THE UNIVERSITY OF MICHIGAN

COLLEGE OF LITERATURE, SCIENCE, AND THE ARTS
Departments of Botany and Zoology

Progress Report

GENETICS OF TETRAHYMENA

January 1, 1963 - December 31, 1967

Sally Lyman Allen

ORA Project 01520

under contract with:

NATIONAL INSTITUTES OF HEALTH
NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES
RESEARCH GRANT GM-15879 (formerly HD-01243)
BETHESDA, MARYLAND

administered through:

OFFICE OF RESEARCH ADMINISTRATION ANN ARBOR

January 1968

TABLE OF CONTENTS

	Page
LIST OF TABLES	iii
LIST OF FIGURES	iv
SUMMARY PAGE	v
I. RESEARCH ACCOMPLISHMENTS TO DATE	1
A. Biological Aspects	1
1. General Comments	1
2. Linkage Studies	1
3. Genomic Exclusion	1
4. Synthesis of New Strains	10
5. Synthesis of Isogenic Strains	10
6. Artificially Induced Mating	11
B. Isozymes in Syngen 1 of *T. pyriformis*	11
1. Esterases	11
2. Phosphatases	13
3. Electrophoretic Variants of Mitochondrial Enzymes	15
4. Electrophoretic Variants of a Microbody Enzyme	16
C. Isozymes in the Syngens of *T. pyriformis* and *Paramecium aurelia*	16
1. *T. pyriformis*	17
2. *P. aurelia*	19
D. Phenotypic Diversity in Heterozygotes of Syngen 1	19
1. Analysis of Esterases	21
2. Analysis of Phosphatases	21
E. Preliminary Studies on DNA Homologies in Strains and Clones of *T. pyriformis*	26
II. PUBLICATIONS	26
III. STAFFING	28
IV. OTHER CONTRIBUTIONS	28

LIST OF TABLES

Table		Page
1	Genes of *T. pyriformis*, syngen 1	2
2	Clonal Performance of AB and C*	6
3	Frequency of Micronuclear Types in Selected Subclones of AB and C*	7
4	Viability, Genetic Ratios and Frequency of Mated Amicronucleates in Crosses of AB to Selected C* Subclones	8
5	Strains in Syngens of *T. pyriformis*	18
6	Stocks of *P. aurelia*	20

LIST OF FIGURES

Figure

1 Scoring Phenotypes in Tetrahymena.

2 Derivation of Homozygous Strains.

3 Relationship of Strains 7, 8, 17, and 21.

4 Intersyngenic Variation in Propionylesterases of T. pyriformis. Code letters at bottom of gels refer to strains listed in Table 5. Mark to left and right of set of gels shows position of E-3. Origin is at "O". Cathode is at top, anode at bottom of photograph.

5 Intrasyngenic Variation in Propiopylesterases of T. pyriformis. Code letters at bottom of gels refer to strains listed in Table 5. In all but gel at extreme left, peptone (P) extracts were inserted on left side, skimmed milk (SK) extracts on right side of gel. Mark beside gels indicates E-3. Note migration of syngen 1 isozymes is slightly greater than those of other syngens. Origin is at "O". Cathode is at top, anode at bottom of photograph.

6 Intersyngenic Variation in Butyrylesterases of T. pyriformis. Code letters at bottom of gels refer to strains listed in Table 5. Mark to left and right of set of gels shows position of E-3. Origin is at "O". Cathode is at top, anode at bottom of photograph.

7 Intersyngenic Variation in Acid Phosphatases of T. pyriformis. Code letters at bottom of gels refer to strains listed in Table 5. Origin is at "O". Cathode is at top, anode at bottom of photograph.

8 Intersyngenic Variation in Propionylesterases of P. aurelia. Order of stocks (from left to right): P, 72, 261, 51, 236, 166, 227, 138, 312, 223, 306, 273, 238, 328, 147, 126. Cathode is at top, anode at bottom of photograph.

9 Intersyngenic Variation in Butyrylesterases of P. aurelia. Order of stocks (from left to right): P, 72, 261, 51, 236, 166, 227, 138, 312, 223, 306, 273, 238, 328, 147, 126. Cathode is at bottom, anode is at top of photograph. (These are slices from the bottom portion of gels shown in Figure 8).

SUMMARY PAGE

(a) GM-15879: Genetics of Tetrahymena

(b) Sally Lyman Allen

(c) University of Michigan

(d) January 1, 1963 - December 31, 1967

(e) December 31, 1967

(f) Summary Statement:

Experiments on syngen 1 of *T. pyriformis* have been directed along the following lines: (1) With the establishment of the cytogenetic basis of genomic exclusion, this abnormal form of conjugation has been used to synthesize new homozygous strains and to initiate the production of isogenic lines. (2) The isozymes of several esterases, phosphatases and oxidoreductases have been characterized as to their inheritance, linkage relations, chemical properties, intracellular localization, and variation under different growth conditions. (3) Phenotypic diversity arises among cell lines of heterozygotes. This phenomenon has been examined as a function of fissions for the esterases and phosphatases. A detailed kinetic study was carried out on the phosphatases on untreated and on lines in which macronuclear retention was induced.

Polymorphisms have been observed for the esterases and phosphatases in representative strains of the 12 syngens of *T. pyriformis*. The properties of these enzymes have been compared to those previously studied in syngen 1.

In order to get at the molecular basis for phenotypic diversity in heterozygotes, studies were initiated of the DNA homologies of strains and clones of different genetic relationship. When the *in vitro* techniques of nucleic acid hybridization were applied, discrimination between DNA's from different sources was achieved.

I. RESEARCH ACCOMPLISHMENTS TO DATE

A. Biological Aspects

1. General Comments

There are 12 breeding groups, or syngens (or varieties) in *Tetrahymena pyriformis*. Within a syngen genes are freely exchanged, but between syngens little, if any, gene flow occurs. Most strains of syngens 2-12 are the fission products of "wild" strains which have been collected from various parts of the world, mainly by Dr. A. M. Elliott (University of Michigan) or his students. Representative strains are maintained in 1% proteose-peptone in my laboratory by routine subculturing.

Most of my work has involved syngen 1. Inbred strains were derived by crosses from wild strains or between derived strains by Dr. D.L. Nanney (University of Illinois) or in my laboratory. Currently some ten inbred strains (A, A1, A3, B, B2, C, C1, D, D1, and E) are maintained by annual bouts of inbreeding in order to keep viability at a maximum. The responsibilities for maintaining the strains are divided between my laboratory and that of Dr. D.L. Nanney (University of Illinois).

Genes identified at 6 of the known loci are shown in Table 1. In addition are two lethals and one semi-lethal which affects maturity (Bleyman and Simon, personal communication). How the phenotypes associated with the 6 genes shown in Table 1 are scored, is diagrammed in Figure 1.

2. Linkage Studies

The linkage relationships of 5 of the 6 loci described above were explored. *H*, *E-1*, *E-2*, and *P-1* segregate independently. *Mt* and *E-2* also segregate independently. *Mt* and *E-1* are linked, recombination being of the order of 25%. These studies are summarized in Allen, S.L. (1964) Genetics 49: 617-627. A reprint is supplied.

3. Genomic Exclusion

Genomic exclusion is an abnormal form of conjugation occurring between cells with defective micronuclei and normal cells with diploid micronuclei. The progeny are heterocaryons; each cell has an old macronucleus but a new diploid micronucleus derived from one meiotic product of the normal mate. Such cells express genes found in the old macronucleus, are sexually mature, and can be specifically selected. When inbred, they give rise to lines genetically homozygous at all known loci.

TABLE 1

GENES OF *T. PYRIFORMIS*, SYNGEN 1

Gene	Alleles	Phenotype	No. Stable Types In A Single Homozygote	No. Stable Types In a Single Heterozygote
mt	A, C-F	Mating Types	I,II,III V,VI	5 in A/C,A/D, etc.
	B		II,III,IV,V,VI,VII	7 in A/B,B/C, etc.
H	A,C,D,E	High Temperature Serotypes(20-30°C), in CA or Peptone	1	2
T	A,B,C	Torrid Temperature Serotypes(38-40°C), in liver-peptone	1	2
E-1	B,C	Variants of a group of 5-6 Esterase Isozymes(Propionate)	1	2
E-2	B,C	Variants of an Esterase(Butyrate)	1	2
P-1	A,B	Variants of an Acid Phosphatase(Interaction in heterozygote, with a total of 5 isozymes in some heterozygotes)	1	3 (2 parentals, 1 hybrid)

Most of our observations on genomic exclusion have been published in a series of papers. The first reported the genetic observations: Allen, S.L. (1963) J. Protozool. 10: 413-420. A reprint is supplied. The abstract follows:

Genomic exclusion is an aberration that occurs during conjugation in variety 1 of Tetrahymena pyriformis. Instead of containing markers from both parents, the outcross pairs are either homozygous for all the genes of one parent (unilateral genomic exclusion); or, some of the pairs are homozygous for the genes of one parent and other pairs are homozygous for the genes of the other parent (bilateral genomic exclusion). This phenomenon was first demonstrated in the C strain: some stocks evoke unilateral genomic exclusion; others, bilateral genomic exclusion. C*, inbred for 5 generations, was used to explore this phenomenon in some detail since unilateral genomic exclusion of C genes occurs in almost all pairs in outcrosses of C*. In a mating of C*, both exconjugants are recovered, both are diploid and similar in phenotype. Using morphological markers, C* can be shown to participate in the mating; therefore, C* does not induce illegitimate matings of the normal mate. When the normal mate is heterozygous for alleles (H^A/H^D) not present in C*, 3 classes of offspring (H^A/H^A, H^A/H^D and H^D/H^D) are produced in a 1:2:1 ratio. These observations indicate that 2 meiotic products of the normal mate unite to form the syncarya. The genetic ratios obtained in 1 and 2 factor crosses limit the possible cytogenetic bases for genomic exclusion. They suggest that 1 of the 4 haploid nuclei replicates and the replica fuses randomly with any 1 of the 4 nuclei. The 2 schemes of nuclear behavior (single fertilization, double fertilization) that would satisfy these requirements have not yet been resolved.

The interpretation of the cytogenetic events turned out to be wrong. When a combined cytological and genetic study was carried out (Allen, S.L. (1967) Genetics 55: 797-822), the following was found:

A cytological and genetic analysis was made on timed matings of a cross of a clone belonging to the heterozygous AB strain and C*, a clone belonging to the inbred C strain in syngen 1 of Tetrahymena pyriformis. Cells of the AB clone have a normal diploid micronucleus, and C* cells are hypodiploid or amicronucleate. The progeny of this mating receive only the genes derived from the AB parent, and, in mass matings, genetic ratios approaching 1:2:1 are observed for genes present in heterozygous condition in the AB strain. Since the genes from C* are excluded, the phenomenon has been termed genomic exclusion. Genomic exclusion includes two consecutive rounds of mating. The first round of conjugation is abnormal. The diploid syncarya of both exconjugants are derived from one meiotic product

of AB. However, since the old macronucleus is regularly retained, the products that arise are heterocaryons, express the macronuclear genes, and are sexually mature. The second round of conjugation is normal, and the syncarya are derived from two meiotic products, one from each conjugant. If exconjugants from different Round 1 pairs are allowed to remate at random, the progeny fall into three phenotypic classes. If, however, the two exconjugants from the same Round 1 pair are remated, the progeny can be shown to be genetically homozygous at all known loci. The establishment of the cytogenetic basis for genomic exclusion accounts not only for all earlier genetic observations, but it also has useful and important implications for future genetic work with this organism. For, by using a clone, such as C* in crosses to heterozygotes, homozygous diploid lines can be synthesized almost instantly.

