GENETIC CONTROL OF AN ACID PHOSPHATASE IN TETRAHYMENA: FORMATION OF A HYBRID ENZYME[1]

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MANY examples of multiple molecular forms of an enzyme are now known (WRÓBLEWSKI 1961). These multiple forms, designated isozymes by MARKERT and MØLLER (1959), vary in number and charge as a reflection of differences between species, tissues, and the state of differentiation. They also differ in their degree of molecular relationship: some have identical substrate, inhibitor and cofactor requirements; others vary in one or more of these parameters. A complex hierarchy of structural relationships probably exists, much in the same manner as the hierarchy of taxonomic relationships between various organisms.

The relationships between multiple molecular forms seem to reflect their genetic relationships. Some multiple forms arise under the influence of single genes—such as the E-1 esterases of *Tetrahymena pyriformis* (ALLEN 1961a) or the alkaline phosphatases of *Escherichia coli* (BACH, SIGNER, LEVINTHAL, and SIZER 1961; LEVINTHAL, SIGNER, and FETHEROLF 1962). Others arise under the joint action of alleles—such as the hybrid esterases of corn (SCHWARZ 1962), or under the joint influence of nonalleles—such as the hybrid lactate dehydrogenases (APPELLA and MARKERT 1961; MARKERT 1961; CAHN, KAPLAN, LEVINE, and ZWILLING 1962). Finally, some arise as a result of the independent action of nonalleles, and are members of a family of enzymes. The relationship of the E-1 and E-2 esterases of *T. pyriformis* is such an example (ALLEN 1961a).

CAHN, KAPLAN, LEVINE, and ZWILLING (1962) suggest that a distinction should be made between hybrid enzymes and isozymes. In this paper, the term isozyme will be reserved for multiple forms that arise under the influence of a single gene. The term hybrid enzyme will be used for multiple forms that are the product of interaction between alleles or nonalleles.

Several acid phosphatases can be separated electrophoretically in starch gels from extracts of various genotypes of *T. pyriformis* (ALLEN, MISCH, and MORRISON 1963). Since these phosphatases can be distinguished genetically, or by their chemical properties, their behavior under different growth conditions, or their intracellular localization, the acid phosphatases appear to be a family of enzymes with different degrees of relationship. Some are different enzymes. Others are more closely related and represent variations of a single enzyme. In this report,

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we shall describe our results with one of these acid phosphatases, the P-1 phosphatases. Some of the variations are mutant forms of P-1, others are hybrid forms produced by gene interaction, and some are isozymes produced by a single gene.

Preliminary work on the chemical characterization of the P-1 phosphatases and the results of a breeding analysis will be reported. A study of the hybrid pattern during phenotypic drift offers some clues as to possible structural relationships of these phosphatases. A hypothetical molecular model will be examined.

MATERIALS AND METHODS

Inbred strains: All nine inbred strains of variety 1 of *T. pyriformis* (A, A1, B, B1, C, D, E and F) were screened for their acid phosphatases. Strains A, B and C, now in their 12th or 13th generation of inbreeding, were used for the genetic analysis. The origins of these strains have been previously described (ALLEN 1960; NANNEY 1959, 1963a; NANNEY, REEVE, NAGEL and DEPINTO 1963).

Crosses: Crosses were made in the bacterized medium (Cerophyl rye grass inoculated with *Aerobacter aerogenes*) at 23°C. Pairs were separately isolated into depression slides, exconjugants being separated in some crosses. When the medium was exhausted (ca. 13 fissions), half of each culture was tubed up in sufficient medium to permit approximately two 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. The serotypes of the progeny of certain crosses were tested using samples of these tubed cultures and the method of NANNEY and DUBERT (1960).

Massive transfers (ca. 5000 cells) were made of each tubed culture through antibiotics (streptomycin and penicillin) and into 1 percent proteose-peptone in test tubes. After two to three days of growth at 30°C, these tubed cultures were dumped into 250 ml Erlenmeyer flasks with 100 ml of proteose-peptone. Whole cell extracts were prepared after five days of growth at 30°C.

A sample of each of the tubed cultures left over in bacterized medium was placed in a depression slide and three or more single cells were isolated into separate depressions. Each subline was carried to maturity by serial transfers of single cells at 13 fission intervals. After approximately 95 fissions the mature cultures were tubed up in bacterized medium and mating type tests were performed. Of the three sublines derived from the same pair, only those that differed in mating type were retained. From most pairs at least two sublines of different mating type were obtained by these procedures. The probability is high that these sublines originated from different caryonides (NANNEY 1963a). Some, however, may be sub-caryonides.

After 120 fissions samples of these sublines were transferred to proteose-peptone through antibiotics. Whole cell extracts of flask cultures were prepared.

Extracts: For routine assays of the phosphatases, whole cell extracts were prepared in sterile glass-distilled water (see ALLEN, MISCH, and MORRISON 1963). In some experiments, extracts were made in 0.1 M Michaelis Veronal buffers of

different pH. In other experiments, samples of the water extracts were heated at various temperatures for 30 minutes.

Electrophoresis: For routine assay of the phosphatases, starch gels were prepared in 30 millimolar boric acid-Tris buffer at pH 7.5 [29 mM boric acid and 1 mM Tris(hydroxymethyl)aminomethane] using commercially prepared hydrolyzed starch (Connaught Medical Research Laboratories), and poured into lucite molds measuring 23 mm wide, 7 mm deep and 22.5 cm long (internal dimensions). Details of this procedure are given elsewhere (ALLEN, MISCH, and MORRISON 1963). In these experiments, two Whatman #1 filter papers, each measuring 10 mm × 7 mm and saturated with different extracts, were inserted in the starch gel, leaving a gap of 3 mm between the extracts. Electrophoresis was carried out in the horizontal position using 8 to 9 v/cm for five hours at room temperature (23 to 26°C). A 0.3 M boric acid-Tris buffer containing 0.246 M boric acid and 0.054 M Tris(hydroxymethyl)aminomethane (pH 7.5) was used in the end trays. In one series of experiments boric acid-Tris buffers or boric acid-NaOH buffers in the pH range 7.0–8.5 were used during the electrophoretic separation of the phosphatases.

Incubation of starch strips: After electrophoresis each gel was removed from its mold and cut horizontally so that a slice 3 mm thick was obtained (see ALLEN, MISCH, and MORRISON 1963, for details). From the 3 mm slice of starch a 9 to 10 mm wide strip was carefully cut from each of the two edges, and the middle piece was discarded. The strips were lubricated with distilled water and slipped into test tubes, containing the incubation mixture, always with the origin oriented to the bottom of the test tube. The cathodal and anodal strips were placed in separate test tubes. The incubation mixture contained 100 mg of sodium alpha-naphthyl acid phosphate (4 mM; Dajac Laboratories), 100 mg of the diazonium salt of Fast Garnet GBC (4-amino-3:1′-dimethyl azobenzene; Dupont De Nemours and Company), and 100 ml of 50 millimolar acetate buffer at pH 5.0. The mixture was made up immediately prior to use and was filtered through Reeve Angel #202 filter paper. The filtrate was poured into the test tubes, and the starch strips were incubated therein for one hour at room temperature. After incubation, the starch strips were washed in several changes of distilled water for one hour or longer and stored in water in the refrigerator until photographed.

Photography: The starch strips were photographed by transillumination using Royal Pan film (Eastman Kodak Company).

RESULTS

The P-1 Acid Phosphatases: The acid phosphatases of cells derived from different inbred strains can be separated into a total of 17–18 bands in starch gels of pH 7.5 (ALLEN, MISCH, and MORRISON 1963). All of these acid phosphatases have an optimal pH of about 5.0 and are inhibited by sodium fluoride or d-tartaric acid. They vary in their ability to hydrolyze various substrates and in their inhibition by Mn^{++}, Zn^{++}, or p-chloromercurobenzoic acid (PCMB). Variations between the phosphatases occur under different growth conditions and in their

distribution in various cell fractions. A major variation divides the inbred strains into two groups.

