seven were $E\text{-}1^C/E\text{-}1^C$. Since some pairs produced in an outcross of C* can be shown to be true heterozygotes, segregation apparently is, in rare exceptions, normal.

The most informative observation was made when heterozygotes (B/C) were crossed to C*. Three classes of pairs were formed: B homozygotes, heterozygotes and C homozygotes. The genotypes of these classes were confirmed by testcrosses.

TABLE 13

GENOMIC EXCLUSION IN OUTCROSSES OF C*

a. Segregation of Alleles at E-1

Cross	E-1B	E-1BC	E-1C	Total Pairs
1. $E\text{-}1^B/E\text{-}1^B \times E\text{-}1^B/E\text{-}1^B$	35	0	0	35
2. $E\text{-}1^C/E\text{-}1^C \times E\text{-}1^C/E\text{-}1^C$	0	0	30	30
3. $E\text{-}1^B/E\text{-}1^B \times E\text{-}1^C/E\text{-}1^C$	0	10	0	10
4. $E\text{-}1^C/E\text{-}1^C \times C^*$	0	0	22	22
5. $E\text{-}1^B/E\text{-}1^B \times C^*$	25	1	0	26

b. Segregation of Alleles at mt (at 30°C)

Cross	Frequency of Mating Types I	II	III	IV	V	VI	VII	Total No. Caryonides
1. $mt^B/mt^B \times mt^B/mt^B$	0	17.8	2.2	62.5	3.4	7.7	6.4	594
2. $mt^C/mt^C \times mt^C/mt^C$	44.8	19.8	1.7	0	0.9	32.8	0	116
3. $mt^B/mt^B \times mt^C/mt^C$	25.8	13.3	0	24.2	3.3	29.2	4.2	120
4. $mt^C/mt^C \times C^*$	48.4	23.3	3.3	0	1.7	23.3	0	60
5. $mt^B/mt^B \times C^*$	0+	17.0	1.5	71.0	3.5	4.0	3.0	200

+Mating type I was derived from a selfer

c. Segregation of Alleles at H

Cross	Ha	Hae	He	Total Pairs
1. $H^A/H^A \times H^A/H^A$	62	0	0	62
2. $H^E/H^E \times H^E/H^E$	0	0	73	73
3. $H^A/H^A \times H^E/H^E$	0	44	0	44
4. $H^E/H^E \times C^*$	0	0	31	31
5. $H^A/H^A \times C^*$	84	2	0	82

TABLE 14

GENOMIC EXCLUSION IN SEROTYPES OF F1 EXCONJUGANTS

Cross	No. Pairs Isolated	Viability of Exconjugants: Both Dead	One Dead	Both Alive	Antigens of Exconjugants			
					Ha	Ha	He	He
A x C*Small	45	25	5	15	14	14	0	0
					5	--	0	-
					0	0	1^{+}	1^{+}
A x C*GIANT	52	2	2	48	48^{++}	48^{++}	0	0
					2	--	0	-

$^{+}$Testcross showed that this pair was $\underline{H}^{A}/\underline{H}^{E}$
$^{++}$Testcrosses of 7 pairs showed them to be $\underline{H}^{A}/\underline{H}^{A}$

TABLE 15

TESTCROSSES OF PAIRS PRODUCED BY GENOMIC EXCLUSION

Cross	No. Pairs	Frequency of Mating Types: I	II	III	IV	V	VI	VII	Total
F1 x F1	4	0	18.2	1.5	62.8	5.3	6.6	5.7	457
F1 x C*	5	0	15.8	3.5	61.5	3.7	9.3	6.2	517
F2 x F2	5	0	20.4	2.4	58.6	3.0	8.0	7.7	338
$\underline{mt}^{B}/\underline{mt}^{B}$ x $\underline{mt}^{B}/\underline{mt}^{B}$		0	17.8	2.2	62.5	3.4	7.7	6.4	594

These classes appeared in frequencies suggestive of a 1:2:1 ratio. This type of cross is demonstrated for E-1 in Table 16 for C* as well as C'(C-6586), a derivative of C*. A total of 16 pairs were E-1B, 37 were E-1BC and 21 were E-1C. This distribution fits a 1:2:1 ratio very closely (p = .8).