A reprint is supplied.

Evidence for the occurrence of genomic exclusion in other strains of syngen 1 was reported in Allen, S.L., S.K. File and S.L. Koch (1967) Genetics 55: 823-837. A reprint is included. The abstract follows:

Genomic exclusion is probably of general occurrence in *Tetrahymena pyriformis*. In syngen 1, it may occur whenever a cell, which is defective in its micronucleus, is mated to a cell with a normal diploid micronucleus. Evidence is reported that there is a high correlation between the presence of a semi-amicronucleate parent in the cross, mature progeny, and the occurrence of genomic exclusion in crosses in syngen 1. These are aspects of a syndrome of associated nuclear phenomena basic to the genetic consequences of genomic exclusion and previously worked out on a cross of AB X C*. Genomic exclusion has, so far, been unequivocally demonstrated in syngen 1 only, although the results of some crosses recorded in the literature of other syngens suggest that it may occur elsewhere. Loss of the micronucleus appears to occur often in this organism, since the frequency of amicronucleate clones in world-wide collections is very high. In syngen 1 this loss is influenced by the macronuclear genotype. Since amicronucleate clones are sexually dead, it is proposed that genomic exclusion represents an evolutionary alternative in which the defective micronucleus is destroyed and replaced by a normal micronucleus in which genic recombination has occurred.

Genomic exclusion can be employed to generate homozygous diploid lines. This approach is outlined in Allen, S.L. (1967) Science 155: 575-577. A reprint is enclosed.

The following is a condensed version of a manuscript entitled: "Defective micronuclei and genomic exclusion in selected C* subclones" (Allen, S.L. and S.L. Weremiuk):

Subclones were initiated from C*, a clone known to be defective in its micronucleus and to induce genomic exclusion when mated to a normal diploid clone (AB). The micronuclear constitution, chromosome number, and ability to induce genomic exclusion of two groups of C* subclones were examined. The two groups differed primarily in the frequency with which amicronucleate animals were present in the cultures. In spite of this difference, the breeding performance of the subclones was similar and the viability of all crosses was high.

No diploid subclones of C* were recovered after selection for "normalcy" by repeated subculture, although almost 50% of the subclones were lost and a gradual decrease in the frequency of crinkled cells (which are amicronucleate) was noted in the survivors (Table 2). Ten subclones were selected for intensive cytological and genetic analysis on the basis of the frequency of crinkled cells in the cultures. One group of five appeared to be nearly normal. The second group of five subclones appeared to be highly abnormal, though viable, and a high frequency of crinkled cells was observed upon serial subculture. Upon cytological examination of stained preparations, the first group had an average of 5% amicronucleate animals in the population (range 1-8%), while the second group had an average of 27% amicronucleate animals (range 20-31%) (Table 3). Many amicronucleate animals were round in appearance and lacked any vestige of an oral apparatus.

The stem-cell of C* appears to be aneuploid. The most normal subclone had one micronucleus that contained 3-5 chromosomes. Other subclones often had fewer chromosomes and contained several small micronuclei. The fact that chromosomes of the micronuclei of C* could be seen during mitosis, and in some cells actually counted, was in itself an unusual finding. The mitotic chromosomes of C* were 2-4x smaller than normal meiotic chromosomes. Apparently the forces which normally cause the formation of the spindle-shaped aggregate of chromosomes have been relaxed in C* cells (Loosening of the chromatin in a clone losing its micronucleus was reported by Wells in 1961, Fig. 1; J. Protozool. 8, 284).

No difference was found in the breeding performance of these subclones when crossed to a normal heterozygote (AB)(Table 4). All subclones induced genomic exclusion and similar ratios were observed in the progeny of late-isolated pairs. Cytological examination of the early pairs of four subclones which differed in the frequency with which amicronucleate animals were present (1%, 7%, 29%, 31%) showed a similar, but low frequency of mating amicronucleate animals (0.5%, 3%, 3.5%, 3%). Thus, amicronucleate animals do not readily mate and are usually excluded during conjugation. Micronucleate animals, regardless of how much chromatin is present, do participate in the mating and appear to be potentially equivalent in their ability to induce genomic exclusion, since the breeding performance of the subclones was similar.

TABLE 2

CLONAL+ PERFORMANCE OF AB AND C*

	Transfers of AB:				Transfers of C*:			
	0	1	2	3	0	1	2	3
Accumulated								
% Dead	0	1.2	1.2	1.2	5.0	29.4	39.5	47.0
% Slows	0	0	1.2	2.4	2.5	3.4	4.2	6.7
% Normal	100	98.8	97.6	96.4	0	2.5#	5.9	9.2
% Crinkled	0	0	0	0	92.5	64.7	51.4	37.1

+Design of experiment: 90-120 morphologically normal single cells were isolated from populations of AB and C*. At 3 day intervals (and after 13 fissions), a single cell, also morphologically normal, was transferred from each subclone to fresh medium.

#Presence of amicronucleate (crinkled cells) looked for in populations that developed after 3 days. These cultures had no detectable crinkled cells.

TABLE 3

FREQUENCY OF MICRONUCLEAR TYPES IN SELECTED SUBCLONES OF AB AND C*

Sample#	Diploid: 1	Diploid: 2	Aneuploid: 1	Aneuploid: 2	Aneuploid: 3+	Amic	Approx. Total No. Chromosomes	Remarks
AB-1	100.0	0	0	0	0	0	(10)	Difficult to observe
2	99.6	0.4	0	0	0	0	(10)	chromo-somes.
3	100.0	0	0	0	0	0	(10)	Compacted into
4	99.8	0.2	0	0	0	0	(10)	spindle-shaped
5	100.0	0	0	0	0	0	(10)	aggregate even at
6	98.8	1.2	0	0	0	0	(10)	metaphase-anaphase.
7	98.6	1.4	0	0	0	0	(10)	
								Nuclei:
C*-27	0	0	98.6	0.4	0.2	0.8	3-5?	Uniform in size
23	0	0	40.8	53.2	8.4	5.6	2-5?	Variable in size
25	0	0	22.6	50.4	22.2	4.8	2-3?	Variable
45	0	0	89.2	3.6	0.4	6.8	3-5?	Uniform
68	0	0	37.0	51.0	4.2	7.8	2-5?	Variable
C*- 3	0	0	63.4	5.2	2.0	29.4	2-5?	Variable
7	0	0	53.8	10.2	5.0	31.0	2-5?	Highly variable
10	0	0	54.0	11.6	4.0	30.4	2-5?	Highly variable
24	0	0	71.4	4.2	1.8	22.6	3-5?	Variable
28	0	0	52.2	23.8	4.2	19.8	2-5?	Highly variable

#Total no. cells observed was 500 per sample.

TABLE 4

VIABILITY, GENETIC RATIOS AND FREQUENCY OF MATED AMICRONUCLEATES IN CROSSES OF AB TO SELECTED C* SUBCLONES

C* Sub clone	% Viability of Pairs Isol. At: 10½ Hrs	36 Hrs	Serotypes of: 10½ Hr Pairs Had	Ha	Hd	36 Hr Pairs Had	Hd	Hd	% Amic Sub-clones	in: Crescents at 4 Hrs#
27	3	87	0	0	1	12	7	7	0.8	0.5
23	3	72	0	0	1	11	3	6	5.6	
25	0	62				8	5	5	4.8	
45	3	62	0	0	1	7	4	5	6.8	3.0
68	27	85	0	5	3	9	7	7	7.8	
Total	7.2	73.5	0	5	6	47	26	30	5.2	1.8
3	0	82				10	7	5	29.4	3.5
7	3	53	0	1	0	8	5	3	31.0	3.0
10	0	82				7	8	7	30.4	
24	0	89				11	6	7	22.6	
28	10	80	0	1	2	11	5	8	19.8	
Total	2.6	75	0	2	2	47	31	30	26.6	3.3

#200 Crescents examined per sample

In addition, we have been concerned with factors affecting mating during the two consecutive rounds of conjugation which occur with genomic exclusion. Some time ago we observed that the frequency of Ha: Had: Hd is closer to a 1:1:1 ratio than a 1:2:1 in unstopped matings of AB x C*. Why is there this distortion? Why does there appear to be an increased number of homozygotes?

First, we wondered if this distortion might occur if there was some tendency for Round 1 exconjugants to go into Round 2 without separating. For this experiment we made a cross between AB x C* and isolated pairs 4 hours after the onset of mating and long before an opportunity existed for the completion of Round 1. 1000 pairs were isolated. After 3 days, the progeny were scored: 30 were dead, 967 were mature, and 3 were immature. All 3 immature progeny were homozygotes. However, this frequency (0.3%) is much too low to account for the distortion seen in the genetic ratios.

Second, we wondered if the distortion affected different phenotypes in the same way. The H antigen is a surface antigen, and mating is a surface phenomenon. Distortion might occur here, whereas it might not for a phenotype <u>not</u> associated with the cell surface. We chose the phosphatases as a non-surface phenotype. We made a cross between AB x C* and isolated 240 pairs at 36 hours after the onset of mating, or an interval sufficient for both Rounds 1 and 2 to occur. After 3 days the progeny were scored: 25 were dead, 44 were mature, and 161 were immature. 100 of the immature progeny were screened for their H antigens and P-1 phosphatases (97 complete tests were made). The distribution of H and P-1 was, as follows:

	P-1a	P-1ab	P-1b	
Ha	10	13	14	37
Had	6	15	7	28
Hd	10	11	11	32
	26	39	32	97

When tested against a 1:2:1 ratio by Chi square, the distribution for P-1 is not significant ($p=.1$) whereas it is significantly different for H ($p<.001$). These observations thus suggest that surface characters are affected in the remating of Round 1 exconjugants, whereas non-surface characters are not.

These observations, and others on mating, will be collected together and written up under the title: "Selective mating in genomic exclusion?" (Allen, S.L. and S.L. Weremiuk).

4. Synthesis of New Strains

Homozygous strains of different genotypes were derived in the summer of 1966 by crosses of heterozygotes (between inbred strains A and C or B and C) to the defective clone C* using genomic exclusion. The method by which this was done is diagrammed in Figure 2. A number of Round 1 pairs were isolated from crosses 231-4 (Figure 2, bottom). From a number of depressions, 6 replicate Round 2 pairs were isolated 3-4 days later. All 6 replicates were dead or mature from some of the strains (72/153 in 231-2; 115/162 in 233-4). Of the remaining strains (128), most replicates were alive, although a few died or were mature (and were discarded). All replicates were typed for H. Duplicates were typed for T and for the enzyme loci. Several clones were initiated from each of the replicates and brought to maturity and tested for mt. Certain strains were then selected and their viabilities upon inbreeding were determined. The strains selected all had viabilities ranging from 93-100%.

The homozygous strains are designated by number and mating type. For example, strain #7, mating type VI, is designated 7/VI. This system may have to be modified to indicate subsequent inbreeding.

"Trios" of homozygous strains were selected on the basis of their genotype. These bear a particular relationship to one another, as shown in Figure 3 (top). Four such trios were saved. The genotypes of the strains included in the trio used for the experiments on nucleic acid hybridization (reported in Allen and Gibson, 1967) are shown in Figure 3 (bottom).