Extracts of strains A, A1, B1, D and D1 possess an intense band (Band 5) that migrates to about 4.5 cm toward the anode after 5 hours at 8 to 9 v/cm. (Figure 1a). Extracts of strains B, C, E and F do not possess this band; instead, they have three isozymes (Bands 1, 2 and 3) that migrate to about 2.5, 3.0 and 3.5 cm toward the anode in decreasing order of intensity (Figure 1b). These bands are usually weaker than Band 5 in extracts of similar protein concentration. The reason for this is that Bands 1, 2 and 3 are probably being slowly inactivated by the pH (7.5) of the boric acid-Tris buffer used during electrophoresis (see below). Mixed extracts of cells from strains A and B or strains A and C possess Bands 1 and 5 (Figure 1c). Bands 2 and 3 usually are not visible in zymograms of a mixed extract unless the mixture contains at least three times the number of B cells relative to A. Extracts of heterozygotes (A × B; A × C), when screened at about 15 fissions after conjugation, have a unique pattern (Figure 1d): Bands 3 and 5 are intense, Band 1 is somewhat less intense, and Bands 2 and 4 are barely visible. Thus, the P-1 phosphatases are separable into five bands (1, 2, 3, 4 and 5) at pH 7.5. Photographs of the P-1 phosphatases in different genotypes appear in Figure 2.

The P-1 phosphatases are resistant to 0.1 mM PCMB, they hydrolyze sodium alpha-naphthyl acid phosphate and sodium beta-glycerophosphate rapidly, glucose-6-phosphate somewhat less rapidly, and a series of naphthol AS compounds more slowly (ALLEN, MISCH, and MORRISON 1963). They appear to be associated with large particles (lysosomes?) and are more active in old cultures. They are active in cells grown in protease-peptone and bacterized medium and are considerably less active in cells grown in synthetic medium.

In a series of experiments, the P-1 phosphatases were separated electrophoretically using buffers of different pH. At pH 7.5 and below, Bands 1, 3 and 5

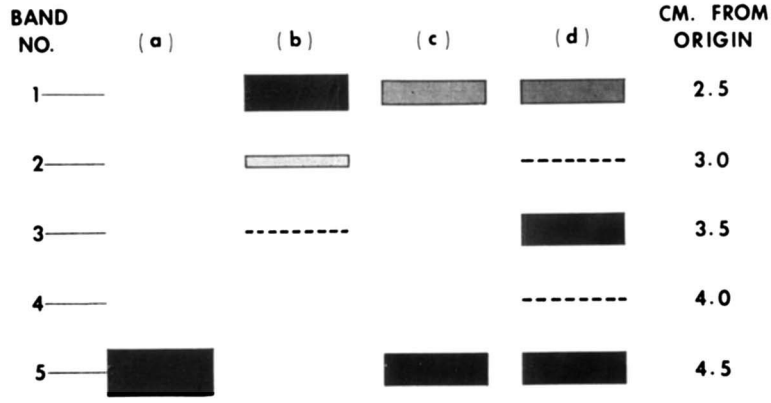

FIGURE 1.—P-1 acid phosphatases in extracts of (a) A strain, (b) B strain, (c) a mixture of A- and B-strain cells, and (d) F_1 (A × B). The distances from the origin toward the anode are indicated.

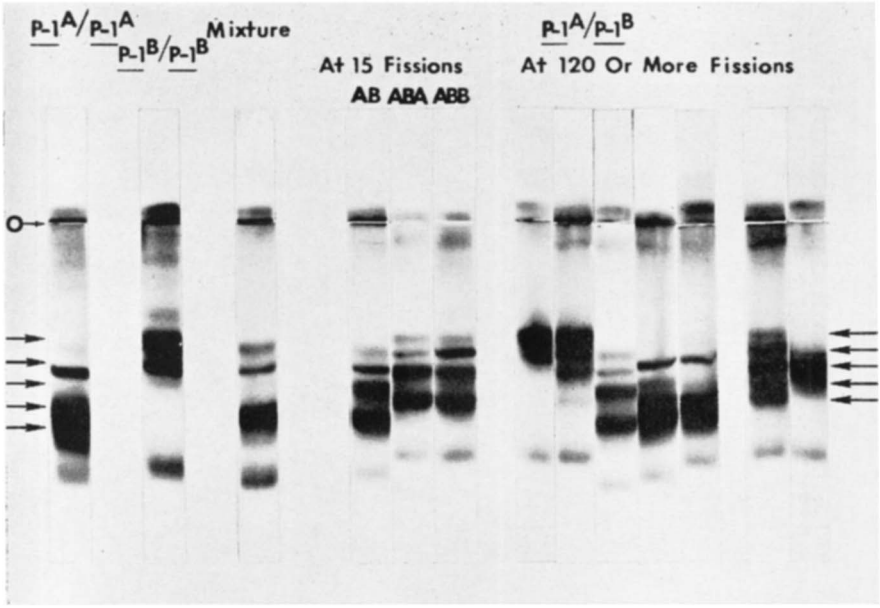

FIGURE 2.—Zymograms of the acid phosphatases in three *P-1* genotypes. The P-1 phosphatases are indicated by arrows. The electrophoretic separations were carried out in starch gels of pH 7.5. The gels were incubated in sodium alpha-naphthyl acid phosphate and Fast Garnet GBC at pH 5.0 for 1 hour. In all the photographs the cathode is at the top, the anode is at the bottom, and the origin is indicated by a "O".

migrate in parallel, the set of bands moving from the anode towards the cathode (Figure 3). At pH 7.1, Band 1 migrates a few millimeters toward the cathode. At pH 7.0, Band 3 migrates only a few millimeters toward the anode. The distances that these bands move at pH's between 7.0 and 7.5 were measured. On the basis of these measurements, we calculate that Band 3 would disappear into the origin around pH 6.9 and Band 5 around pH 6.7. At pH 7.0–7.2, A-strain zymograms possess additional faint P-1 bands (Bands 6 and 7?). Thus, it is possible that isozymes are formed in A extracts under conditions other than those usually used.

At pH's higher than 7.5, the P-1 phosphatases are progressively "inactivated." Band 1 is much less intense when separated at pH 7.6; at pH 7.8 and above, it is missing from the zymograms (Figure 3). Band 3 is less intense at pH 7.7 and even more so at pH 7.8; at pH 8.0, it is barely visible. All the P-1 bands are missing from the zymograms when they are separated at pH 8.5. Since we were concerned that the inactivation might be effected by inhibition by Tris rather than the higher pH, some starch gels were prepared with boric acid-NaOH buffers. With minor differences, comparable results were obtained in the absence of Tris.

This suggested that the P-1 phosphatases might be sensitive to slightly alkaline pH's. In the routine preparative procedure, extracts were made in glass distilled

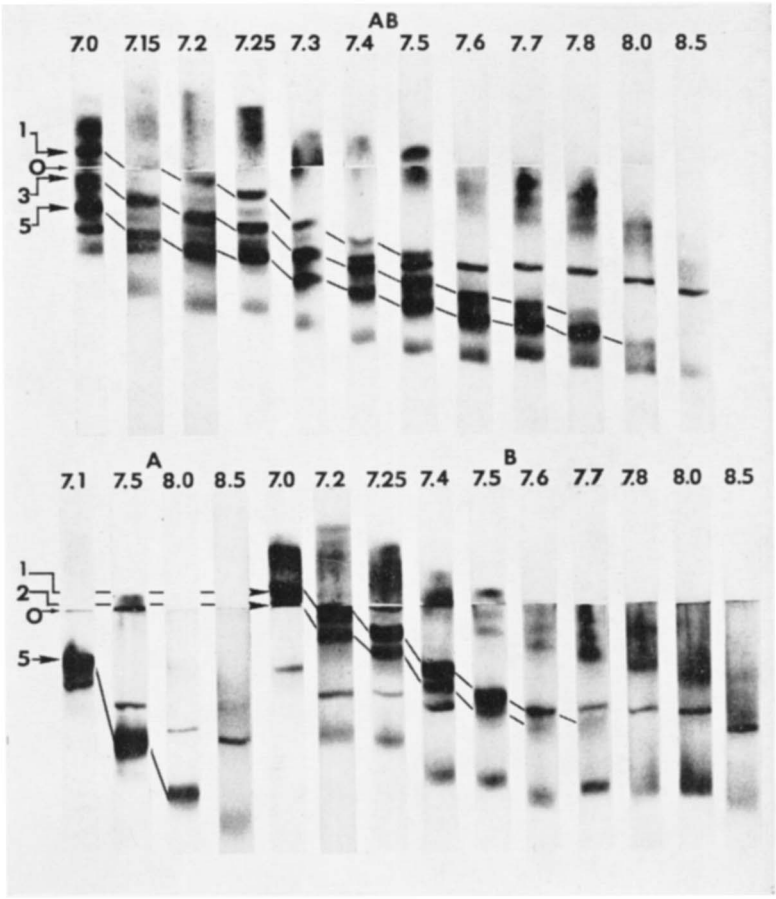

FIGURE 3.—Migration of P-1 phosphatases in gels of various pH: AB zymograms at top, A and B zymograms at bottom. The separations were carried out in starch gels of pH 7.0 to 8.5, as indicated. The P-1 phosphatases are marked.