A very simple interpretation of this observation is that genomic exclusion arises as a consequence of induced selfing of normal cells by C*. This kind of observation has been made by L. L. Larison and R. W. Siegel in Paramecium bursaria (1961 J. Gen. Microbiol. 26: 499). According to this interpretation, the abnormal pairs would arise as a result of illegitimate matings induced by the presence of C*. True matings between C* and a normal cell would give rise to normal pairs. This interpretation accounts for all of the observations. Thus, in a mating of C* and a B homozygote only B homozygotes would be expected; whereas, in a mating of C* and a B/C heterozygote a 1:2:1 ratio of B/B, B/C and C/C pairs would be expected.

TABLE 16

SEGREGATION OF E-1 ALLELES IN
PROGENY OF F1 (B x C) CROSSED TO C* OR C'

Cross	E-1B	E-1BC	E-1C	Total
F1(B x C) x C*	10	15	9	34
F1(B x C) x C'	6	22	12	40
Total Observed	16	37	21	74
Expected 1:2:1	18.5	37	18.5	p = .8

In order to test this hypothesis, it was necessary to find out whether the mates of a pair formed in a mating of C* by a normal cell were both normal cells or whether one was a normal cell and the other was C*. This problem was solved by experiments in which the C* cell was marked.

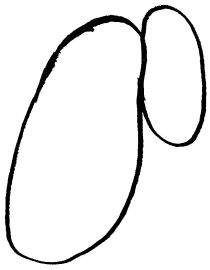
Two lines of evidence show that C* does not induce selfing of normal cells but that C* is one of the mates. Ten to 20 pairs were isolated into separate depressions and serotype tests performed (Table 17). As controls, tests were made on pairs obtained from matings within inbred strains (A x A; B x B; C x C) and in matings between a normal C and A or B (A x C; B x C). These tests were then compared to matings of C* and A, B or C (A x C*, B x C*, C x C*). In the inbred series and in the mating of C x C* immobilization of pairs occurred only with homologous antiserum, whereas in the outcrosses (A x C, B x C) immobilization of pairs occurred with both parental antisera. Significantly, immobilization occurred with anti-He in the outcrosses of C* (A x C*, B x C*).

TABLE 17

IMMOBILIZATION TESTS ON PAIRS

Mating	Antiserum to Ha	Hd	He
A x A	+	-	-
B x B	-	+	-
C x C	-	-	+
A x C	+	-	+
B x C	-	+	+
A x C*	+	-	+
B x C*	-	+	+
C x C*	-	-	+

A second line of evidence involved a morphological marker for C*. A subline of C* pure for GIANTS was used in a mating to normal-sized cells of strains A or B. The pairs contained one GIANT and one small member. The size difference diminished with time. However, when GIANT-small pairs were isolated and checked at two-hour intervals until separation, the size difference gradually decreased but was usually still noticeable at the time the exconjugants were separated. This was a viable mating, and genomic exclusion was observed in the separately tested exconjugant cultures (Table 14).



Genomic exclusion must, therefore, require some other explanation than induced selfing. Somehow, more than one meiotic product of the normal cell must participate in reconstituting the diploid nucleus to account for the unexpected classes of pairs. The 1:2:1 ratio of pairs observed in a cross of a heterozygote and C* could only arise if one out of four choices is made in forming the diploid nucleus. This condition would be met if a replica of one of the four meiotic products fused randomly with one of them. Such a scheme is outlined below:

Haploid Nuclei:	Diploid Nuclei:	Classes of Pairs:		
		AA	Aa	aa
A^1 — A^1	A^1A^1	A^1A^1	A^1a^1	a^1a^1
A^2	A^1A^2	A^1A^2	A^1a^2	a^1a^2
a^1	A^1a^1		A^1a^1	
a^2	A^1a^2		A^2a^1	
a^1 — A^1	A^1a^1			
A^2	A^2a^1			
a^2	a^1a^2			
a^1	a^1a^1			

By this mechanism a 1:2:1 ratio of pairs would be expected for locus A : 2 AA, 4 Aa and 2 aa. Notice that half of the AA and half of the aa pairs involve the reunion of identical nuclei (A^1A^1 and a^1a^1). Cells possessing such nuclei should, therefore, be "pseudoautogamous," or homozygous for alleles at all loci.