5. Synthesis of Isogenic Strains

Two of the three strains from the trio were selected as the progenitors of the isogenic strains. These are strains #7 and 8, and their genotypes are shown in Figure 3 (bottom). Genes from strain #8 are being introduced into strain #7, and during the backcrosses, progeny which are heterozygous at the H, T, E-1, E-2, and P-1 loci are selected - this being the order of the selection of the genes. That is, only progeny which are heterozygous for H are tested for T; then, the T heterozygotes are tested for their esterases; and finally the E-1, E-2 heterozygotes are tested for P-1. It was decided to give up on the mating type locus since it was not practical to include it. The viabilities of the backcrosses have (fortunately!) been high. We have been routinely obtaining about 130 to 140 good progeny from the isolation of 150 pairs. We end up with about 4-5 progeny which are quintuply heterozygous. At present (Dec. 31, 1967) we have reached the eleventh backcross generation and plan to go through a total of 12 backcrosses. To obtain homozygotes and a strain which is isogenic with #7, a cross will be made to C*. An isogenic strain which contains the alleles of strain #8

at the five loci will be selected in a manner similar to the procedure used in the backcrosses: i.e., by serial selection. (It should also be possible to obtain other isogenic strains with single gene differences from the discards for future interesting and obvious uses.)

6. Artificially Induced Mating

Chemical induction of mating within and between species of Paramecium has been carried out by Miyake (J. Protozool. 7 (Suppl.): 15). In some instances the mating results in successful conjugation. So far as I am aware, the chemical basis for the action of the agents employed (proflavin or acriflavin, ± KCl, ± acetamide) is not known. Obviously, overcoming the natural barriers to mating may be especially useful if gene flow between species is desired.

So far as I am aware, this method has not been previously applied to Tetrahymena. In preliminary studies we have been able to take immature clones or clones pure for mating type in syngen 1 and induce a small number of pairs with acriflavin plus acetamide. An increased number of pairs is observed if a mating mixture is so treated. KCl appears to inhibit the mating reaction in syngen 1 whereas it stimulates it in clones pure for mating type in syngen 4. We have not, as yet tried to induce an intersyngenic mating, since we feel we need to find conditions which are common for the intrasyngenic matings.

B. ISOZYMES IN SYNGEN 1 OF T. PYRIFORMIS

1. Esterases

The esterase isozymes are resolved by starch gel electrophoresis. Structural genes (E-1 and E-2) have been identified for esterase-1 and esterase-2 (Allen, S.L., 1961, Annals N.Y. Acad. Sci. 94: 753-773). There are two alleles at each of these loci, distributed among the various inbred strains. There is no interaction in the heterozygote, and the heterozygous pattern resembles a mixed extract of the parental genotypes.

Esterase-1 is a propionylesterase, is inhibited by eserine sulfate, is activated by sodium taurocholate, and each homozygote has 5-6 conformers, depending upon the conditions of electrophoresis and the conditions under which the cells are grown (Allen, S.L., 1960, Genetics 45: 1051-1070). The pattern of conformers shifts during the growth cycle. Complicated changes in pattern are observed if the composition of the growth medium is altered. New conformers can be generated *in vitro* by addition of iodoacetamide. One isozyme in each homozygote is present in microsomes, and this form is the first to

appear in logarithmically grown cells. The other isozymes are present in particles sedimenting at lower speeds of centrifugation and are found in older cells. Centrifugation in sucrose density gradients results in their partial separation, and they appear to be associated with membranes of different size. Esterase-1 has been partially purified using a combination of column chromatography and electrophoresis.

The esterase-2 isozymes are butyrylesterases, insensitive to eserine, but are inactivated by p-chloro-mercuribenzoic acid. Little variation is observed in cells of different age or if the cells are grown in various media.

The chemical properties of these esterases are summarized in Tables 1 and 2 in Allen, S.L. (1965) Brookhaven Symp. Biol. 18: 27-51. A reprint is supplied.

Triton X-100 activates certain of these esterases. Its effects are complicated and are discussed in Allen, S.L., J.M. Allen, and B.M. Licht (1965) J. Histochem. Cytochem. 13: 434-440. A reprint is included. The abstract follows:

> Triton X-100, a non-ionic detergent, was incorporated into reaction mixtures used for the visualization of esterases and acid phosphatases separated by electrophoresis in starch gels. Its effects were tested, in combination with 12 different substrates, on enzymes derived from <u>Tetrahymena pyriformis</u> and rat liver. The effects of Triton X-100 were complex. It promoted the solubilization of some substrates, notably the α-naphthyl fatty acid esters. It also altered the color of the enzymatically produced end product. The net effect was apparent enhancement of enzymatic activity with certain substrates and apparent inhibition of enzymatic activity with other substrates. Differential activation and inhibition of some of the electrophoretically resolved enzymes was observed. Both quantitative and electrophoretic studies indicated that Triton X-100 is an activator of certain esterases. A cathodally migrating acid phosphatase of rat liver was activated by Triton X-100 in the presence of naphthol AS, naphthol AS-BI, or naphthol AS-MX phosphates.

The centrifugation and growth cycle studies of esterase-1 appeared in Allen, S.L. (1964) J. Exptl. Zool. 155: 349-370. A reprint is included, and the abstract appears below.

> The esterase isozymes of variety 1 of <u>T</u>. <u>pyriformis</u> can be separated by electrophoresis in starch gels. At maximal resolution there are two groups of six isozymes and these groups are under the control of alleles at the E-1 locus. In this study cell fractions were prepared by differential centrifugation. In each genotype one isozyme (Isozyme 3) appears to be localized to the

microsomes, another may be present in somewhat larger particles, while the remaining isozymes appear in fractions that sediment with low centrifugal forces. Intensive analysis of the isozymes in cells of different age and in cells grown in enriched media suggests that each isozyme has a characteristic time of appearance during logarithmic growth and a characteristic time of peak activity during the growth cycle. Certain isozymes have peak activities during the logarithmic phase and others have peak activities during the stationary phase. Homologous behavior is observed in different genotypes. Since Isozyme 3 is the first to appear during logarithmic growth and is present in microsomes, the molecular form of Isozyme 3 may be close to that of the newly synthesized enzyme. The other isozymes may be derived from this form during their incorporation into cellular structures.

2. Phosphatases

The acid phosphatase isozymes are resolved by electrophoresis in starch gels. The general properties of these phosphatases are discussed in Allen, S.L., M.S. Misch and B.M. Morrison (1963) J. Histochem. Cytochem. 11: 706-719. A reprint is enclosed. The abstract of this paper follows:

The acid phosphatases of variety 1 of <u>Tetrahymena pyriformis</u> can be separated into 17 zones by electrophoresis in starch gels of pH 7.5. All of these acid phosphatases have an optimal pH of about 5.0 and are inhibited by 10 mM sodium fluoride or d-tartaric acid. With one exception, all hydrolyze sodium α-napthyl acid phosphate. Differences between the acid phosphatases are observed in their ability to hydrolyze other substrates using either the coupling technique or a modification of the Gomori-lead method. Inhibition of 2 of the acid phosphatases occurs in the presence of 1 mM of Mn^{++} or Zn^{++}; 0.1 mM of p-chloromercurobenzoic acid inhibits these and others. Variations between the acid phosphatases were observed under different growth conditions and in their distribution in various cell fractions. A major variation in the acid phosphatases that is under genetic control occurs in extracts of different genotypes.

The results suggest that the electrophoretically separated acid phosphatases are a family of enzymes that vary in their degree of relationship. Some are different enzymes. Others are more closely related and represent variations of a single enzyme; i.e., mutant forms produced by different alleles, hybrid forms produced by interaction of alleles, and isozymes produced by a single gene.

Most of the acid phosphatases are probably associated with lysosomes, although one appears to be associated with microsomes.

This acid phosphatase is much more active in synthetically grown cells than in cells grown in proteose-peptone or in bacterized medium. It does not hydrolyze sodium β-glycerophosphate.

The effects of Triton X-100 on the phosphatases are discussed in Allen, S.L., J.M. Allen and B.M. Licht (1965) J. Histochem. Cytochem. 13: 434-440. A reprint is supplied.

A structural gene (P-1) has been identified for phosphatase-1. There are two alleles, and interaction occurs in the heterozygote. As many as three hybrid isozymes may occur in heterozygotes, but variation occurs in different cell lineages. The genetic studies appeared in Allen, S.L., M.S. Misch and B.M. Morrison (1963) Genetics 48: 1635-1658. A reprint is supplied, and the abstract appears below:

Alternative forms of an acid phosphatase (P-1) of variety 1 of *Tetrahymena pyriformis* may be separated by electrophoresis in starch gels. The P-1B enzyme is less heat stable and is inactivated at a lower pH than is the P-1A enzyme; P-1B also forms isozymes. The P-1 phosphatases are controlled by alleles at a single locus or by linked genes. One exceptional hybrid may have arisen as a result of a crossover or a micronuclear mutation: one cell lineage was hybrid in phenotype but bred as if it were a homozygote; another line maintained at a lower temperature bred as if it were a heterozygote.

Variations are observed at five electrophoretic positions in heterozygotes. The pattern is identical immediately following conjugation in all hybrid crosses. Bands 1, 3, and 5 are prominent in the zymograms. Band 3 exhibits temperature and pH stabilities intermediate between P-1B (Band 1) and P-1A (Band 5) in the hybrid. After phenotypic drift, cell lineages with distinctly different phenotypes are formed. Some lines retain the three banded pattern; others show five bands. From each type of cell lineage a different set of subclones may be derived. Besides the stable cell types that are parental in phenotype (P-1A and P-1B), a third stable cell type may occur. This cell type has Band 3 almost exclusively. The observations on phenotypic drift are discussed with reference to changes in expression at a single locus or at two linked loci. A hypothetical molecular model is also discussed.

The chemical properties of phosphatase-1 are discussed in the preceding papers as well as in Allen, S.L. (1965) Brookhaven Symp. Biol. 18: 27-51, and in Allen, S.L. (1968) Annals N.Y. Acad. Sci. (in press). Reprints are enclosed. Phosphatase-1 appears to be a lysosomal enzyme, and it is found when cells are grown in media which induce the formation of food vacuoles. Minor variations in the isozyme pattern can also be induced by the type and composition of the medium.

Perhaps the most interesting aspect of the phosphatase-1 isozymes are their distribution in heterozygous cell lines. There appear to be seven cell types, one which has five isozymes, three which have three isozymes (1,3,5; 1,2,3; and 3,4,5), and three which have a single isozyme (1; 3; and 5). Cells which have a single isozyme are stable (See section D for a discussion of phenotypic diversity in heterozygotes). Clonal analyses on these seven cell types gave indirect evidence for the structure of phosphatase-1 and its pattern of synthesis. These studies suggested that phosphatase-1 is a tetramer; however, it appears to be assembled in two stages. First, dimers are formed; then, tetramers. There appear to be three kinds of dimers (AA, BB and AB). The varying patterns of the different cell types can be explained if cells vary in which dimers are present. The clonal studies are discussed at length in Allen, S.L. (1965) Brookhaven Symp. Biol. 18: 27-51, and in Allen, S.L. (1968) Annals N.Y. Acad. Sci. (in press).

3. Electrophoretic Variants of Mitochondrial Enzymes

Analysis of the electrophoretic pattern in acrylamide gels has shown that at least 10 of 12 mitochondrial enzymes examined in _T. pyriformis_ occur in multiple molecular form. The patterns of four of these enzymes are shown in Figure 2 in Allen, S.L. (1968) Annals N.Y. Acad. Sci. (in press; copy enclosed). Although we have looked very carefully, so far we have _not_ observed variations which have a genetic basis (or else they are too complicated to resolve easily). Most of the variation can be attributed to clonal variation in growth rate. If all clones - of different strains - are in a similar stage of growth (logarithmic growth), similar patterns are observed in the gels. If any one clone is observed as a function of the growth cycle, shifts in the patterns of four different enzymes have been observed. Some of the shifts in pattern definitely affect multiple forms present in the mitochondria, as determined by preparing mitochondrial fractions.