water. The pH of these water extracts was 6.4 to 6.6. Therefore, a series of experiments was initiated in which whole cell extracts were prepared in 0.1 M Michaelis Veronal buffers of varying pH. To 1.0 ml of buffer, 0.3 ml of packed cells was added. The pH was read and adjusted by adding known volumes of dilute HCl or NaOH. The total volumes were adjusted to 1.5 ml with the addition of water. Extracts were prepared of A-strain and of B-strain cells. Electrophoresis was carried out in starch gels of pH 7.4.

A typical experiment is illustrated in Figure 4. In extracts of A- or B-strain cells prepared at pH 3.0, the P-1 phosphatases are virtually absent from the zymograms. They are present in the zymograms of the pH 4.0, 5.0, and 6.0 extracts. At pH 7.0 they are less active, and at pH 8.0 they are almost missing from the zymograms of strain B. In strain A, the P-1 phosphatase is present in the pH 7.0 extract, less active in the pH 8.0 extract and missing from the zymograms

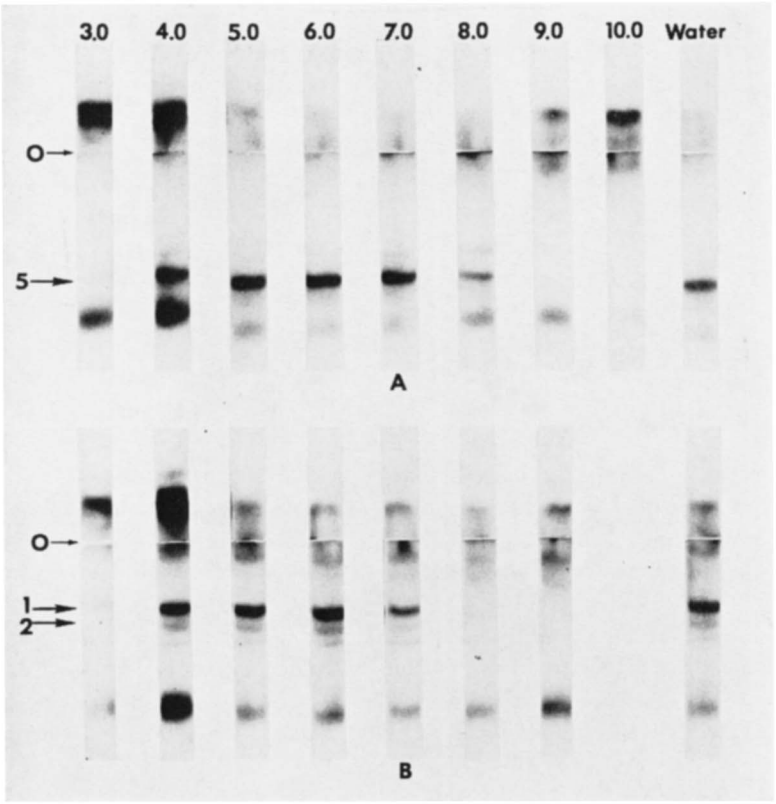

FIGURE 4.—Zymograms of extracts prepared at various pH's: A, at top; B, at bottom. The separations were carried out in starch gels of pH 7.4. The P-1 phosphatases are indicated by arrows.

of the pH 9.0 and pH 10.0 extracts. This experiment suggests that these phosphatases are tolerant of a pH range from 4.0 to almost neutrality. Above pH 7.0 they are progressively inactivated. Inactivation of P-1 in B-strain extracts occurs at a lower pH (7.0–8.0) than in the A-strain extracts (pH 8.0–9.0). The pattern of inactivation is under investigation at more closely spaced pH intervals. Experiments are also in progress to determine if the inactivation can be reversed. So far, we have achieved partial restoration of enzymatic activity of pH 3.0 and pH 8.0 extracts (B strain) by dialysis against buffers of pH 5.0 or 6.0.

The P-1 acid phosphatases are stable if stored in the deep freeze. Even after a year, and with repeated thawing and refreezing, no deterioration or changes in electrophoretic pattern have been noted. They are not sensitive to room temperature (23 to 26°C) when exposed, sometimes repeatedly, for ½- to 1-hour periods—or for as long as one 24-hour period.

The P-1 phosphatases are fairly heat stable. They survive a 30 minute exposure to 38°C. Band 1 is slightly inactivated at 40°C and is destroyed at 42°C. Band 3 is stable at 40°C and is almost completely inactivated at 45°C. Band 5 is stable at

42°C, slightly inactivated at 45 to 48°C, and is destroyed at 50°C. These experiments suggest that each band has a distinct temperature at which it is inactivated: 40 to 42°C for Band 1 and 45 to 50°C for Band 5. Band 3 appears to have an intermediate temperature stability. In extracts of cell lines containing only Band 3 (P-1$\overline{AB}$), Band 3 is inactivated at 45 to 48°C.

Since Band 5 survives a higher temperature and a higher pH than Band 1, the tertiary structure of the enzyme migrating as Band 5 is probably tighter than that of the Band 1 enzyme. This difference in structure is borne out by the fact that the B-strain enzyme exists in multiple form (i.e., as isozymes), while the A-strain enzyme usually does not. It is also interesting that Band 3 possesses temperature and pH stabilities (inactivation occurs in starch gels of about pH 7.8) intermediate to Bands 1 and 5. This is especially interesting in view of the possibility that in heterozygotes Band 3 may be a hybrid enzyme.

Inheritance: For the genetic analysis strain A possessing Band 5, or P-1A in phenotype, was selected as representative of one of the groups of inbred strains. Strains B and C, possessing Bands 1, 2 and 3, or P-1B in phenotype, were selected as representative of the second group. All the progeny of an A × A cross were P-1A, while all the progeny of B × B or C × C crosses were P-1B in phenotype (Table 1).

Crosses between strains A and B or between strains A and C and their hybrid generations are shown in Table 1. Exconjugants from several F_1 and F_2 pairs were separated, and in all cases examined exconjugants from the same pair were alike in phenotype. Each entry in Table 1 represents an observation on a pair culture. All the F_1 pair cultures were P-1AB in phenotype; that is, they possessed the pattern shown in Figure 1d. In the F_2, three types of pair cultures were ob-

TABLE 1

Inheritance of the P-1 acid phosphatases

Crosses	Distribution of pairs				Expected ratio			
	P-1A	P-1AB	P-1B	Total	One locus		Two loci	
1. A × A	20	0	0	20	1:0:0	P > .99‡	1:0:0	P > .99
2. B × B	0	0	20	20				
C × C	0	0	18	18				
	0	0	38	38	0:0:1	P > .99	0:0:1	P > .99
3. A × B	0	18	0	18				
A × C	0	10	0	10				
	0	28	0	28	0:1:0	P > .99	0:1:0	P > .99
4. AB × AB*	11	19	10	40				
AC × AC	13	25	12	50				
	24	44	22	90	1:2:1	P = .95	1:3:1	P = .05–.1
5. AB × A	10	11	0	21	1:1:0	P = .9	1:1:0	P = .9
6. AB × B†	0	12	8	20	0:1:1	P = .5	0:1:1	P = .5

* For additional crosses of heterozygotes, see Table 2.
† For additional crosses of heterozygotes × B, see Table 3.
‡ P-value from chi-square.

served: 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. Several P-1A or P-1B F_2 or backcross pairs, presumed to be homozygotes, were selfed. All of these pairs gave rise to progeny that were P-1A or P-1B, depending upon the parent. Thus, recombinant lines are formed that breed as if they were homozygotes.