A corollary of this hypothesis consequently emerges that is most important, since it can be challenged by experimentation. If pairs homozygous for alleles at locus A are selected and are screened for alleles at a second locus, B, a statistically predictable distortion towards homozygosis can be computed. Provided neither locus is closely linked to its centromere, a 3:2:3 ratio of pairs would be expected from the following consideration:

	BB	Bb	bb	
A^1A^1	.25	0	.25	.5
A^1A^2	.125	.25	.125	.5
	.375	.25	.375	

By the same reasoning, among pairs doubly homozygous at A and B, a 5:2:5 ratio of pairs would be expected for alleles at a third locus, and so on.

To test this hypothesis, a heterozygote between the A and B strains was used as the normal mate in a cross to C*. The respective genotypes of the A/B cell and C* are shown in Table 18 for the H, P-1 and mt loci. The H serotypes were screened in 138 pairs, and in all but one of these pairs segregation of the H^A and H^D alleles (contributed from the A/B heterozygote) was observed. The exconjugants from 47 of these pairs were tested separately. The H serotypes were identical in the exconjugants of each pair. The observed distribution of pairs—34 Ha, 60 Had and 43 Hd, fits a 1:2:1 ratio ($p = .2$) and does not fit a 3:2:3 ratio ($p < .0001$).

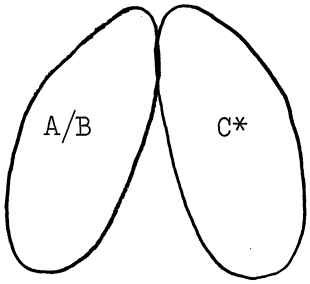
Twenty-five Ha pairs and 25 Hd pairs were selected and their P-1 phosphatases examined. Exconjugant cultures of ten of these pairs, when screened separately, showed identical phenotypes within a pair. The observed distribution of pairs—20 P-1A, 13 P-1AB and 17 P-1B, fits a 3:2:3 ratio ($p = .9$) but does not fit a 1:2:1 ratio ($p < .01$) nor a 7:29:7 ratio ($p < .0001$), expected if two unlinked genes controlled the acid phosphatases.

Caryonides were randomly selected from each pair culture and carried to maturity from 15 Ha, P-1A or P-1B pairs and 15 Hd, P-1A or P-1B pairs. Test-crosses of the mating type genotype of only three pairs have been completed; others are in progress.

To date, the observed distributions have most satisfactorily fulfilled those proposed by the genetic theory. Hence, a genetic basis for genomic exclusion is established. But, by what sequences of nuclear divisions, fusions and migrations are the requirements of the genetic theory carried out? Fortunately, these requirements are rigorous enough to restrict the sequences of nuclear events that can be considered. Two schemes meeting these requirements can be envisioned depending upon whether single or double fertilization occurs (Figure 7). If all the prezygotic divisions (MI, II and III) took place in the normal mate and random fusion of one MIII product with one of the other four haploid nuclei occurred to produce a diploid nucleus, the diploid nucleus must divide and one of the products must migrate to C* in order to fulfill the requirement that the C* mate becomes diploid after mating. This is the "single fertilization" scheme outlined in Figure 7. Alternatively, C* could gain a haploid nucleus after the third prezygotic division (MIIIa) by transfer from the normal mate; then, a second mitotic division (MIIIb) could take place. In C* the transferred haploid nucleus could divide again. In the normal mate

TABLE 18

CROSSES OF A/B x C*



a. H^A/H^D H^E/H^E

b. $P\text{-}1^A/P\text{-}1^B$ $P\text{-}1^B/P\text{-}1^B$

c. mt^A/mt^B mt^C/mt^C

a. Segregation of H Alleles:

Cross	Exconjugants Separated	Ha	Had	Hd	Total	Hde
1.	No	21	39	30	90	1
2.	Yes	13	21	13	47	0
Total Observed		34	60	43	137	
Expected 1:2:1		34.25	68.5	34.25	p = .2	
Expected 3:2:3		51.4	34.2	51.4	p < .0001	

b. Segregation of P-1 Alleles in H Homozygotes

	P-1A	P-1AB	P-1B	Total
Ha	9	7	9	25
Hd	11	6	8	25
Total Observed	20	13	17	50
Expected 1:2:1	12.5	25	12.5	p < .01
Expected 3:2:3	18.75	12.5	18.75	p = .9

any one of the four haploid nuclei might undergo this division. The products of MIIIb might function as migratory and stationary nuclei, permitting reciprocal fertilization as in a normal mating. This is the "double fertilization" scheme outlined in Figure 7.