Initially we had great difficulty in achieving repeatability in some of these patterns. The principal sources of variation turned out to be the conditions of growth of the cultures and the method of enzyme extraction.

We spent much time, effort and frustration in trying to separate the isozymes of NAD linked malate dehydrogenase (MDH). MDH is exceedingly active in this organism, and several times we thought we had variations which were strain-specific, only to find that upon repetition of the extraction the original differences disappeared, and other differences in pattern appeared. We prepared mitochondrial fractions and found a variable number of isozymes in a region close to the origin (with disc electrophoresis). This pattern changes upon storage in the cold or with freeze-thawing. I suspect we are dealing with a very unstable enzyme that breaks down easily into a

number of components depending upon pH, the buffer used in extraction, and the conditions of electrophoresis. We examined several systems of electrophoresis. On starch gel electrophoresis (both horizontal and vertical) and with several different buffer systems, MDH from Tetrahymena appears as one long smear, while in the same runs mouse MDH separated nicely into two groups of isozymes. With the Raymond acrylamide system, Tetrahymena MDH was, again, one long smear. With agar gels, multiple sites of activity could be resolved (5-8 zones); however, much of the resolution seemed to depend upon endosmotic flow rather than charge - and altering the molarity of the bed and/or tank buffer had profound effects on the pattern observed. With disc acrylamide electrophoresis, a variable number of sites of activity - often two closely spaced sets - appeared, but, more often, we obtained one horrible smear. When mercaptoethanol or iodoacetamide were added separately during extraction, the activity was not affected and the smeariness decreased. When both were added, a single sharp zone - or two closely spaced zones - were resolved, but enzymatic activity was severely affected.

4. Electrophoretic Variants of a Microbody Enzyme

Studies on α-hydroxy acid oxidase, a microbody enzyme, were carried out by Frances J. Malinoff, a student at Michigan. Multiple forms of this enzyme are observed (See Figure 2 in Allen, S.L., 1968, Annals N.Y. Acad. Sci., in press). The multiple forms vary in number and appearance depending upon the growth cycle, medium and the method of extraction of the enzyme. A substrate profile was run on these multiple forms, and Miss Malinoff found that the greatest number of isozymes appeared with α-valeric acid. An assay system was developed, and a microbody fraction prepared by sucrose density gradient centrifugation. This fraction contained α-hydroxy acid oxidase activity, but, so far, we have not been able to obtain sufficient quantities of this enzyme (with a small rotor) to resolve the multiple forms by electrophoresis and thus determine if all the forms are present in a single fraction.

C. ISOZYMES IN SYNGENS OF <u>T. PYRIFORMIS</u> AND <u>PARAMECIUM AURELIA</u>

The isozymes of the esterases and acid phosphateses of the 12 syngens of <u>T. pyriformis</u> were compared by starch gel electrophoresis in studies carried out by Sharon Koch (Weremiuk), now a student at Michigan. The studies on the esterase isozymes of the 14 syngens of <u>P. aurelia</u> were carried out in collaboration with Bruce C. Byrne and Donald L. Cronkite, students at Indiana University. The results of these studies were reported at the Isozyme Conference, December 8-10, 1967, at Sanibel Island, Florida.

1. *T. pyriformis*

We have examined the propionylesterases (Figures 4,5), butyrylesterases (Figure 6) and acid phosphatases (Figure 7) of representative strains from all 12 syngens, listed in Table 5. Our reference group is syngen 1, and our approach has been to compare the isozymes of the other groups to those which we know about in our reference group--in electrophoretic mobility, enzymatic properties and the ability of these molecules to form hybrids *in vitro*. In all of these studies we have used the same electrophoretic conditions we have found optimal for syngen 1. One esterase, which we call E-3 in syngen 1 (and indicated by a mark beside the gels) appears in most of the other syngens with the same electrophoretic mobility. It splits both α -naphthyl propionate and α -naphthyl butyrate and is eserine insensitive. There appear also to be E-1 esterases in some of the other syngens, namely, syngens 2,3,7,9 and 11 (Figure 4). This statement follows from the observation that they split α -naphthyl propionate and not α -naphthyl butyrate, that they are activated by sodium taurocholate, and that they are inhibited by eserine sulfate. They also seem to have conformers. Currently we are testing the effects of iodoacetamide on some of these enzymes, since it does affect the E-1 esterases in syngen 1. Some of the syngens have little propionylesterase activity (syngens 8,10, and 12) although overnight incubations do raise faint smudges. Figure 5 shows the intrasyngenic variation in what we believe to be the E-1 esterases. Except for the first gel, side by side on the same gels, are compared extracts made from cells grown in 1% proteose-peptone (on the left) and in skimmed milk medium (on the right). With some exception, the activity of the esterases is greater if the cells are grown in skimmed milk, and more conformers appear. Little variation was observed in the patterns of strains within syngens 3 and 9, but in syngen 7, none of the three strains tested had the same pattern. None of the syngens seem to show very much activity with α -naphthyl butyrate (Figure 6). Certain esterases in syngens 2,4,7,9, and 12 appear to be similar to the E-2 esterases in syngen 1, since they split α -naphthyl butyrate and not α -naphthyl propionate, and they are inactivated by p-chloromercuribenzoic acid (PCMB).

There is considerable acid phosphatase activity in all the syngens (Figure 7). The same extracts (skimmed milk) that were used for resolving the esterases were used here. The incubation times had to be cut to one-half hour. Thus, our failure to see esterase activity in some of these extracts does not appear to be due to some technical artifact but represents a real difference between these syngens. There appears to be considerable polymorphism in the acid phosphatases. Some seem to have similar mobilities to ones observed in syngen 1. PCMB seems to inhibit the phosphatases in the other syngens we have examined, such as syngens 4,7 and 9, so we have not as yet obtained any presumptive evidence for the P-1 phosphatases of syngen 1, which are insensitive to PCMB.

TABLE 5

STRAINS IN SYNGENS OF T. PYRIFORMIS

	Strain		Geographic Origin	Code
Syngen 1	#7/V,VI		U.S.A.--Derived	1a
	#8/I		" "	b
	#(7/8)7		" "	c
Syngen 2	UM-3	2/1	Massachusetts	2a
	UM-457	2/II	Oklahoma	b
	H	2/III	Derived	c
	UM-351	2/IV	Michigan	d
	HAM-3	2/IX	Derived?	e
Syngen 3	UM-700	3/I	Mississippi	3a
	UM-705	3/II	"	b
	F_2-666	3/IV	Derived	c
	F_2-665	3/V	"	d
	UM-787	3/VII	Michigan	e
Syngen 4	UM-913	4/I	U.S.A.	4a
	In3T	4/II	India	b
	T Ray	4/III	U.S.A.	c
Syngen 5	R	5/I	U.S.A.	5a
	UM-30	5/II	Massachusetts	b
Syngen 6	UM-1060	6/I	Michigan	6a
	UM-1091	6/II	Florida	b
	UM-1147	6/III	Derived?	c
Syngen 7	UM-1215	7/I	North Carolina	7a
	R	7/II	U.S.A.	b
	UC-651	7/III	California	c
Syngen 8	O	8/II	Derived	8a
	UM-1286	8/III	Minnesota	b
	Alp-1	8/III	Michigan	c
	R	8/IV	U.S.A.	d
Syngen 9	JK	9/I	Panama	9a
	TC-148	9/II	"	b
	TC-89	9/V	"	c
Syngen 10	EN	10/I	England	10a
	EN	10/II	"	b
Syngen 11	AU-1-2x	11/I	Australia	11a
	AU-50-1	11/II	"	b
	AU-94-10	11/III	"	c
Syngen 12	AU-$F_1$2	12/I	Australia	12a
	AU-$F_1$4	12/I	"	b
	AU-3-4	12/II	"	c
	AU-$F_1$1	12/III	"	d
	AU-115-3	12/IV	"	e
	AU-$F_1$3	12/IV	"	f

We have done some preliminary work in attempting to create intersyngenic hybrid enzymes in vitro. For this work we have concentrated our attention on mixed extracts of one strain of syngen 1 (strain 8) and one strain of syngen 4 (T-Ray). We have tried freeze-thawing in salt with and without iodoacetamide. With iodoacetamide, we hoped we might observe some interaction between the E-1 esterases. Our one attempt with freeze-thawing was not successful in affecting the phosphatases. These experiments will be extended, and other approaches examined.

2. P. aurelia

So far, we have examined some 75 stocks among the 14 syngens of P. aurelia (Table 6). A "representative" stock was selected for each syngen, and a comparison made between the syngens for the propionyl-esterases (Figure 8) and for the butyrylesterases (Figure 9). Some esterases are found in all syngens and others are restricted to particular syngens. We can identify some of the syngens by their unique patterns, such as syngen 9. Syngens 4 and 8, which mate but produce inviable F2, have patterns which are more similar to each other than those of other syngens. Syngens 1, 3, and 5 show cross-reactions in mating, and these syngens have rather similar patterns. Some syngens show little intrasyngenic variation--despite the wide geographic origin of the stocks compared. This is true for syngens 1 and 4, although we have observed an occasional stock with a markedly variant pattern. On the other hand, there is extensive intrasyngenic variation in syngen 2, and of the 11 stocks examined 7 different patterns could be detected. What the meaning of this difference in intrasyngenic variation is, is not clear at the moment.

With P. aurelia we have not looked at any of the inhibitors we have used in T. pyriformis so we cannot say how the esterases compare between species complexes. All we can point to in Paramecium is the considerable polymorphism we observe and that we can order some of these polymorphisms with regard to other characteristics which have been examined in Paramecium.

We were unsuccessful in resolving the acid phosphatases in Paramecium by the electrophoretic conditions which work for Tetrahymena. There is phosphatase activity, but it remains at the origin. Obviously, we need to experiment with the technical aspects to find the appropriate conditions for their migration.

D. PHENOTYPIC DIVERSITY IN HETEROZYGOTES OF SYNGEN 1

Phenotypic changes have been observed within heterozygous clones of Tetrahymena. These changes are highly stable and lead to cell lines of diverse phenotype. This phenomenon has been observed for

TABLE 6

STOCKS OF P. AURELIA

	Stocks:	Geographic Origin:
Syngen 1	P	Maryland
	147	Japan
	175	Peru
	220	Hawaii
	257	Mexico
	285	California
	320	Poland
	337	U.S.S.R.
Syngen 2	50	Oregon
	71	Indiana
	72	Texas
	91	Pennsylvania
	149	Florida
	160	Minnesota
	179	Chile
	193	Germany
	206	Norway
	234	Japan
	305	Arizona
Syngen 3	M	Maryland
	79	New Jersey
	152	Connecticut
	261	Quebec
	275	Alaska
Syngen 4	29	Maryland
	32	Maryland
	47	California
	51	Indiana
	126	Florida
	139	Florida
	148	Japan
	163	Pennsylvania
	172	Peru
	173	Chile
	230	Australia
	280	Virginia
	298	Panama
	315	Italy
	316	Holland
	329	Poland

	Stocks:	Geographic Origin:
Syngen 5	63	Indiana
	210	Ohio
	236	Nevada
	311	Australia
Syngen 6	166	India
	309	Thailand
	326	Kenya
Syngen 7	38	Florida
	227	Florida
	253	Florida
	325	Florida
Syngen 8	31	Maryland
	138	Florida
	214	Florida
	276	Texas
	299	Panama
	327	Florida
	330	Georgia
Syngen 9	204	Scotland
	312	Germany
	317	France
	338	U.S.S.R.
Syngen 10	223	Florida
Syngen 11	306	Texas
Syngen 12	270	Florida
	273	Louisiana
	274	Louisiana
Syngen 13	209	France
	238	Madagascar
	321	Mexico
Syngen 14	328	Australia

heterozygotes at the H and T antigen loci and also for heterozygotes at the E-1, E-2 and P-1 enzyme loci. Similar observations were also made with the mating type locus on homozygotes as well as heterozygotes. Thus, this phenomenon affects all the genes that have been studied in this organism. For the heterozygotes, the observations can be simply stated: immediately after the genesis of a heterozygote of hypothetical genotype A^1/A^2 the phenotype associated with both A^1 and A^2 is observed within all cells of the clone. When subclones are initiated and propagated for several hundred fissions, most of these cell lines no longer express both A^1 and A^2, but some lines express the A^1 phenotype and other lines the A^2 phenotype. A few lines still do express both A^1 and A^2 but these are unstable and and give rise to new lines which express only one of the alleles and which are stable. These phenotypic changes appear to be controlled by the macronucleus, since the missing alleles reappear during normal conjugation when the old macronucleus is destroyed. The source of these alleles is, of course, the micronucleus. During genomic exclusion the old macronucleus is retained, and, under these conditions, the phenotypic change persists.