Two genetic explanations for these data will be considered. 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 separate loci. If a single locus is involved, the P-1A phenotype would arise in cells homozygous for a *P-1*^{A} allele, the P-1B phenotype in cells homozygous for a *P-1*^{B} allele, and the P-1AB phenotype in heterozygotes. Thus, a 1:2:1 ratio would be expected in the F_2 generation and 1:1 ratios in the backcrosses. The data fit this hypothesis well.

On the other hand, if genes at separate loci are involved, the P-1A phenotype 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. If these two loci are unlinked, then 6.3 percent of the F_2 pairs should die from this cause alone. The observed lethality of the F_2 was even greater—16.8 percent in the F_2 (A × B) and 52.4 percent in the F_2 (A × C). Assuming no linkage, the viable F_2 classes that would be expected under this hypothesis would be 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 data in Table 1 are not incompatible with this hypothesis.

Two types of tests were made in order to differentiate between control exerted at a single locus and at two loci. First, additional F_2 were raised so that a statistically significant distinction could be made between a 1:2:1 and a 1:3:1 ratio. Backcross hybrids (ABA or ABB) that had been classified as heterozygotes were used in these crosses. They were also selected on the basis of their final phenotype after phenotypic drift had occurred. We made two crosses of ABA heterozygotes (Crosses *2* and *4*, Table 2) and two crosses of ABB heterozygotes (Crosses *1* and

TABLE 2

Crosses of heterozygotes differing in phenotype

	Source of heterozygote	Pair nos.	Phenotypes of parents	Distribution of pairs P-1A	P-1AB	P-1B	Total
1.	ABB	1 × 1	P-1A × P-1A	3	7	6	16
2.	ABA	2 × 3	P-1A × P-1A	5	7	4	16
				8	14	10	32
3.	ABB	1 × 4	P-1B × P-1B	3	9	4	16
4.	ABA	3 × 2	P-1B × P-1B	6	7	3	16
				9	16	7	32
			Totals	17	30	17	64

3, Table 2). Crosses *1* and *2* utilized heterozygotes that were P-1A in phenotype, while Crosses *3* and *4* involved heterozygotes that were P-1B in phenotype. The results of these crosses suggest that differences in genetic background or in final phenotype of the heterozygote do not influence the types of progeny observed in this type of cross. The total data—17 P-1A, 30 P-1AB and 17 P-1B, may be added to the F_2 data obtained in Cross *4*, Table 1. This yields a new F_2 distribution of 41 P-1A, 74 P-1AB and 39 P-1B. This distribution fits a 1:2:1 ratio ($P = .9$) and does not fit a 1:3:1 ratio ($P < .01$). The observed values thus eliminate the hypothesis that *unlinked* loci control the P-1 phosphatases.

A second type of test was devised to differentiate between one locus and two locus control of P-1. This involved testing the genotypes of the backcross heterozygotes. This is a much more sensitive test and gave us the opportunity of investigating the possibility that separate, but loosely linked, loci were involved in determining the P-1 phenotypes. If separate loci controlled P-1, two genotypes would be present among the backcross heterozygotes. For example, ABA heterozygotes would be either *AABb* or *AaBb* in genotype. These could be distinguished by crossing them 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 should be genetically identical ($P\text{-}1^A/P\text{-}1^B$) and, when crossed to the B strain ($P\text{-}1^B/P\text{-}1^B$) all heterozygotes would produce P-1AB and P-1B pairs.

Eleven ABA heterozygotes were selected. Six of these were heterozygous for their H serotypes (i.e. H^A/H^D); five were homozygous for H^A. The ABA heterozygotes were crossed to the B strain (H^D/H^D) and tests were made of the phosphatases and serotypes of a sample of the resulting pair cultures. The inclusion of a second marker (H) was helpful in ascertaining that no aberrant pairs were included in the significant P-1B group. Occasionally pairs indulge in genomic exclusion rather than true conjugation in variety 1 of *T. pyriformis* (ALLEN 1963; NANNEY 1963a). Thus, if genomic exclusion of the ABA mate's genes occurred in a mating to the B strain, P-1B progeny would result that could only be Hd.

Ten of the ABA heterozygotes gave rise to some P-1AB and to some P-1B pairs when crossed to the B strain (Table 3). Actually, the P-1B pairs were slightly in excess, the departure from a 1:1 ratio bordering on significance ($P = .05$–$.1$). From each cross at least one P-1B pair that was Had was observed, indicating that genes had been transmitted from the ABA parent.

The eleventh ABA heterozygote, ABA-6d, which was H^A/H^A, proved to be an exception. This heterozygote was P-1$\overline{\text{AB}}$ (almost P-1A) in phenotype (see Figure 7 for a diagram of the various P-1 phenotypes found in heterozygotes; see Figure 5 for photographs of the phenotypes of ABA-6d). When crossed to the B strain, all 33 progeny were P-1AB (Table 4). The viability of this cross was 82.5 percent. Three different subclones of ABA-6d that were P-1$\overline{\text{AB}}$ in phenotype (Figure 5) were selected. When crossed to strain B, ten pairs from each cross were P-1AB (Table 4). The viability of these crosses was 100 percent.

TABLE 3

Results of crosses of ABA heterozygotes with B strain

Heterozygote		Distribution of pairs	
clone	phenotype	P-1AB	P-1B*
ABA-6a	P-1A	2	3
3b	P-1A	1	4
2b	P-1A	2	2
ABA-2d	P-1$\underline{\text{A}}$B	2	3
6b	P-1$\overline{\text{AB}}$	3	6
6c	P-1$\overline{\text{AB}}$	4	6
2e	P-1A$\underline{\text{B}}$	3	2
ABA-3	P-1B	2	8
2	P-1B	8	2
3d	P-1B	2	8
		29	44

* Some or all of the P-1B pair cultures of each cross were serotype Had.

These results suggested that ABA-6d had been formed as a result of a crossover between two linked genes controlling the P-1 phosphatases. Its genotype should, therefore, be *AABb*. An additional test was made by crossing ABA-6d to a heterozygote, known not to be a crossover by its behavior in a cross to the B strain; that is, it was *AaBb* in genotype. A cross of *AABb* × *AaBb* should give rise to 1 P-1A : 3 P-1AB. Instead of a 1:3 ratio, a 1:1 ratio of pairs was observed (Table 4). The viability of this cross was 100 percent, suggesting that a lethal factor was *not* involved. Thus, ABA-6d behaved as if it were genetically

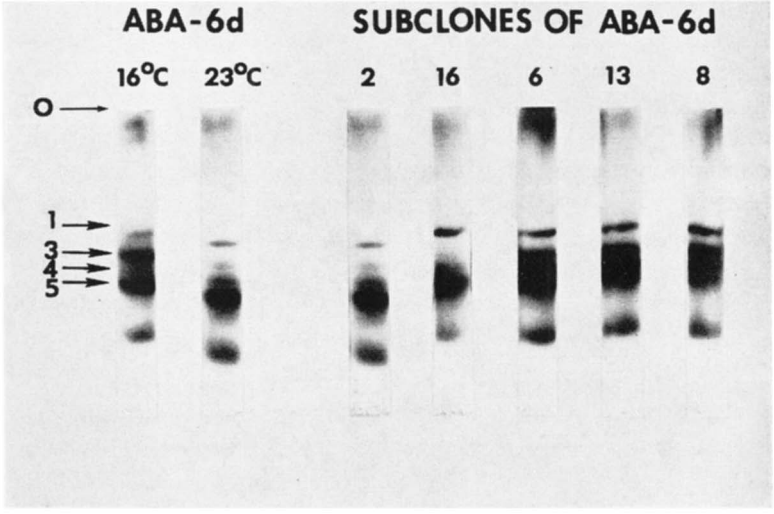

FIGURE 5.—Zymograms of ABA-6d: at left, those of the 16° and 23°C lines; at right, those of five subclones (2, 16, 6, 13 and 8) derived from the 23°C line. The separations were carried out in starch gels of pH 7.5. The P-1 phosphatases are marked by arrows.

