INHERITED VARIATIONS IN THE ESTERASES OF TETRAHYMENA[1]

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Received February 23, 1960

THE technique of zone electrophoresis in starch gels was developed by SMITHIES (1955) and used in genetic studies of the serum proteins of man and cattle (SMITHIES and WALKER 1955; SMITHIES and HICKMAN 1958). To this technique HUNTER and MARKERT (1957) coupled certain histochemical procedures for identifying the enzymatic properties of electrophoretically resolved proteins from tissue homogenates. The combined electrophoretic and histochemical techniques have recently been applied to an analysis of the esterases of variety 1 of the Protozoan, *Tetrahymena pyriformis* (ALLEN 1959). One class of these esterases is eserine sensitive and activated by sodium taurocholate. Since this class shows strain differences, a genetic analysis of these differences was initiated. The present report is concerned with a description of these esterases and the manner in which differences among them are inherited within the clone and in breeding experiments. The results of this analysis show that a complex system is involved and that the system is under genetic as well as epigenetic (NANNEY 1958) control.

MATERIALS AND METHODS

Material: Variety 1 of *T. pyriformis* contains four inbred strains (families), A, B, C, and D, each of which was derived from a wild isolate by selection and inbreeding for various mating type alleles (NANNEY 1959b). Strains A and B were derived from a cross of wild strains WH–6 and WH–14; each has been inbred for at least eight generations. The origins of strains C and D are not clear. Strain C was supposedly derived from a cross of wild strain Alp–4 and strain B and then inbred for five generations. Strain D was supposedly derived from a cross of wild strain UM–226 and strain B and inbred for seven generations. However, the esterase patterns and the distribution of serotypes (ALLEN, unpublished; LOEFER, unpublished) show similarities between strains C and UM–226 and similarities between strains D and Alp–4. This observation suggests that strain C probably arose from UM–226 and strain D from Alp–4. A resolution of this problem awaits further investigation. In the work to be reported below crosses were performed between strains using the most inbred member of each strain. Most of the work concerns strains B and C and their hybrids.

Maintenance of clones: Each clone was maintained on bacterized medium and on a sterile peptone medium. The bacterized medium was prepared by inoculating Cerophyl (rye grass) infusions with *Aerobacter aerogenes*. Tubed cultures

[1] Supported by a research grant (C-3545) from the National Cancer Institute, U.S.P.H.S.

of each clone were maintained in the bacterized medium at 16°C; these served as the source of the parents in the earlier crosses. The sterile medium contained one percent proteose-peptone broth adjusted to *p*H 7.0. Tubed cultures of each clone were stored in the peptone medium at 16°C; these served as the source of flask cultures, which were grown at 30°C and used in making extracts for the esterase analysis.

Crosses: The most recent crosses have been performed under sterile conditions using washed animals grown in one percent proteose-peptone. All isolations and transfers were made into peptone medium. The earlier crosses were carried out in bacterized medium. In either case the crosses were performed at 30°C and the general procedures of NANNEY and CAUGHEY (1955) were followed. Non-conjugants were eliminated and the exconjugants were carried to maturity by serial transfers of single cells (unless otherwise specified). Mature cells, when grown on the bacterized medium, were then transferred by aseptic technique to the sterile medium by a series of transfers in sterile glass-distilled water and antibiotics (streptomycin and penicillin).

Starch gel electrophoresis: Erlenmeyer flasks (250 ml) containing 100 ml of sterile peptone medium were inoculated with 1 to 5 ml of a tubed plateau culture and grown for five to seven days at 30°C (plateau) unless otherwise specified. Crude extracts of concentrated cells were prepared by repeated freeze-thawing. A filter paper (Whatman #1) of known dimensions was dipped into the crude extract and inserted into a transverse cut in the starch block. Electrophoresis was then carried out in the horizontal position (SMITHIES 1955) for five hours at room temperature using a borate buffer system (0.03 M in the starch; 0.3 M in the end trays) at *p*H 7.0–7.5. An eight-volt drop per centimeter through the starch was generated by a Heathkit Variable Voltage Regulated Power Supply Kit (Model PS-3).