Clonal analyses were performed on heterozygous cells newly generated by conjugation for E-1, E-2 and P-1. In addition, data were obtained on subclones of cell lines which had been propagated for 100, or more, fissions.

1. Analysis of Esterases

Cell lines which were quadruply heterozygous for E-1, E-2, H and mt were followed as a function of fissions. Stable lines appeared by 13 fissions for H but not until 40 fissions for E-1 and E-2. The two stable types for E-1 (or E-2) had the phenotypes of homozygotes and appeared in equal frequencies. The distribution of phenotypes appeared to be independent for unlinked (E-1 vs E-2) as well as for linked loci (mt and E-1).

These studies are discussed more fully in two papers:
Allen, S.L. (1965) Brookhaven Symp. Biol. 18: 27-51.
Allen, S.L. (1968) Annals N.Y. Acad. Sci. (in press).
Reprints are supplied.

2. Analysis of Phosphatases

The same phenomenon was originally reported for the phosphatases in Allen, S.L., M.S. Misch and B.M. Morrison (1963) Genetics 55: 823-837. A reprint is included. The situation is more complicated for the phosphatases, since there is a third stable phenotype (called P_3) in addition to the two types with homozygous phenotypes (P_1 and P_5).

This third type is much rarer in occurrence and has a unique phenotype, which is unlike the heterozygote immediately following conjugation ($P_{1,3,5}$). These observations are discussed in Allen, S.L. (1965) Brookhaven Symp. Biol. 18: 27-51 and Allen, S.L. (1968) Annals N.Y. Acad. Sci. (in press).

In addition to the published observations, three additional experiments on the phosphatases have been completed. These are summarized below:

Experiment I: Analysis of $\underline{P\text{-}1}^A/\underline{P\text{-}1}^B$; $\underline{H}^A/\underline{H}^D$ caryonides from a cross of inbred strains A and B. All 4 caryonides were obtained from 25 pairs. Each caryonide was carried through approximately 117 fissions by serial transfers of single cells at 13 fission intervals (9 transfers). The phosphatases and serotypes were examined after each transfer. Note late appearance and low frequency of stable phosphatases (P_1 and P_5) compared to the serotypes. No stable P_3's appeared. A lag of 52 fissions in the appearance of the stable phosphatase types can be calculated from the expected MIDAC percentages.

Cumulative Frequencies of Caryonides of Various Phosphatase Types and Serotypes at Successive Fission Intervals:

Depressions	Fissions	Expected % Stable MIDAC 1:1	P_1	P_5	$P_{1,3,5}$	$P_{1,2,3,4,5}$	P_3 $P_{1,2,3}$ $P_{3,4,5}$	Ha	Hd	Had
1	0	0			100			2		98
2	13	0.17			100			8		92
3	26	3.85			96	4		18	1	81
4	39	12.10			94	6		41	1	58
5	52	21.98	1		94	5		58	1	41
6	65	31.70	3	2	88	7		67	2	31
7	78	40.57	5	5	84	6		73	5	22
8	91	48.40	8	7	76	9		74	6	20
9	104	55.25	11	10	72	6		76	7	17
10	117	61.16	18	14	61	9	0	77	8	15

Experiment II: Analysis of selected $\underline{P\text{-}1}^A/\underline{P\text{-}1}^B$ caryonides from a cross of inbred strains A and B. 32 sublines were initiated from tubes of "First Depression" (1) cultures from Experiment I, (At the initiation of this experiment tubed cultures were 6 months old and had been fed every other week, or about 5 fissions per month). Each subline was carried through 9 transfers, or approximately 43+117 fissions. The phosphatases of each subline were examined on the initial culture and after the 9th transfer. Note that the frequency of stable types P_1 and P_5 is much higher both in the initial and final cultures than in Experiment I, but that there are no stable P_3's (there are, however, a few $P_{1,2,\underline{3}}$ and $P_{\underline{3},4,5}$). Note that the distributions of types in different caryonides do not differ significantly after 9 transfers.

Initial Cultures

Expt.+ I	Caryonide	P_1	P_5	$P_{1,3,5}$ $P_{\underline{1},3,5}$ $P_{1,3,\underline{5}}$	$P_{\underline{1},2,3,5}$ $P_{\underline{1},2,\underline{3},4,5}$	P_3 $P_{1,2,\underline{3}}$ $P_{\underline{3},4,5}$	Total No.
P_1	54_a	0	0	31	1	0	32
$P_{1,3,5}$	b	1	0	28	3	0	32
$P_{1,3,\underline{5}}$	c	3	0	25	4	0	32
P_1	d	1	0	29	2	0	32
$P_{1,2,3,4,5}$	43d	4	1	23	4	0	32
P_5	44b	1	1	30	0	0	32
P_1	45c	0	1	30	1	0	32
$P_{1,2,3,4,5}$	59c	3	0	26	3	0	32
Total		13	3	222	18	0	256
%		5.1	1.2	86.6	7.2	0	

+ Phenotype of single subline of caryonide after 9 transfers in Exp't I. Basis for selection in Exp't II.

Experiment III: Analysis of $\underline{P-1}^A/\underline{P-1}^B$; $\underline{H}^A/\underline{H}^D$ cell line with phenotype of heterozygote immediately following conjugation ($P_{1,3,5}$). This cell line had, however, undergone 200 fissions. In this experiment part of the population was mated to C* to induce macronuclear retention. Exconjugants which were $\underline{P-1}^A/\underline{P-1}^B$ were selected by their H phenotype and 140 cell lines were initiated. These were carried by 3 additional transfers of single cells at 13 fission intervals. From the untreated part of the population (control series) 140 cell lines were also initiated and carried for 3 additional transfers. The phosphatases were examined in all 4 x 140 lines from each of the two series, and the number of stable cells which were either P_1, P_3 or P_5 were scored.

Purpose of experiment on macronuclear retention: During macronuclear retention, the old macronucleus becomes morphologically smaller and intensely stained. Thus, there was some reason to believe that material *may* be lost, although whether it is DNA, RNA, protein or water, is *not* known. *If* it were DNA, reduction in the DNA might result in a different pattern of phenotypic diversity being observed in the experimental series. If there are subnuclei, then reduction in their number would only be expected to be temporary, since the full complement is restored before division of the macronucleus. No differences in the pattern of phenotypic diversity would be expected. A negative experiment, however, does not prove much, since the reduction in size of the macronucleus might not effect either the subnuclei or the DNA, but other components. The observations were, as follows:

Transfer	Exconjugant Series: P_1	P_5	P_3	Control Series: P_1	P_5	P_3
1	3	0	0	2	0	0
2	7	2	0	6	1	0
3	10	4	0	13	5	0
4	8	8	4	10	7	1

Total Rates of New Stable Types Per Fission (Exconjugant Series + Control Series):

Transfer	Nos. Obs.	Rate Per Transfer	Rate Per Fission(÷13)
1	5/278	.0180	.0014
2	16/273	.0586	.0045
3	32/257	.1245	.0096) .0113
4	38/225	.1688	.0130)

Results: (1) No differences between the experimental and control series were observed. (2) When the data are pooled, the total rates of production of stable types (P_1 + P_3 + P_5) in the first two transfers are lower than expected. However, by the 3rd and 4th transfer the rate observed for other loci (*mt*, *H* and *T*) *is* observed: .0113 per fission. (3) There are differences in the rate and time of appearance of the stable types: P_1, P_5 and P_3 --in this order.

E. PRELIMINARY STUDIES ON DNA HOMOLOGIES IN STRAINS AND CLONES OF T. PYRIFORMIS.

One way in which phenotypic diversity could arise in heterozygotes would be by loss of the DNA representing the unexpressed allele from the macronucleus during fission. This possibility could be examined directly by means of the *in vitro* techniques of nucleic acid hybridization by comparing the DNA homologies of cell lines, which express different phenotypes, but which are derived from heterozygotes between isogenic lines. Provided, of course, that the techniques could be made sensitive enough to detect allelic differences, even at 5 loci. Nothing had been done with these techniques in Tetrahymena, and so the level of discrimination that might be achieved in this organism was not known. Therefore, strains of Tetrahymena which varied in their genetic relationships--and theoretically in their degree of DNA homologies--were tested in the initial experiments.

Comparisons were made of the DNA homologies between syngens, within a syngen and within a homozygous strain. Syngens 1, 9 and 10, which show the widest variation in %G+C (25, 28 and 33%, respectively) were included in the study, and DNA's from representative strains of these syngens were compared. The homologies of DNA's from four homozygous strains (#7, 8, 17 and 21; see Figure 3) were compared as well as DNA from a partially isogenic strain and from clones (which differed in mating type) of one of the homozygous strains. The experiments were carried out in the summer of 1967 in collaboration with Dr. Ian Gibson at the University of East Anglia, England. We found that (1) we could discriminate between the DNA's from different sources; (2) we could show that discrimination parallels the genetic relationship of these strains; and (3) we could discriminate between the DNA's of clones of a homozygous strain.

The results of these experiments were reported at the Fall Meetings of the National Academy of Sciences, October 23-25, 1967, held in Ann Arbor, Michigan. The abstract (Allen, S. and I. Gibson, 1967, Science 158: 523-524) of this report is included in the application. A preliminary draft of a manuscript of this work is included with the reprints.

II. PUBLICATIONS

Allen, S.L., 1963 Genomic exclusion in *Tetrahymena*: Genetic basis. J. Protozool. 10: 413-420.

Allen, S.L., 1964 The esterase isozymes of Tetrahymena: their distribution in isolated cellular components and their behavior during the growth cycle. J. Exptl. Zool. 155: 349-370.

Allen, S.L., 1964 Linkage studies in variety 1 of _Tetrahymena pyriformis_: a first case of linkage in the ciliated protozoa. Genetics 49: 617-627.

Allen, S.L., 1965 Genetic control of enzymes in _Tetrahymena_. Brookhaven Symp. Biol. 18: 27-51.

Allen, S.L., 1967 Genomic exclusion: a rapid means for inducing homozygous diploid lines in _Tetrahymena pyriformis_, syngen 1. Science 155: 575-577.

Allen, S.L., 1967 Cytogenetics of genomic exclusion in Tetrahymena. Genetics 55: 797-822.

Allen, S.L., 1967 Chemical genetics of protozoa. In _Chemical Zoology_, Vol. I., M. Florkin and B. Scheer, Editors, Academic Press, N.Y. (pp?; published November 1967; copy not received yet.)