P-1^{A}/*P-1*^{A} or *AAbb*—instead of a crossover. ABA-6d-8 and ABA-6d-6 were crossed to two different A strain cultures. Twenty progeny tested as P-1A. This is the result expected of a cross of two *P-1*^{A} homozygotes.

A duplicate culture of ABA-6d had been maintained at 16°C. Its phenotype was P-1AB (Figure 5). When crossed to the B strain, six pairs were P-1AB and four pairs were P-1B. Thus, the 23° and 16°C lines of ABA-6d did not behave as if they were genetically alike. Either the 23°C culture is really strain A by contamination with a culture that was also mating type VI; or, environmental conditions have in some unknown manner changed the genetic potential of ABA-6d. Tests of the mating type genotype of the 23°C line showed that it was *mt*^{A}/*mt*^{B}. Therefore, this line does, indeed, possess some B-strain genes and it cannot have originated by contamination with an A-strain culture.

We are still faced with the observation of subclonal variation in phenotype of ABA-6d. Out of 28 isolates of ABA-6d, 19 were P-1A, 4 were P-1AB, and 5 were P-1AB (see Figure 5). Is the phenotype of subclones ABA-6d-6, -8 and -13 really P-1AB, or is it a modified form of P-1A? We have never observed isozymic variation among homozygotes in either the inbred strains or in recombinant lines, although we have looked for this type of variation. Further subcloning of ABA-6d-6, -8, or -13 is contemplated. Perhaps by repeated rounds of phenotypic selection for heterozygosity, a P-1B subclone could be extracted. If such selection were possible, it would suggest that mutation may have occurred in the *micro*nucleus of the 23°C line of ABA-6d.

At present the distinction between a single locus and two linked loci has not been made. Only 11 ABA heterozygotes and two ABB heterozygotes have been tested genetically (for ABB, see Table 2, Pairs 1 and 4). Thus, the data presented here and elsewhere (ALLEN 1963) are compatible with either type of control. Both hypotheses will be considered in the next section, which deals with phenotypic drift. Until proved otherwise, the single locus hypothesis must be given preference. Accordingly, we would assign the genotypes *P-1*^{A}/*P-1*^{A} and *P-1*^{B}/*P-1*^{B} to the two homozygotes, responsible for the P-1A and P-1B phenotypes, respectively, and *P-1*^{A}/*P-1*^{B} to the heterozygote with the P-1AB phenotype. The alternative hypothesis—linked loci—would lead to the genotypic assignments of *AAbb*, *aaBB*, and *A–B–* to the above phenotypic classes.

Phenotypic drift in the heterozygote: In the routine assay of the products of a cross, the pair cultures were screened approximately 15 fissions after conjugation had occurred. At this time the phenotype of the heterozygote shows very little variation, regardless of genetic background or of the phenotypes of the parents used in the cross (Figure 2, *P-1*^{A}/*P-1*^{B} at 15 fissions). All extracts possess Bands 1, 3 and 5 in a pattern similar to Figure 1d. Strong extracts of a heterozygote also possess Bands 2 and 4, weak in intensity and intermediate in mobility. These intermediate bands are much stronger in extracts of some sublines. This gives a total of *five* electrophoretic positions in starch gels for the P-1 acid phosphatases of heterozygotes.

After 120 fissions had occurred, a number of the sublines from heterozygous

TABLE 4

Crosses of ABA-6d (P-1$\overline{AB}$; Ha)

Cross	Percent viable (no. pairs)	Distribution of pairs P-1A	P-1AB	P-1B	Expected if ABA-6d is: $P\text{-}1^A/P\text{-}1^B$	*AABb*	$P\text{-}1^A/P\text{-}1^A$	Distribution of pairs Ha	Had	Hd	Expected if cross is:
23°C Line:											
ABA-6d × B	82.5(85)	0	33	0				0	33	0	
ABA-6d-6 × B	100.0(30)	0	10	0				0	10	0	
ABA-6d-8 × B	100.0(30)	0	10	0				0	10	0	
ABA-6d-13 × B	100.0(30)	0	10	0				0	10	0	
		0	63	0	0:1:1	0:1:0	0:1:0	0	63	0	$H^A/H^A \times H^D/H^D$
ABA-6d × ABA-2d	100.0(60)	27	33	0	1:2:1	1:3:0	1:1:0	31	29	0	$H^A/H^A \times H^A/H^D$
ABA-6d-8 × A	93.4(60)	10	0	0				10	0	0	
ABA-6d-6 × A	85.3(54)	10	0	0				10	0	0	
		20	0	0	1:1:0	1:1:0	1:0:0	20	0	0	$H^A/H^A \times H^A/H^A$
16°C Line:											
ABA-6d × B	100.0(30)	0	6	4	0:1:1	0:1:0	0:1:0	0	10	0	$H^A/H^A \times H^D/H^D$

pairs were transferred to proteose-peptone and tested for their phosphatases. In contrast to the lack of variation in the pattern observed at 15 fissions, at 120 fissions the heterozygote is capable of expressing one of a spectrum of phenotypes. Ordered variations in intensity of all five bands are observed (Figure 2, *P-1^A/P-1^B* at 120 or more fissions). Some sublines are parental in phenotype (P-1A or P-1B); others are P-1AB and others are intermediate, P-1$\underline{\overline{AB}}$ and P-1A$\underline{\overline{B}}$. The phenotypic distribution of *P-1^A/P-1^B* sublines derived from three types of crosses (F_1 or AB and the two backcrosses, ABA and ABB) at 120 fissions is shown in Table 5. The frequency of sublines that were parental in phenotype was 14.3 percent in the F_1 and 35.0 percent and 35.7 percent in the two backcrosses.

The stability of these phenotypically distinct cell lines varies. Subclones derived from cell lines that are P-1A in phenotype are all P-1A. Those derived from P-1B cell lines are all P-1B in phenotype, and the isozymic pattern does not depart from the "standard" P-1B pattern (Figure 1b). Cell lines that are hybrid or intermediate in phenotype are not stable. If single cells are isolated from such lines and the subclones are immediately assayed, a much narrower distribution of phenotypes is observed. A P-1AB cell line gives rise to P-1AB, P-$\underline{\overline{AB}}$ and P-1A$\underline{\overline{B}}$ subclones. A P-1$\underline{\overline{AB}}$ cell line gives rise to P-1AB, P-1$\underline{\overline{AB}}$ and P-1A subclones, while a P-1A$\underline{\overline{B}}$ cell line gives rise to P-1AB, P-1A$\underline{\overline{B}}$ and P-1B subclones.

These observations suggest that the P-1AB heterozygote undergoes phenotypic drift, the extent of drift being a function of the number of fissions that have taken place 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 and DUBERT 1960), *E-1* and *E-2* (ALLEN 1961a). A macronuclear basis has been established for the H variations (NANNEY, REEVE, NAGEL, and DEPINTO 1963). In this case, phenotypic drift seems to arise as a consequence of changes that occur in the composition of the macronucleus. The macronucleus is hypothesized to contain 45 diploid subnuclei (ALLEN and NANNEY 1958). In *H* heterozygotes interallelic interactions occurring in separate subnuclei give rise to a heterogeneous macronucleus (NANNEY 1963b). 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. Such cell lines are highly stable.

TABLE 5

Distribution of P-1^A/P-1^B *sublines at 120 fissions*

Cross	No. of sublines with P-1 phenotypes					Total	Percent parental phenotypes
	A	$\underline{\overline{AB}}$	AB	A$\underline{\overline{B}}$	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

* $\overline{AB}$ in phenotypes.