Histochemical techniques: The starch blocks were divided into three equal strips by horizontal cuts and a different histochemical test was made on each strip (HUNTER and MARKERT 1957; MARKERT and HUNTER 1959). The substrates employed were alpha naphthyl acetate, beta naphthyl acetate, alpha naphthyl propionate, and alpha naphthyl butyrate; the stable diazotate of 4′-amino–2′,5′–dimethoxybenzanilide (Fast Blue RR, General Dyestuff Co.) was used as dye coupler. The substrate (0.01 percent) and dye coupler (1 gm/L) were added to 0.1 M phosphate buffer at *p*H 7.4 and the reaction carried out at room temperature (PEARSE 1953). At the sites of enzymatic activity in the starch strips the naphthol molecule is split off and coupled to the diazonium salt to give rise to a colored band. For inhibition studies, eserine sulfate, at a final concentration of 10^{-4} M, was added one hour before the substrate and dye coupler were added to the reaction mixture. Sodium taurocholate, at a final concentration of 10^{-2} M, was added to the reaction mixture at the same time as the substrate and dye coupler. After four hours of incubation, the starch strips were removed from the reaction mixture, washed in distilled water, and photographed using a Polaroid Land camera. The intensity of staining of the various bands, which is roughly proportional to the activity of the enzyme, was determined directly from some of

the starch strips with a modified Photovolt Densitometer (Model 525, modified by DR. ROBERT L. HUNTER in cooperation with the Photovolt Corporation).

Characterization of the esterases

About 20 bands with esterase activity can be separated in the starch gel from extracts of variety 1 of *T. pyriformis*. The esterases can be divided into two classes by their response to 10^{-4} M eserine sulfate (Figure 1). The eserine insensitive class will be treated in more detail elsewhere (ALLEN, unpublished); it includes a motley array of esterases, some with restricted specificities and others with broader specificities. Some of these esterases are activated by 10^{-2} M sodium taurocholate and others are unaffected. Some of the enzymes of this class are weak and appear only in preparations of high protein concentration; hence, their detection tends to be somewhat erratic. With certain exceptions, most of them are found in all of the strains and hybrids. The relationship between esterases of this class and the total pattern of esterases is not clear.

In the present paper attention will be confined to the class of esterases which is sensitive to eserine, and which, by this criterion, should contain the cholinesterases. Nine to ten bands possessing this type of activity migrate as two groups of bands, labelled B and C in Figure 1. The B group contains five bands and migrates toward the cathode at *p*H 7 to 7.5, while the C group contains four or five bands and migrates toward the anode. Figure 2 shows that the B group is found in strains A, B and D, but that the C group is limited to strain C. For further characterization of these groups of bands, strain B was selected as representative of the B group and strain C as representative of the C group. Except for a very few crosses, no further work has been done with strains A and D.

All of the bands of this class respond in a similar manner to inhibitors and activators—behavior which makes these esterases difficult to classify. Thus, as shown in Figure 4, *all* of the bands (B, on the right; C, on the left, of the same starch strips) are inhibited by 10^{-4} M eserine sulfate and *all* are enhanced by 10^{-2} M sodium taurocholate. The inhibition by eserine would result in their being classified as cholinesterases, while the activation by taurocholate would result in their being classified as lipases (GOMORI 1952). The chemical basis of the enhancement of these bands by taurocholate is not known and it may involve changes in the starch matrix itself. Unfortunately, the ability of these enzymes to hydrolyze choline esters cannot be tested directly with these techniques since the available compounds cannot be employed in the coupling reaction. Thus, a classification of these esterases cannot be satisfactorily made at present, and, in order to avoid controversy, they will be designated class I esterases, or eserine sensitive esterases. The eserine insensitive class will be referred to hereafter as the class II esterases.

In addition to their similar patterns of inhibition and activation, all of the class I esterases have similar affinities for the substrates used, as shown in Figure 3. They can split alpha naphthyl acetate, beta naphthyl acetate and alpha naphthyl propionate, but they cannot split alpha naphthyl butyrate. If the class I esterases are, in fact, cholinesterases, these specificities would suggest that their natural