Allen, S.L., 1968. Genetic and epigenetic control of several isozymic systems in _Tetrahymena_. Annals N.Y. Acad. Sci. (in press).

Allen, S. and I. Gibson, 1967 Genetic homologies and drift within populations of DNA molecules. Science 158: 523-524 (Abstract).

Allen, S.L., J.M. Allen, and B.M. Licht, 1965 Effects of Triton X-100 upon the activity of some electrophoretically separated acid phosphatases and esterases. J. Histochem. Cytochem. 13: 434-440.

Allen, S.L., S.K. File and S.L. Koch, 1967 Genomic exclusion in Tetrahymena. Genetics 55: 823-837.

Allen, S.L., M.S. Misch and B.M. Morrison, 1963 Variation in the electrophoretically separated acid phosphatases of _Tetrahymena_. J. Histochem. Cytochem. 11: 706-719.

Allen, S.L., M.S. Misch and B.M. Morrison, 1963 Genetic control of an acid phosphatase in Tetrahymena: formation of a hybrid enzyme. Genetics 48: 1635-1658.

Manuscripts

Allen, S.L. Acid phosphatases in heterozygotes of Tetrahymena. To be submitted to J. Exptl. Zool.

Allen, S.L. and I. Gibson. Genetic homologies and drift within populations of DNA molecules. To be submitted to J. Mol. Biol. or Biochem. Genetics.

Allen, S.L. and I. Gibson. Genetics of Tetrahymena, Chap. 14, in Biology of Tetrahymena, A.M. Elliott, Editor, Appleton, Century and Crofts, Inc., New York.

Allen, S.L. and S.L. Weremiuk. Defective micronuclei and genomic exclusion in selected C* subclones. To be submitted to J. Protozool.

Allen, S.L. and S.L. Weremiuk. Selective mating in genomic exclusion? To be submitted to J. Protozool.

III. STAFFING

Sally Lyman Allen, Research Associate, 1963-1966, 100%
Research Assoc. and Visiting Prof. of Genetics, 1966-1967, 58.3%
Research Assoc. and Assoc. Prof. of Botany, 1967-1968, 62.5%

Assistant Research Zoologists:

Barbara Morrison Licht	1963 - 1964(Sept.)	100%
Sharon K. File	1964 - 1965(Sept.)	100%
La Donna Fleming	1965(July-Oct.)	100%
Sharon L. Koch (Weremiuk)	1965 - 1967	100%
Patricia H.T. Lee	1967(Dec.)--	100%

IV. OTHER CONTRIBUTIONS

Direction of projects performed by students:

1) Frances J. Malinoff, 1965-1967, Investigations of α-hydroxy acid oxidase in *Tetrahymena pyriformis*.

2) Bruce C. Byrne and Donald Cronkite, Winter 1967 at Indiana University, Investigations of the esterases in the syngens of *Paramecium aurelia*.

3) Sharon L. Weremiuk, 1967-1968, Investigations of the esterases and phosphatases in the syngens of *Tetrahymena pyriformis*.

Figure

1 Scoring Phenotypes in Tetrahymena.

2 Derivation of Homozygous Strains.

3 Relationship of Strains 7, 8, 17, and 21.

4 Intersyngenic Variation in Propionylesterases of _T. pyriformis_. Code letters at bottom of gels refer to strains listed in Table 5. Mark to left and right of set of gels shows position of E-3. Origin is at "0". Cathode is at top, anode at bottom of photograph.

5 Intrasyngenic Variation in Propiopylesterases of _T. pyriformis_. Code letters at bottom of gels refer to strains listed in Table 5. In all but gel at extreme left, peptone (P) extracts were inserted on left side, skimmed milk (SK) extracts on right side of gel. Mark beside gels indicates E-3. Note migration of syngen 1 isozymes is slightly greater than those of other syngens. Origin is at "0". Cathode is at top, anode at bottom of photograph.

6 Intersyngenic Variation in Butyrylesterases of _T. pyriformis_. Code letters at bottom of gels refer to strains listed in Table 5. Mark to left and right of set of gels shows position of E-3. Origin is at "0". Cathode is at top, anode at bottom of photograph.

7 Intersyngenic Variation in Acid Phosphatases of _T. pyriformis_. Code letters at bottom of gels refer to strains listed in Table 5. Origin is at "0". Cathode is at top, anode at bottom of photograph.

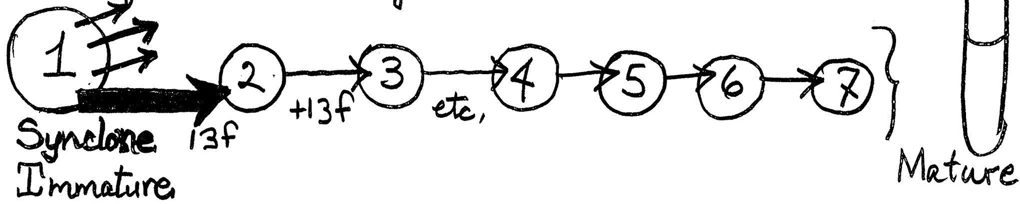
8 Intersyngenic Variation in Propionylesterases of _P. aurelia_. Order of stocks (from left to right): P, 72, 261, 51, 236, 166, 227, 138, 312, 223, 306, 273, 238, 328, 147, 126. Cathode is at top, anode at bottom of photograph.

9 Intersyngenic Variation in Butyrylesterases of _P. aurelia_. Order of stocks (from left to right): P, 72, 261, 51, 236, 166, 227, 138, 312, 223, 306, 273, 238, 328, 147, 126. Cathode is at _bottom_, anode is at _top_ of photograph. (These are slices from the bottom portion of gels shown in Figure 8).

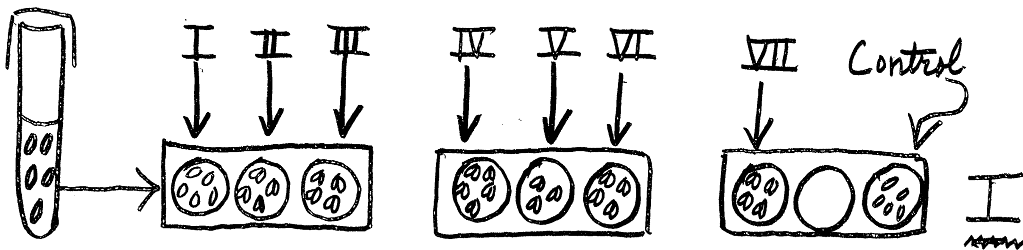
SCORING PHENOTYPES IN TETRAHYMENA

A. Mating Types (We use 23°C)

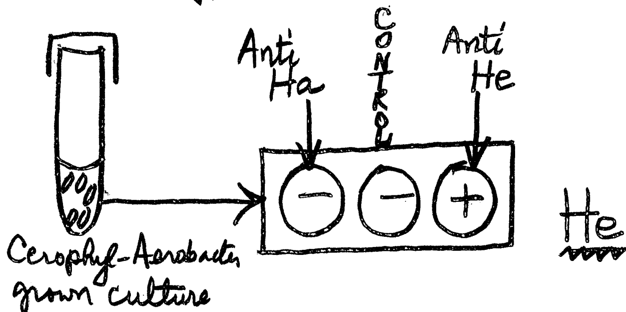
1. Transfers to maturity



2. Mating Type Tests

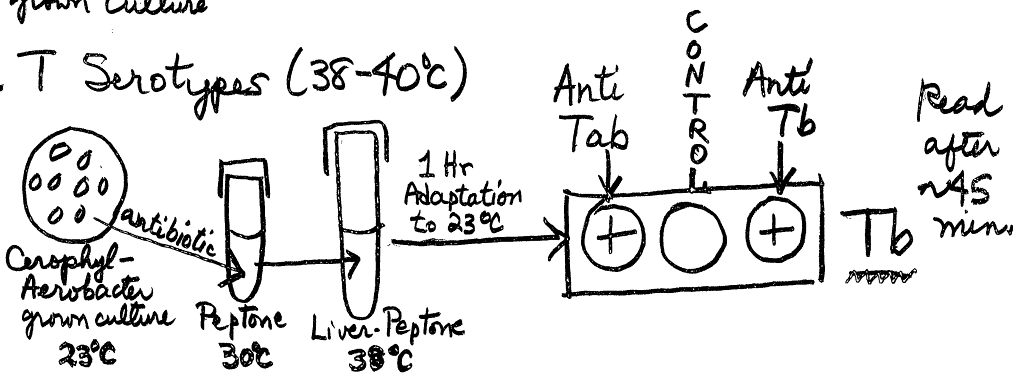


B. H Serotypes (23-26°C)



Titer of antiserum adjusted so that tests are read after about 45 minutes

C. T Serotypes (38-40°C)

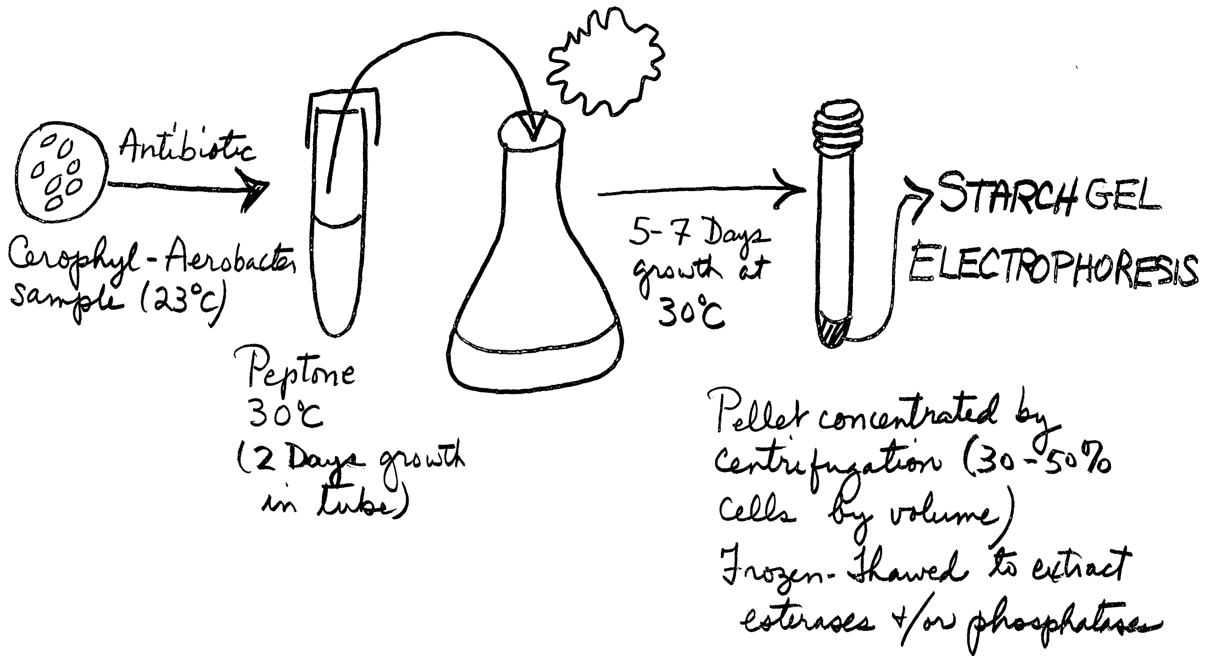


Read after ~45 min.