The changes that took place in the macronucleus of cells heterozygous for *H* were found to be removed once conjugation occurred; this was also true for *E-1*. If phenotypic drift has a macronuclear basis, this result would be expected since the macronucleus disappears during conjugation and is replaced by products of the syncaryon. Crosses of heterozygotes, both of which were P-1A, or both of which were P-1B, resulted in normal F_2 distributions (Table 2). In addition, similar distributions of P-1AB and P-1B pairs were obtained when heterozygotes of different phenotype were crossed to the B strain (Table 3). Thus, the phenotypic changes that occur in P-1 heterozygotes also do not survive after conjugation. This observation is our strongest evidence that phenotypic drift in P-1 heterozygotes has a macronuclear basis. However, without adequate kinetic data and cell lineage studies, this basis cannot be established unequivocally.

Most observations on phenotypic drift of a heterozygote have involved traits that have been interpreted in terms of single locus control (NANNEY 1963b). According to this hypothesis genic interaction in $P\text{-}1^A/P\text{-}1^B$ cells would result in two possible states for each subnucleus: it could express either $P\text{-}1^A$ or $P\text{-}1^B$. If the interaction occurs independently for each of the subnuclei, then some subnuclei would express $P\text{-}1^A$ and others would express $P\text{-}1^B$. After assortment only two stable cell types would be expected.

If, instead, drift involved a heterozygote for traits that were controlled by linked genes, *Ab/aB*, the genic interactions occurring in the subnuclei might be more complex and lead to additional stable cell types. Four states would be expected if genic interaction occurred independently for A and for B: $\frac{Ab}{aB} \longrightarrow \frac{Ax}{xB} \quad \frac{Ab}{xx} \quad \frac{xx}{aB} \quad \frac{xb}{ax}$. If the last type, *xb/ax*, were lethal, then three viable stable cell types would be expected after phenotypic drift: P-1AB, P-1A and P-1B.

The additional complexities offered by the second hypothesis may be relevant in understanding certain features of phenotypic drift peculiar to the phosphatases. In addition to variations that could be interpreted as arising from differences in gene dosage—of $P\text{-}1^A$ versus $P\text{-}1^B$ under the single locus hypothesis—variations were also noted in the extent to which the intermediate Bands, 2 and 4, were present. Some of the P-1AB sublines from each type of cross examined possessed five bands of nearly equal intensity, although Band 3 was often relatively more intense than the other bands. Such cell lines will be referred to as P-1$\overline{\text{AB}}$ in phenotype. A comparison of the P-1AB and P-1$\overline{\text{AB}}$ phenotypes is illustrated in Figure 6.

Three P-1AB and three P-1$\overline{\text{AB}}$ sublines were selected from two AB, two ABA and two ABB heterozygotes. At the time the clonal analysis was initiated the ABA and ABB lines had completed approximately 175 fissions, the AB lines, 200 fissions. Thirty subclones were initiated from each of the six lines. Each subclone was separately maintained for 120 fissions by serial transfers of single cells at 13-fission intervals (or nine transfers). Then, 20 of the subclones were tubed up, and samples transferred to proteose-peptone, and whole cell extracts were made.

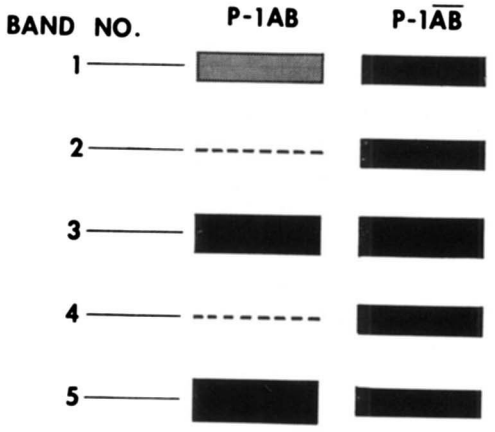

FIGURE 6.—A comparison of the patterns of a P-1AB and a P-1$\overline{AB}$ cell line.

From both the P-1AB and P-1$\overline{AB}$ lines, subclones were derived that were P-1A or P-1B in phenotype; no differences in phenotype among those that were P-1A or among those that were P-1B were apparent. On the other hand, the intermediate and hybrid subclones from the two types of cell lines were distinctly different in phenotype. The differences are illustrated in a diagram (Figure 7). Subclones derived from the P-1AB lineages showed variations in intensity of Bands 1, 3, and 5. Those derived from the P-1$\overline{AB}$ lineages showed a different pattern of variation. The intermediates possessed bands of equal intensity: 1, 2, and 3 in zymograms of P-1$\overline{AB}$, and 3, 4, and 5 in P-1$\underline{AB}$ zymograms. A new cell type was derived from the P-1$\overline{AB}$ lines: P-1$\underline{\overline{AB}}$. In zymograms of such a cell type Band 3 is present almost exclusively, with trace amounts of Bands 2 and 4. This type of cell appears to be quite stable, since subclones initiated from a P-1$\underline{\overline{AB}}$ line were all P-1$\underline{\overline{AB}}$. Whether it is as stable as the P-1A and P-1B cell lines has not yet been determined. Photographs of representative subclones derived from the two types of sublines reveal some of the gradations in phenotype observed between categories of common origin (Figure 8).

A tabulation of the subclones from each of the six sublines shows that the difference in the types of intermediate subclones originating from the two types of cell lines is not absolute (Table 6); thus, one P-1$\underline{\overline{AB}}$ and one P-1$\underline{\overline{AB}}$ subclone were derived from P-1AB cell lines. Other differences between the two groups of subclones are revealed in this table. In general, the frequency of parental types was higher among subclones derived from the P-1AB lines (61.8 percent) than from the P-1$\overline{AB}$ lines (22.1 percent). Moreover, the two parental types were produced in equal frequency from the P-1AB lines, while more P-1B than P-1A subclones originated from the P-1$\overline{AB}$ lines.

The differences between these two groups cannot be explained on the basis of *genetic* differences. The P-1$\overline{AB}$ sublines were AB (or F_1) heterozygotes, and these should genetically be *Ab/aB*. Both ABA sublines and one of the ABB sublines