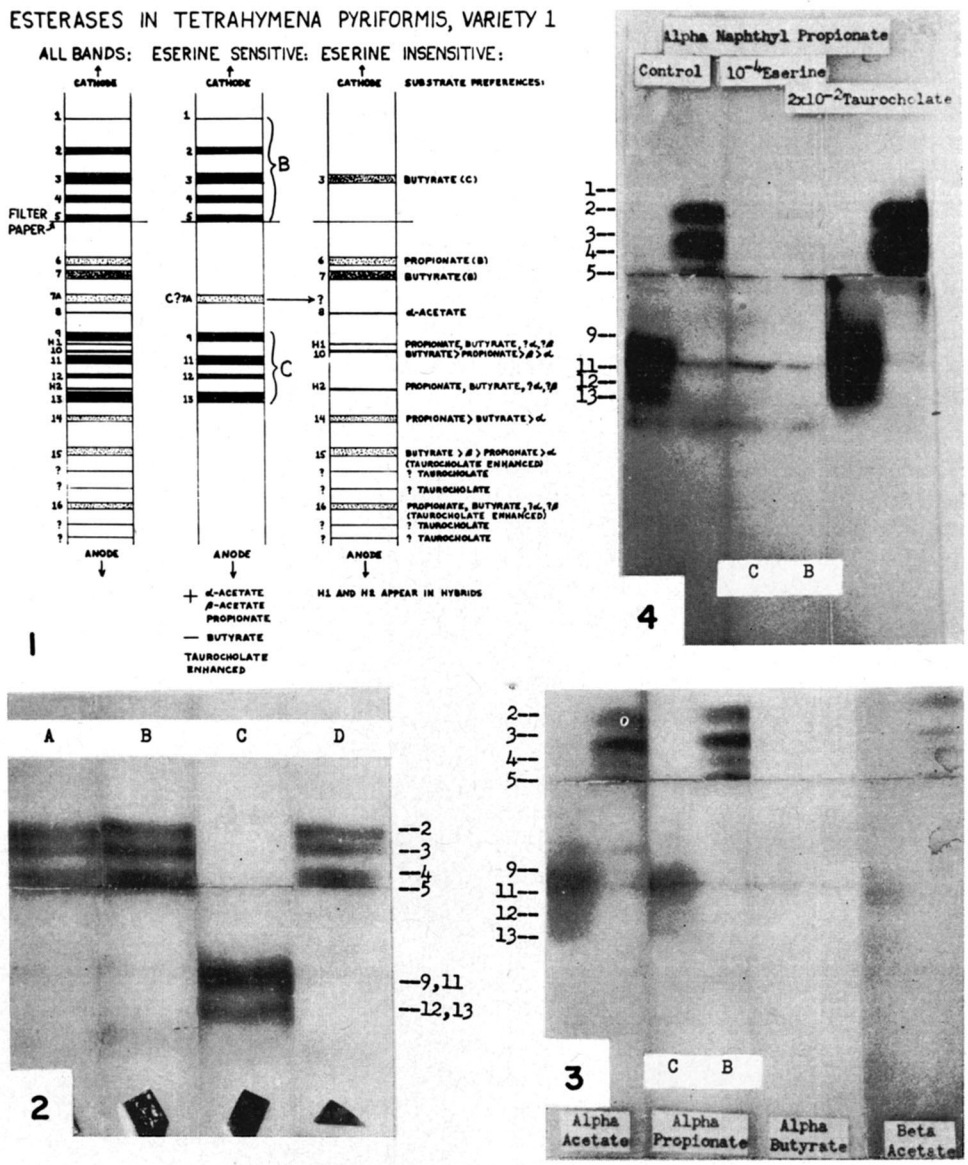


FIGURE 1.—Diagram of esterases in *Tetrahymena pyriformis*, variety 1. FIGURE 2.—Distribution of B and C groups of class I esterases in strains A, B, C and D. FIGURE 3.—Affinities of B and C groups of class I esterases for four substrates: alpha naphthyl acetate, alpha naphthyl propionate, alpha naphthyl butyrate, and beta naphthyl acetate. Two preparations were placed on each starch column—B on the right and C on the left. FIGURE 4.—Effect of eserine sulfate and sodium taurocholate on B (right) and C (left) groups of class I esterases with alpha naphthyl propionate as substrate.

substrate may be acetylcholine, since acetylcholinesterase splits butyrylcholine very poorly (see p. 278, DIXON and WEBB 1958, for a discussion of work by ADAMS and WHITTAKER).

An eserine sensitive esterase, identified by SEAMAN (1951; SEAMAN and HOULIHAN 1951) in a related strain of *T. pyriformis* as an acetylcholinesterase, was found to be associated with the cortex of the cell. The activity of this enzyme was concentrated in a pellicular fraction obtained by cell fractionation procedures. Eserine sulfate, at a concentration of 10^{-4} M, was found to block ciliary movement *in vivo*. SEAMAN (1951) speculated that the fibrillar system in Ciliates acts as a primitive conducting mechanism analogous to nerve fibers.

Cell fractionation procedures have not, as yet, been applied to the present system. However, unsuccessful attempts were made to determine the intracellular distribution of these class I esterases using localization techniques with the above substrates and inhibitors (ALLEN 1958; and unpublished). Although characteristic distributions are obtained, these techniques did not reveal any strain differences nor did they distinguish between the two classes of esterases.