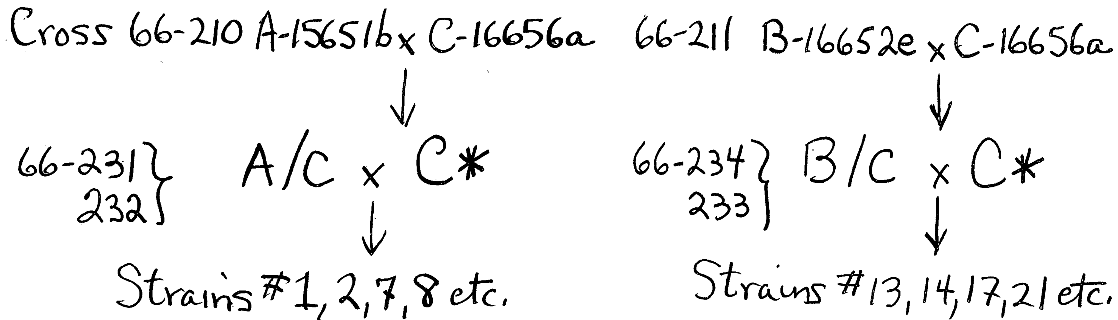
FIGURE 1

FIGURE 1 (Continued)

D. Esterase-1, Esterase-2, and Phosphatase-1



Derivation of Homozygous Strains



Sequence of Isolations

Single pairs are isolated into depressions 1, 2, 3, etc. These go through Round 1 of Genomic Exclusion, come apart and give rise to a population of exconjugants which remate after 3-4 days. These Round 2 pairs are separately isolated into depressions A, B, C, etc.

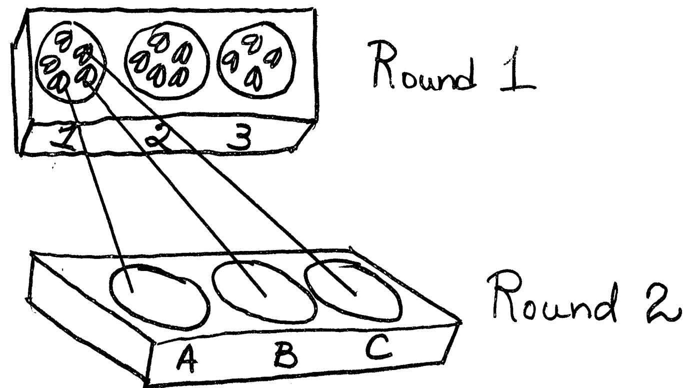
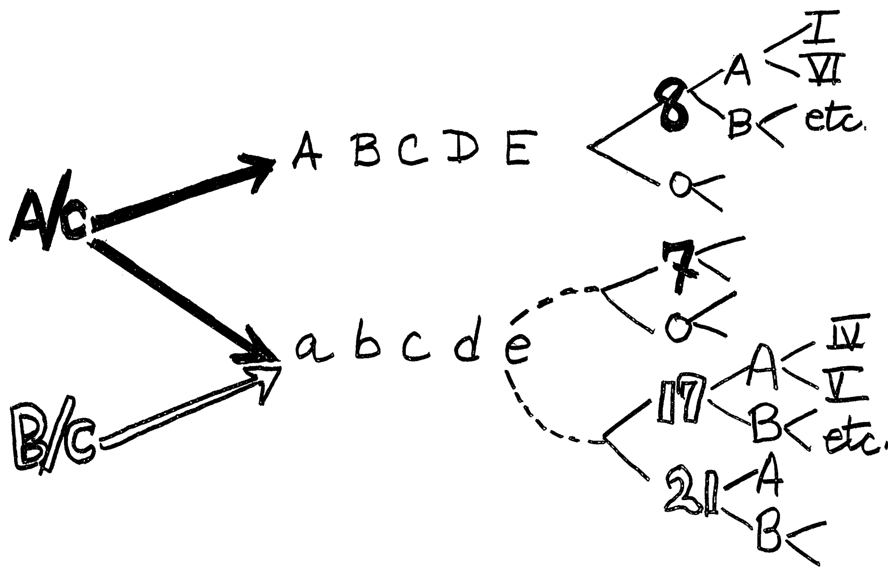


Figure 2



Strain	mt	H	T	E-1	E-2	P-1
7	A	E	A	B	C	B
8	C	A	B	C	B	A
17	B	E	A	B	C	B
21	B	E	A	B	C	B.