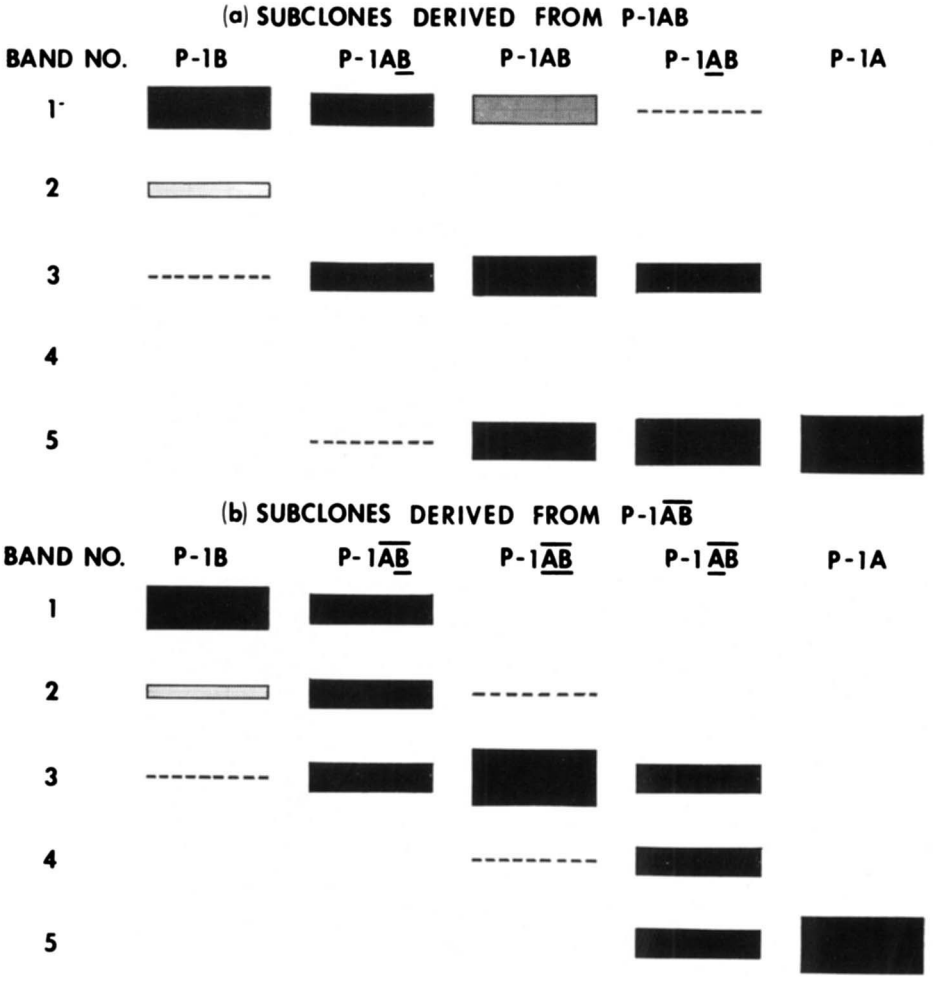

FIGURE 7.—A comparison of the patterns of subclones derived from a P-1AB cell line (a) with those derived from a P-1$\overline{AB}$ cell line (b).

were tested genotypically and found *not* to be crossovers. Since ABA-3 was P-1AB and ABA-6c was P-1$\overline{AB}$, the phenotypic differences of their subclones cannot be attributed to differences in genotype.

If the two groups differed in their macronuclear composition, the observations might fit with the expectations proposed for phenotypic drift involving linked loci. If, in the first group, the subnuclei were largely ones expressing P-1A, or *Ab/xx*, and P-1B, or *xx/aB*, then the results should be very similar to that expected for allelic interaction, since only two stable states would be involved. Theoretically, 62 percent of the subclones should be "pure" for P-1A or P-1B by 120 fissions, if the proportion of subnuclei expressing P-1A is similar to that expressing P-1B (derived from MIDAC computation made by SCHENSTED 1958).

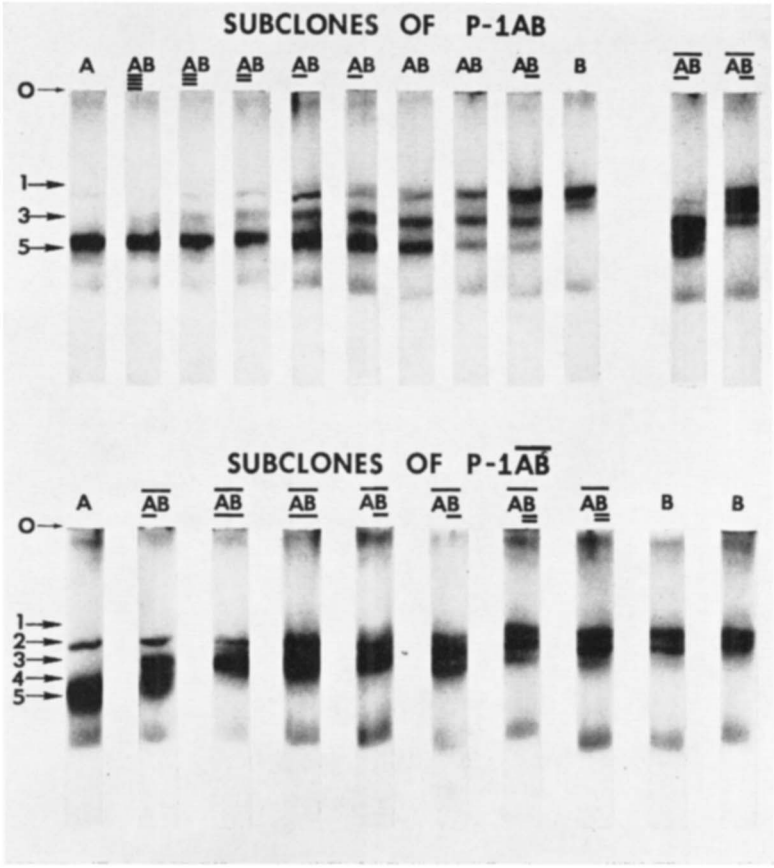

FIGURE 8.—Zymograms of selected subclones of a P-1AB cell line (at top) and of a P-1$\overline{AB}$ cell line (at bottom). The separations were carried out in starch gels of pH 7.5. The P-1 phosphatases are marked by arrows.

TABLE 6

Distribution of subclones from selected P-1^{A}/P-1^{B} *sublines*

AB sublines	No. of subclones with P-1 phenotypes					Total	Percent 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
$\overline{AB}$ sublines	A	$\overline{AB}$	$\overline{AB}$	$\overline{AB}$	B		
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

* AB in phenotype.

The average percentage of the P-1AB group was 61.8 percent—and the two types, P-1A and P-1B, were about equal in frequency.

The percentage of parental phenotypes was lower in the P-1$\overline{AB}$ group: only one P-1A subclone was produced, the others all being P-1B. If the P-1$\overline{AB}$ type is composed of cells containing subnuclei all of which are expressing $Ax/\overline{xB}$, it could represent a third stable cell type. In this case, the original macronuclear composition of the AB sublines might have been Ax/xB and xx/aB. The macronucleus of ABA-6c must have contained subnuclei of three sorts: Ax/xB, Ab/xx, and xx/aB. The fact that the P-1A cell type is rare may only be a sampling phenomenon. It is possible that selection of other cell lineages might have led to a low frequency of P-1B cell types.

The results obtained with an unselected group of sublines (Table 5) could not be explained on the basis of the linked gene hypothesis unless the probabilities of the four states are not equal. No P-1$\overline{AB}$ sublines were found, and the frequency of P-1A and P-1B sublines was very similar but low. These results could be explained if the interaction leading to Ax/xB occurs much less frequently than that leading to Ab/xx or xx/aB. Phenotypic drift seems to proceed independently for the mating types (*mt*) and certain esterases (*E–1*) even though *mt* and *E–1* are linked (ALLEN 1961b and unpublished). However, in the case of the phosphatases, it is possible that genic interaction does not proceed completely independently since the same function is involved.

The linked gene hypothesis with respect to phenotypic drift gives rise to certain expectations that are subject to experimentation. Some, though not all of these expectations, are not excluded under the single gene hypothesis. Thus, a third stable nuclear type could result from some type of intraallelic interaction. Both of these hypotheses will be considered in the discussion of possible synthetic pathways of the phosphatases.

Environmental alteration of the course of phenotypic drift has not been explored. Although certain standard procedures were routinely employed in growing the cultures, slight differences in growth rate between sublines were unavoidable. If slightly different growth conditions affected the various phenotypes differentially, selection of certain cell types might have occurred by accident. In view of the differences in genetic performance between replicate cultures of ABA-6d maintained at different temperatures, such an analysis is warranted.

DISCUSSION

Alternative forms of the P-1 acid phosphatases are controlled by alleles at a single locus or by linked genes. 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. In heterozygotes, variations occur at five electrophoretic positions. Immediately following conjugation the electrophoretic pattern of heterozygotes is identical and independent of genetic background. Bands 1, 3 and 5 are prominent in these zymograms. In the hybrid, Band 3 exhibits temperature and pH stabilities intermediate between P-1B (Band 1) and P-1A (Band 5). After phenotypic drift, cell

lineages with distinctly different phenotypes are formed. Some lines retain the three banded condition; others show five bands. From each type of cell lineage a different set of subclones was derived. Parental types (P-1A and P-1B) were derived from both cell lineages but in different frequencies. These are stable cell types. The hybrid and intermediate types derived from each lineage were strikingly different in pattern. A new type, P-1$\overline{\text{AB}}$, in which Band 3 is present almost exclusively, was derived from the five banded line. It may be a third stable cell type.