A cytological study of C* during conjugation was made before the genetic basis of genomic exclusion was worked out. At the time we had no clear idea at all as to what to look for. Moreover, the techniques used in making the crosses resulted in a panorama of pairs in different stages of conjugation, making the assessment of sequences in the stages exceedingly difficult. This was particularly true of the critical intermediate stages.

In this study the crosses were made in the Cerophyl-Aerobacter medium. Samples were removed at regular intervals (6, 12, 24, 48, 72 hours) and Feulgen preparations were made. In some of the crosses the C*GIANT subline was used to mark C*.

With this protocol new pairs form over the entire period of observation. However, we could conclude that the very early stages of conjugation were normal: crescents form in both mates and both mates undergo MI and MII. The products of meiosis seemed to disintegrate in C*. What could not be determined at that time was the extent to which intermediate stages occur in C*. The stage that often showed irregularity was the third prezygotic division. Normally, one of the products of the second prezygotic division ends up in the paroral cone but in these crosses often one of the products failed to end up in this region in C*. In samples of pairs after 24 hours the third prezygotic division seemed to be taking place in only the normal mate. In the 72 hour sample the third prezygotic division also occurred in both mates of some pairs. The late stages of conjugation appeared to be normal: the postzygotic divisions and macronuclear enlargement take place in both mates.

This study provided confirmatory evidence for the fact that both exconjugants end up with a normal complement of nuclei. However, at the time these observations were made, the peculiarities of the third prezygotic division were very puzzling. In light of our present knowledge, the observation that some pairs did seem to undergo a third prezygotic division in both mates infers that the double fertilization scheme may be applicable. On the other hand, since these pairs were observed only in late samples, they could represent an accumulated fraction of pairs that was normal. Before any definite conclusions can be drawn, a more detailed analysis of these critical stages is needed. In repeating this study we plan to use a technique that will prevent new pairs from forming, making observation of the sequence of stages somewhat simpler.

C* provokes unilateral genomic exclusion in almost all pairs. This characteristic was invaluable for making possible an analysis of the genetic basis of genomic exclusion. Other members of the C strain behave abnormally in outcrosses to a lesser degree and the types of distortions may be different. Some provoke unilateral genomic exclusion but, instead of recovering markers from the other parent, only those of the C parent are recovered. Others provoke bilateral genomic exclusion. For example, a cross of B-8572 x C-6586 resulted in 22 normal pairs that were E-1BC, three E-1B that were also Hd, and three E-1C pairs that were He. These unusual pairs were testcrossed. Those that were E-1B were homozygous for $\underline{\text{E-1}}^{B}$, $\underline{\text{E-2}}^{B}$, $\underline{\text{mt}}^{B}$ and $\underline{\text{H}}^{D}$, while those that were E-1C were homozygous for $\underline{\text{E-1}}^{C}$, $\underline{\text{E-2}}^{C}$, $\underline{\text{mt}}^{C}$ and $\underline{\text{H}}^{E}$.

Genomic exclusion is not confined to the C strain. D. L. Nanney (1963a, in press) has found aberrant segregation in over half the crosses of highly inbred derivatives of several strains. He has observed both unilateral and bilateral genomic exclusion in these crosses. The frequency of genomic exclusion seems to be higher if crosses are made at temperatures other than "standard" (23-26°C). Some strains, like B1 and D, are particularly prone to behave abnormally. He believes that the abnormalities in segregation may be a late manifestation of inbreeding degeneration, since they seem to occur more frequently in stocks that are now in the 12th or 13th generation of inbreeding.