Homozygous for allele shown

FIGURE 3

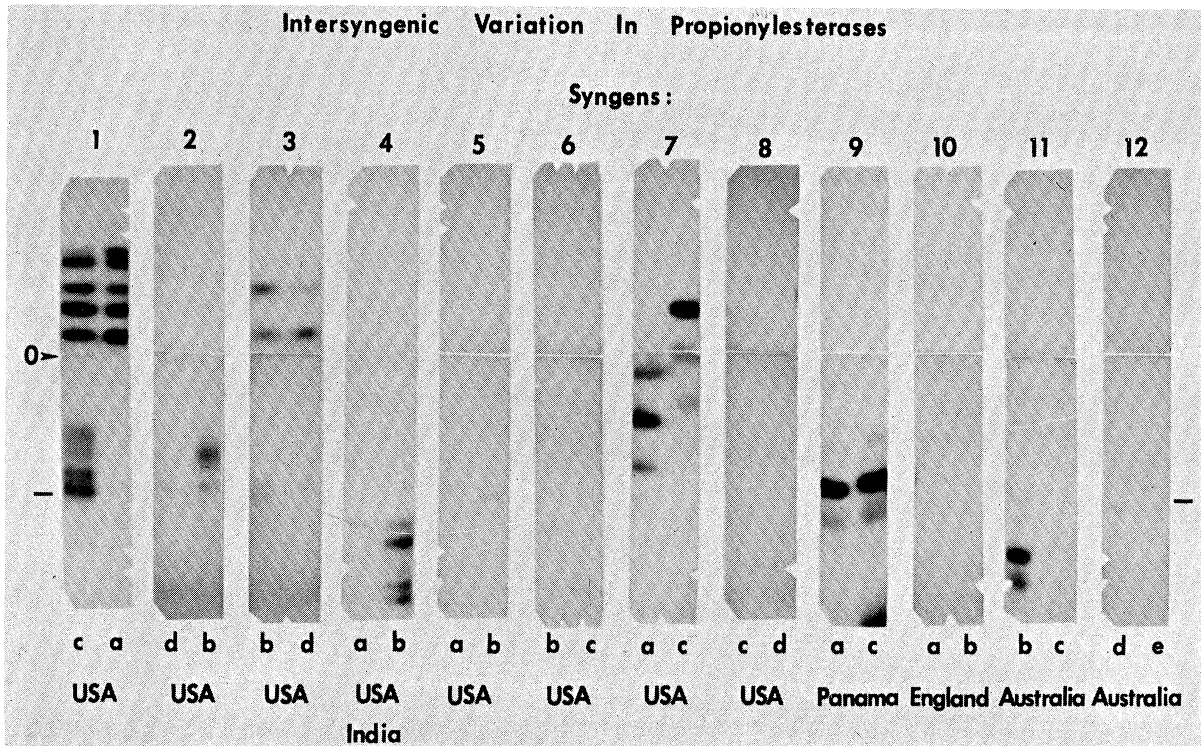


Figure 4

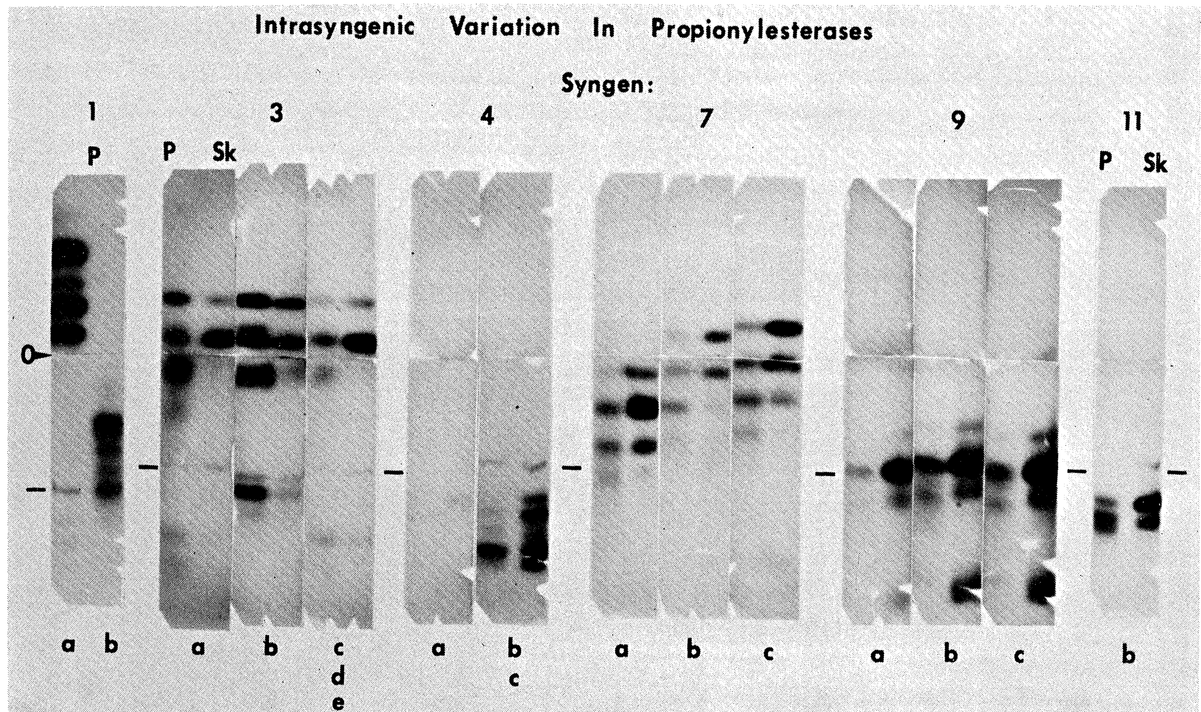


Figure 5

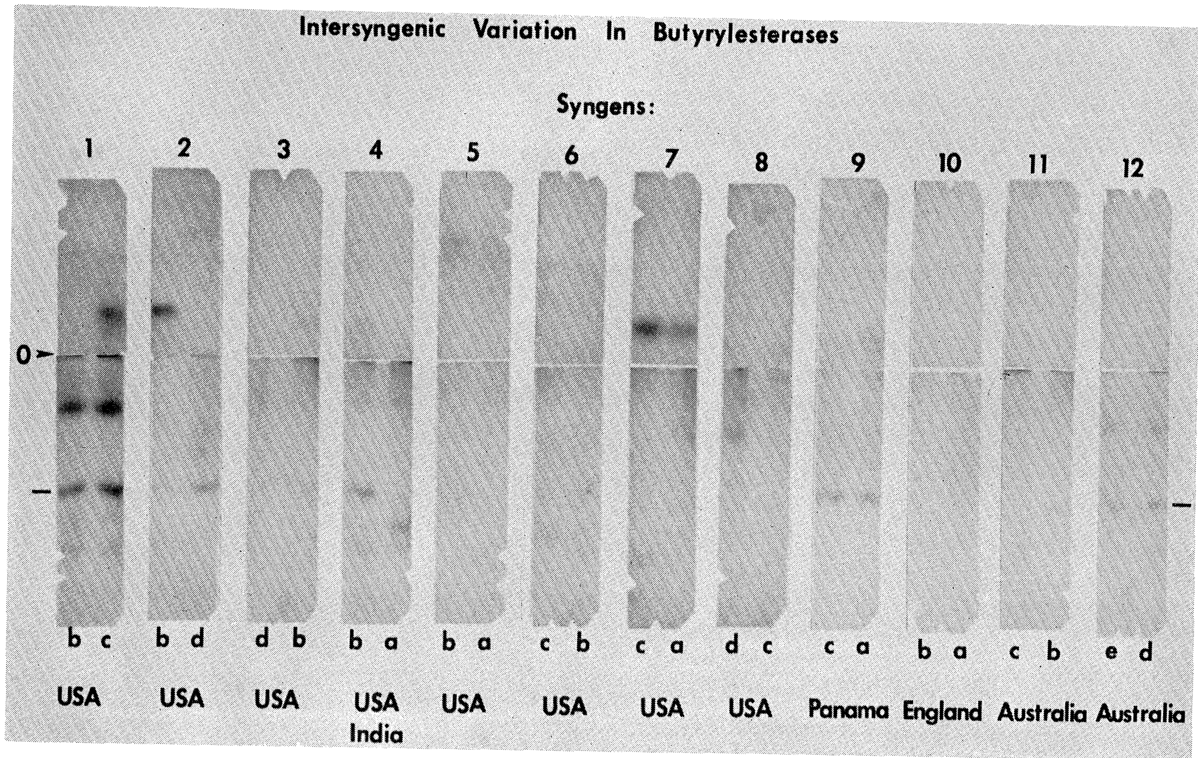


Figure 6

Intersyngenic Variation In Acid Phosphatases

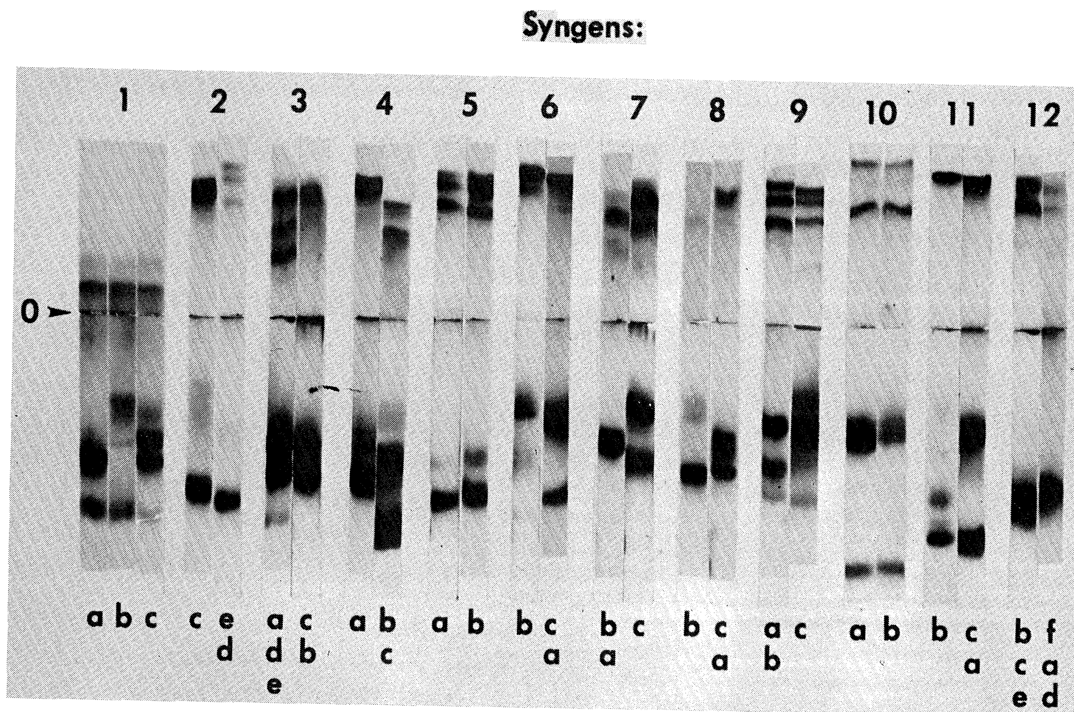


Figure 7

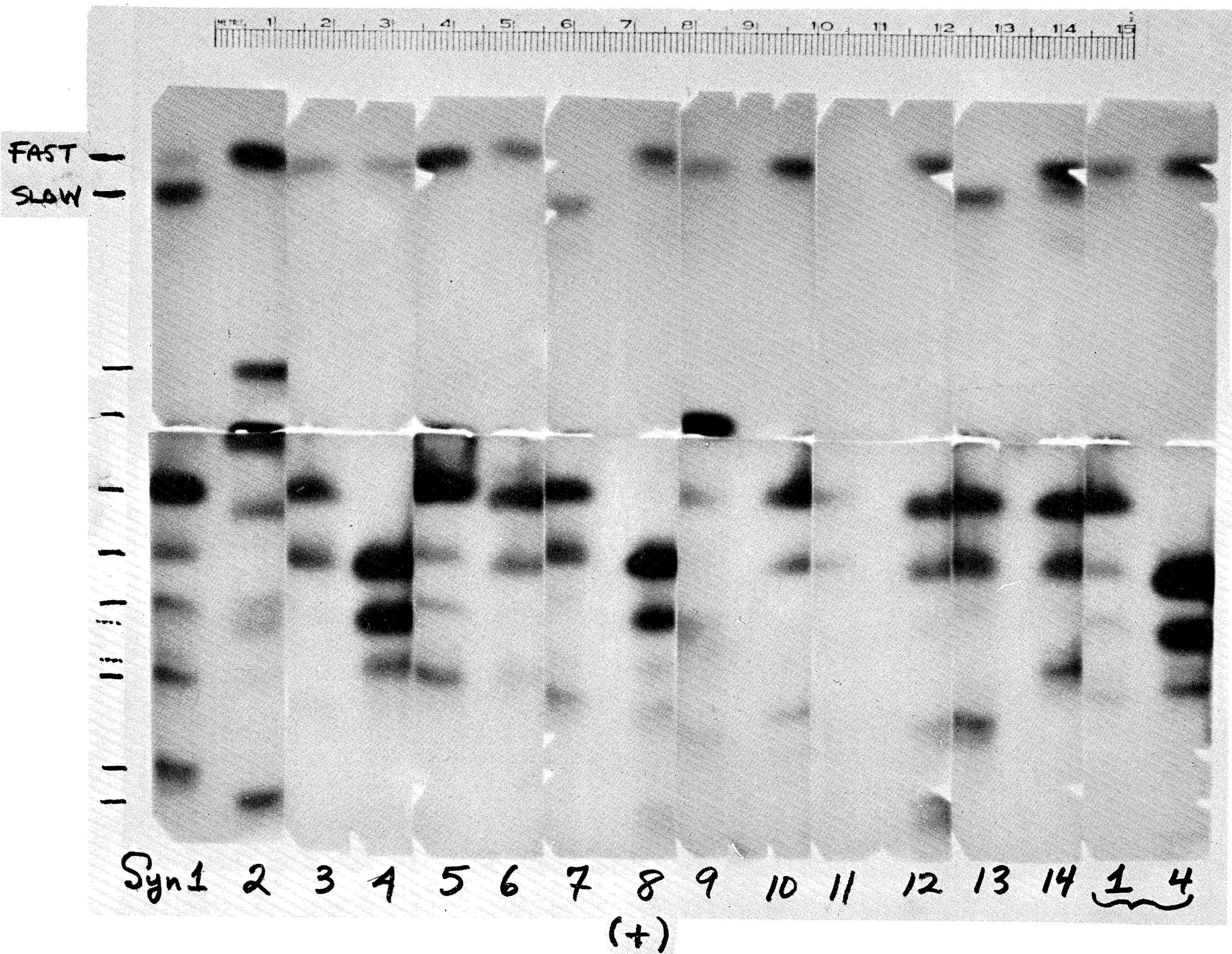


Figure 8

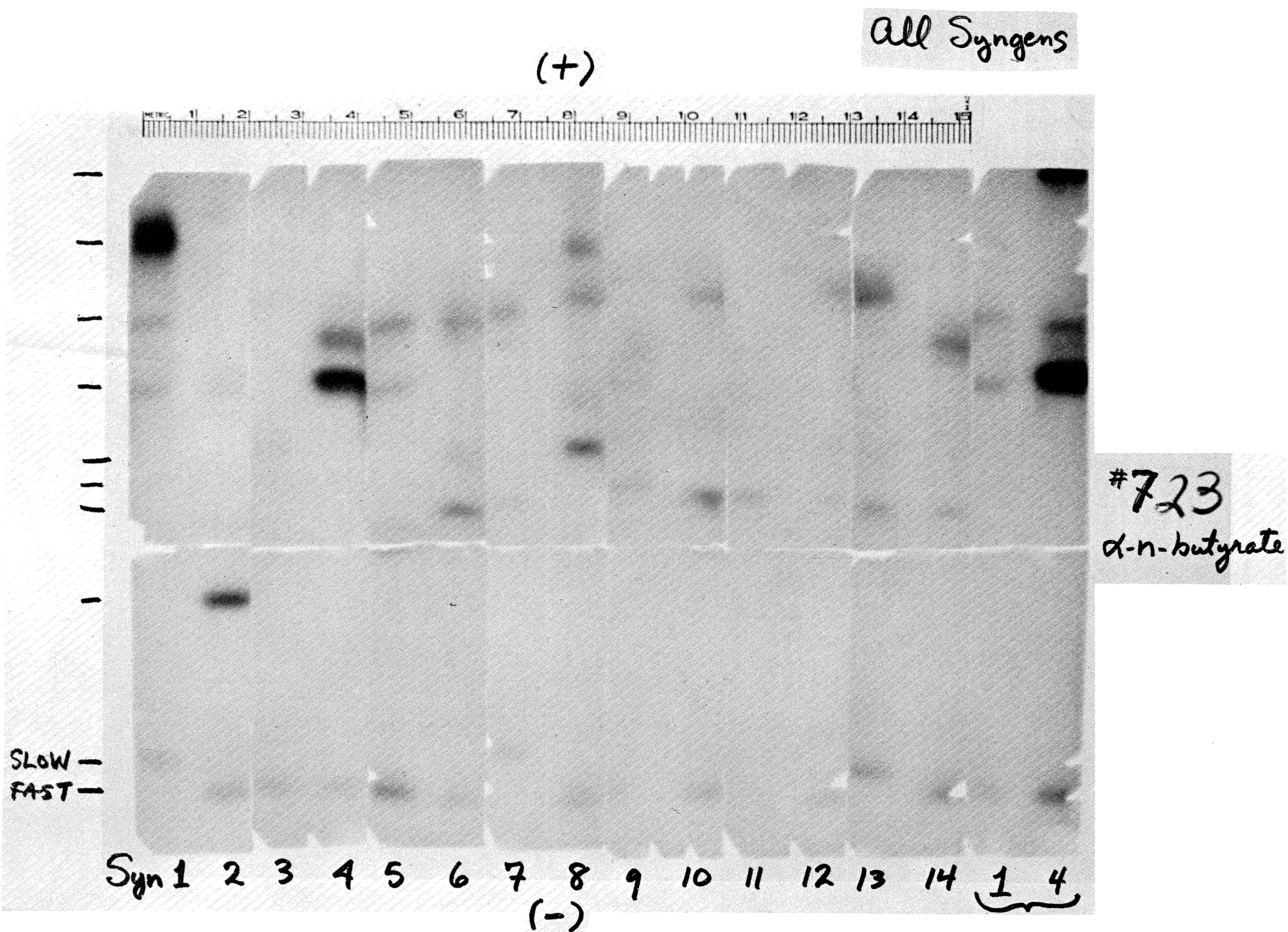


Figure 9

UNIVERSITY OF MICHIGAN
3 9015 02493 7958