Five electrophoretically distinct P-1 acid phosphatases are observed in heterozygotes. Since they are more or less equidistant and migrate in parellel as a function of pH, at least within a limited range, they seem to differ from each other in charge in some regular fashion. The two extreme types (Bands 1 and 5) differ in temperature and pH stability, while Band 3 appears to have intermediate properties. During phenotypic drift the variations in pattern observed in the heterozygote appear to follow a certain sequence. All of these properties must be taken into consideration in searching for a hypothetical scheme that will apply to the formation of these acid phosphatases.

The observations are most compatible with a molecular model, based on that hypothesized for the lactate dehydrogenases (APPELLA and MARKERT 1961; CAHN, KAPLAN, LEVINE, and ZWILLING 1962). Here, it was postulated, and later confirmed by physical and chemical analysis, that the five major LDH's that were distinguished electrophoretically represented tetramers that arise by recombination of two protein subunits. All five LDH's may be generated from purified AAAA and BBBB types by dissociation and recombination of the monomers in 1M NaCl (MARKERT 1963). The variations observed in these LDH's between tissues or at different stages in the development of a tissue have been interpreted as arising from variations in the proportions of the two protein subunits synthesized by a given cell line or cell lines (MARKERT and URSPRUNG 1962; CAHN, KAPLAN, LEVINE, and ZWILLING 1962).

In the case of the P-1 phosphatases a hybrid molecule could be formed through recombination of subunits synthesized under the control of *P-1*^{A} (or *A*) and *P-1*^{B} (or *B*). Further recombinations might give rise to the intermediate molecules. If such a model is applied to the phosphatases and we are dealing with an "A" protein determined by *P-1*^{A} (or *A*) and a "B" protein determined by *P-1*^{B} (or *B*), the following sets of subunits might appear in the three genotypes:

One locus:	*P-1*^{B}/*P-1*^{B}	*P-1*^{A}/*P-1*^{A}	*P-1*^{A}/*P-1*^{B}	
Two loci:	*aaBB*	*AAbb*	*A–B–*	
	BBBB		BBBB	BBBB
	(?)			BBAB
	(?)		ABAB ⟶	ABAB
				AAAB
		AAAA	AAAA	AAAA

Recombination of subunits would take place in two steps: (a) formation of a dimer; (b) formation of a tetramer. Initially, either nonrandomness occurs at step (b) in the heterozygote, or the macronuclear composition is so balanced that equal amounts of "A" and "B" are determined.

If "A" and "B" are determined by linked genes, the types of tetramers that are formed might reflect the macronuclear composition of the heterozygote, i.e., the "quality" and "quantity" of subnuclear types that are present depending upon genic interaction. The relation between macronuclear composition, phenotype and the types of tetramers that would be expected in heterozygotes is illustrated below:

Macronuclear composition:	$\frac{Ab}{aB}$ OR $\frac{Ab}{xx};\frac{xx}{aB}$	$\frac{Ab}{xx};\frac{xx}{aB};\frac{Ax}{xB}$	$\frac{xx}{aB}$	$\frac{xx}{aB};\frac{Ax}{xB}$	$\frac{Ax}{xB}$	$\frac{Ax}{xB};\frac{Ab}{xx}$	$\frac{Ab}{xx}$
Phenotype:	P-1AB	P-1$\overline{AB}$	P-1B	P-1$\underline{\overline{AB}}$	P-1$\underline{\overline{AB}}$	P-1$\underline{\overline{AB}}$	P-1A
Types of tetramers:	BBBB	BBBB	BBBB	BBBB			
		BBAB	(?)	BBAB			
	ABAB	ABAB	(?)	ABAB	ABAB	ABAB	
		AAAB				AAAB	
	AAAA	AAAA				AAAA	AAAA

In order to account for the P-1$\overline{AB}$ type it seems implicit in this hypothesis that the dimerization of "A" and "$\overline{B}$" must occur nonrandomly, possibly at the level of the ribosome. This suggestion has been made for the hybrid esterases of maize by SCHWARZ (1962). There he visualizes a hybrid messenger RNA. In the present situation, genic interaction would result in the nuclear type *Ax/xB*. This might direct the formation of a hybrid m-RNA or the exclusive production of a polyribosome (WARNER, KNOPF, and RICH 1963) containing m-RNA for "A" and m-RNA for "B".

If "A" and "B" are determined by alleles at a *P-1* locus, other factors in the cellular environment besides "gene dosage" must operate in order to account for the subclonal differences observed between three banded and five banded heterozygotes. These factors might influence the degree of randomness in the formation of the tetramer. Initially, we would suggest that nonrandomness occurs at step (b) in the heterozygote. If in certain cell lineages, a shift occurred during step (b) from nonrandomness to randomness, a shift from three to five bands would be expected in the zymograms. Schematically, the following temporal relationships might exist:

P-1^A and *P-1*^{B} are active:

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

The various types of cells that result with phenotypic drift would reflect the amount of "A" or "B" protein synthesized before or during step (a):

	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	
	(?)			
ABAB ⟶	(?)	ABAB	ABAB	
AAAA		AAAA	AAAA	AAAA
(2)	(4)	(5a)	(6a)	(7)
BBBB	BBBB	BBBB		
BBAB	(?)	BBAB		
ABAB ⟶	(?)	ABAB	ABAB	
AAAB			AAAB	
AAAA			AAAA	AAAA

All the cell types except cell type (3) fit very neatly into this scheme. Cell type (3), or P-1$\overline{\text{AB}}$, poses the greatest difficulty in interpretation under this hypothesis. In order to make it fit into the above scheme, it is necessary to invoke nonrandomness in the formation of the dimer in step (a), as was necessary in the linked gene hypothesis. 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.

The relationship of the molecular configurations of the P-1B isozymes to the molecular forms observed in the heterozygote is puzzling, especially in view of their similar migration. Although we have tended to ignore the isozymes in discussing the hybrid pattern, it is not because we are unaware of this problem. We have also ignored the exceptional ABA hybrid since we do not, as yet, understand its genetic performance. If it is *P-1^A/P-1^A* (and the micronucleus is not genetically unlike the macronucleus) then a part of the spectrum of the hybrid phenotype is potentially capable of being expressed by a homozygote, and phenotypic drift must also occur in *P-1* homozygotes. This would necessitate a re-evaluation of the hypothetical model proposed for the heterozygote.

Several types of molecular interactions might result in the formation of isozymes. Isozymes could arise by combination of a protein subunit with a varying number of polysaccharide units, as in the case of the human transferrins (PARKER and BEARN 1962). Or, they could arise by combination of the protein subunit with a cofactor, as in the case of the minor LDH's (FRITZ and JACOBSON 1963). In the latter case, 15 electrophoretically separated LDH's appeared when the starch or polyacrylamide gel had been pretreated with a low concentration of beta-mercaptoethanol. Since a different number of minor bands was observed at each major LDH site, FRITZ and JACOBSON (1963) suggested that normally a cofactor is attached to each of the monomers of LDH and that beta-mercaptoethanol removes the cofactor from only one type of monomer. Notice that in this case the isozymes are electrophoretically distinct and represent "sub-fractions" of a major

component. Not all the additional LDH bands may be explained in this fashion. Human sperm possess a unique LDH (BLANCO and ZINKHAM 1963; GOLDBERG 1963). In this case a third protein subunit has been inferred, possibly under the control of a gene at a third LDH locus.

The alkaline phosphatases of *E. coli* occur in isozymic form, and, when artificial hybrids are made (LEVINTHAL, SIGNER, and FETHEROLF 1962), the hybrid enzyme also appears as isozymes. A hybrid P-1 phosphatase has not, as yet, been made artificially in *T. pyriformis*. However, the results with partial reactivation of the "native" acid phosphatases encourage us in the belief that this goal may ultimately be reached. Artificial production of parental enzymes from Band 3 (from P-1$\overline{\text{AB}}$ cultures) would go a long way towards establishing the existence of a "hybrid" enzyme. The properties of Bands 2, 3, and 4 in cells of different genotype may help to elucidate the relationship of the isozymes of homozygotes to the hybrid enzymes observed in heterozygotes.

SUMMARY

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.

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