Some of our data provides weak, but suggestive, evidence for the idea that genomic exclusion may be partially controlled by genetic factors. Such control, if it does occur, is probably multifactoral. We also have the impression that the frequency of genomic exclusion may rise as cultures age; i.e., after they are removed many fissions from a previous sexual reorganization. Therefore, some measure of control over the frequency of genomic exclusion could conceivably be exerted. If young stocks were used in making crosses and if selection were directed against this trait, its frequency might be kept at a minimum.

I. SEROTYPE S

A new high temperature serotype, S, was inadvertently discovered in immobilization tests of certain F1(A x B) caryonides also tested for their acid phosphatases (Allen, S. L., 1962a). Two caryonides, both from the same pair, did not react with either anti-Ha or anti-Hd. A third caryonide from the same pair was Had. An antiserum specific for S was prepared. It reacted with the two non-H caryonides, when grown at 23°C but not when grown at 16°C, and with some of their progeny. It did not react with cells of any of the previously established serotypes: Ha, Hb, Hc, Hd, He, L or I. In the presence of homologous antiserum both H and S cells transformed to I.

Serotype S is stable in culture. In crosses of the three caryonides, S1, S2 and Had, S segregates with H in the progeny of the S x S cross or in the H x S crosses. The first crosses were made before an antiserum to S was prepared. The results of tests of pair cultures using anti-Ha and anti-Hd are shown in Table 19. In subsequent testing the "minus" category showed immobilization with anti-S. All three crosses gave rise to some Had, Ha, Hd and some "minus" (S) pairs, but the distributions of the classes were significantly different ($p = < .0001$). Cross 1 gave rise to a 3 S to 1H pair ratio, cross 2 to a 2 S to 1 H pair ratio, and cross 3 to a 1 S to 2 H pair ratio. If segregation at only the H locus is considered, a 2:1:1 ratio of Had, Ha and Hd pairs would be expected since these were F1 x F1 matings. However, out of 176 pairs only 57 were Had, four were Ha and 12 were Hd. The number of Had pairs showed extreme variability in the three crosses.

S cells may very likely have been present in some of the H cultures but were not detected, since an anti-S was not yet available. The degree of immobili-

TABLE 19

DISTRIBUTION OF PAIR CULTURES AT 15 FISSIONS

Cross	Had	Ha	Hd	"Minus"	Total
1. S1 x S2	7	2	5	46	60
2. H x S1	17	1	2	37	57
3. H x S2	33	1	5	20	59
	57	4	12	103	176

zation with anti-Ha and with anti-Hd was determined for the Had cultures. Usually the degrees of reactions were very similar, but not all were complete. Of 57 Had cultures, 26 showed complete immobilization and 31 showed partial immobilization. The chances are great (especially in view of recent observations) that S could have been present in some of the 31 cultures showing partial reactions to the H antisera.

When an antiserum to S became available, the first 20 pair cultures of crosses 1, 2 and 3 were retested. All "minus" cultures were S. In cross 2 one of the Had cultures tested as HdS and one of the Ha cultures as S. In cross 3 one of the Had cultures tested as HadS. At the time of retesting, these cultures had undergone approximately 60 fissions. Therefore, some of these cultures were approaching maturity. The appearance of S in the H cultures could represent different mature types; or, they could have arisen as a result of recombination within the culture. Subclones of the HS cultures were initiated and the various lines tested for mating type. In those cultures containing both H and S the H type was associated with a mating type that differed from that of the S type. This observation suggests that H and S are associated with different cell lineages within these pair cultures.

Recently, a cross of H and S was set up, and exconjugents and caryonides were obtained from some of the pairs. A total of 43 pairs were examined at 15 fissions: 18 were H, 17 were HS and 8 were S. The distribution of Had, Ha and Hd among H and HS pairs was peculiar: 24 were Had, 10 were Hd and only one was Ha. A peculiar distribution with respect to Ha was also noted among the caryonides: 36 were Had, 8 were Hd, 6 were Ha, 12 were HadS, 9 were HdS, 0 were HaS, and 33 were S. Most of the caryonides were either H (52.6%) or S (34.7%); only 12.7% were HS. Thus, H and S tend to be exclusive of one another at the level of the caryonide.

Ordered caryonides were obtained from only 13 pairs (Table 20). Here, 59.7% were H, 28.8% were S and only 11.5% were HS in type. Again, H and S tend to be exclusive of each other. Caryonides 1 and 2 are sisters (i.e., they were derived from the same exconjugant) and caryonides 3 and 4 are sisters. It was of interest to examine the possibility that 1 and 2 or 3 and 4 are more often alike than 1 and 3 or 2 and 4. With this sample, the difference is not significant, but it

is also not significant if all the caryonidal data are used. Thus, it looks as if the H and S phenotypes are determined at the level of the caryonide.

A few caryonides (11.5—12.7%) were HS. However, we do not know yet if both types can be produced by the same cell. It is entirely possible that these caryonides are made up of a heterogeneous population of cell types by the time the cultures were examined (15 fissions). Subclonal analysis will provide an answer.

TABLE 20

ORDERED CARYONIDES FROM A CROSS OF H x S

Pair	1	2	3	4
1	Had	Had	S	S
2	Had	Had	Had	Had
3	HdS	Had	S	S
4	Ha	Ha	S	HadS
5	Had	Hd	Hd	Hds
6	Had	Had	Had	Had
7	Had	Had	Had	HdS
8	Had	Had	Had	Had
9	S	HadS	S	S
10	S	S	S	HdS
11	Had	Had	Had	Had
12	Had	Had	Had	Had
13	S	S	S	S

If control of the H-S difference is caryonidal, in view of previous work on H and other loci, it will be of interest to follow several different cell types during their course of replication. The technique of subclonal analysis has turned out to be a powerful tool in other systems; hence, its use here may provide insight into the nature of the interrelationship between H and S.

One observation that comes out of this cross is the low frequency of Ha cell type. Only 1/43 pairs was Ha, and none of the caryonides were HaS. Thus, we have a suspicion that Ha and S may be particularly exclusive of one another. Testcrosses of the eight S pairs will be made. It would be interesting if these pairs were genotypically H^A/H^A.

At present the interrelationship between S and H is not clear, but we believe that they are mutually exclusive of one another—perhaps within a single cell. As a working hypothesis, we believe that S may be controlled by a locus other than the H locus. However, not ruled out is the possibility that S represents some type of rearrangement within the H locus.

II. PUBLICATIONS

Allen, S. L., 1958 Cytochemical localization of enzymes in sexual strains of the protozoan Tetrahymena pyriformis. Anat. Rec. 131: 526-527 (Abstract).

Allen, S. L., 1958a Cytochemical studies of a morphological mutant of Tetrahymena pyriformis. J. Protozool. 5 (Suppl.): 12 (Abstract).

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Allen, S. L. and M. S. Misch, 1962 Variations in the electrophoretically separated acid phosphatases of Tetrahymena pyriformis, variety 1. J. Protozool. 9 (Suppl.): 23 (Abstract).

Allen, S. L. and M. S. Misch, 1962a Genetic and epigenetic factors affecting the acid phosphatases of Tetrahymena. Genetics 47: 938 (Abstract).

Manuscripts

Allen, S. L. Genomic exclusion in Tetrahymena: Genetic basis. To be submitted to J. Protozool.

Allen, S. L. Esterase isozymes in Tetrahymena. To be submitted to J. Cell Biol.

Allen, S. L. A first case of linkage in Tetrahymena pyriformis. To be submitted to Genetics.

Allen, S. L. Regulation of phenotypic drift in Tetrahymena. To be submitted to Am. Nat. or Developmental Biol.

Allen, S. L. and M. S. Misch. Variations in the electrophoretically separated acid phosphatases of Tetrahymena. To be submitted to J. Protozool.

Allen, S. L., M. S. Misch, and B. M. Morrison, Genetic control of the acid phosphatases in Tetrahymena: formation of a hybrid enzyme. To be submitted to Genetics.

III. STAFFING

Sally Lyman Allen, Research Associate 1957—, 100%

IV. FOREIGN TRAVEL

To Pernambuco, Brazil for International symposium on "The function of esterases in animals and plants." Sponsored by the University of Recife, September 8-15, 1963 (contingent upon receiving travel funds from Supplemental CA-03545-06).

V. OTHER CONTRIBUTIONS

Direction of special projects performed by students:

a. Robert A. Berkoff, 1961-1962, A preliminary immunological study of the esterases.

b. John C. Hegenaur, 1962-1963, Genetic studies of serotype S.