Although no differences in *specificity* could be found between bands having class I esterase activity, differences in the *amount* of activity were observed as a reflection of different growth conditions. A systematic investigation of these quantitative differences was undertaken using two variables: the growth cycle and the conjugation sequence.

Under the conditions employed, the various stages of the growth cycle were reached after the following days of growth: logarithmic phase—zero to two days; plateau—five to seven days; stationary—ten days; decline—14 days. Samples of strains B and C were obtained at (a) 0.5 or one day, (b) two days, (c) five days, (d) seven days, (e) ten days, and (f) 14 days. These samples were adjusted to a similar concentration of cells using equal volumes of material with the same optical density (using a Photovolt Lumetron Colorimeter). A photograph of the results with the two strains (Figures 5 and 6) shows that differences in the amount of activity of these esterases did occur at different stages of the growth cycle. These differences were semiquantitated with the Densitometer. The starch strips were thin and transparent, thus permitting a setting of 100 percent transmission of light (zero percent absorption of light). The stained areas were scanned for their maximum absorption of light. It is these values which make up the "density" curves of Figures 7 and 8. Throughout the growth cycle a characteristic curve may be drawn for each band. In strain B, band 1 changed little, band 3 had a high value during the logarithmic phase of the growth cycle, bands 2 and 5 achieved their highest values during plateau, and band 4 reached its maximum value during the stationary phase. In strain C, two bands (12 and 13) changed little, while two other bands (9 and 11) reached their maximum values during the early part of the growth cycle.

The ordered sequence of activities of the members of each group of esterases during the growth cycle might occur under two different circumstances. The changes could reflect shifts in the composition of the population of cells—and for each esterase there could be a different cell type. Or, the changes could occur in

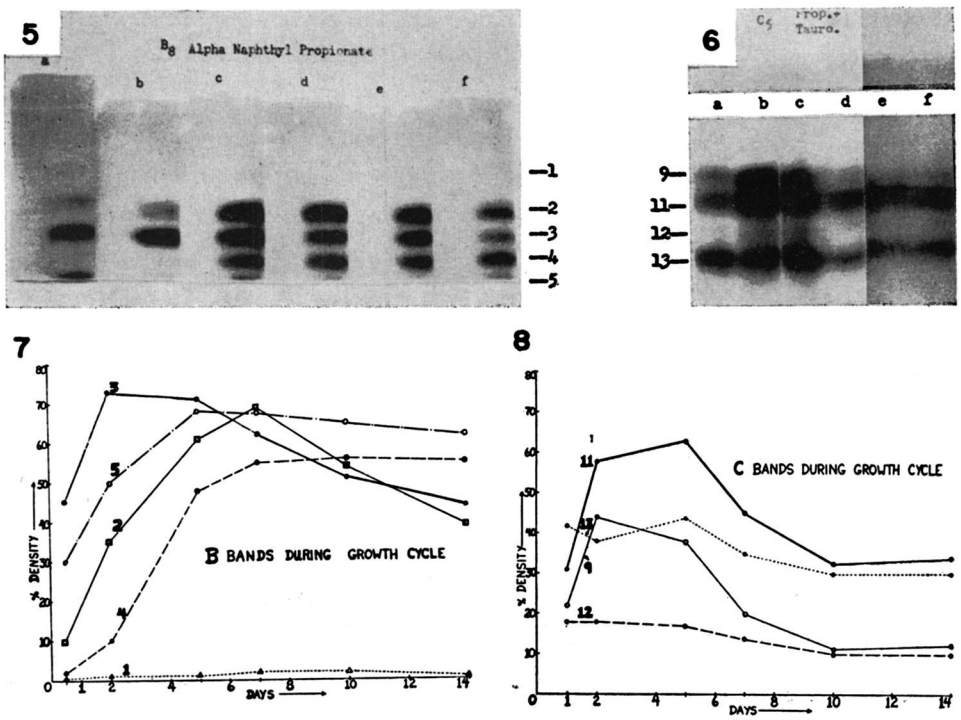


FIGURE 5.—B bands during growth cycle: (a) 0.5 days (b) two days (c) five days (d) seven days (e) ten days (f) 14 days. Substrate: alpha naphthyl propionate. FIGURE 6.—C bands during growth cycle: (a) one day (b) two days (c) five days (d) seven days (e) ten days (f) 14 days. Substrate: alpha naphthyl propionate, with sodium taurocholate. FIGURE 7.—Curves of B bands during growth cycle based on Densitometer readings. Percent density refers to percent absorption of light. Substrate: alpha naphthyl propionate. FIGURE 8.—Curves of C bands during growth cycle based on Densitometer readings. Substrate: alpha naphthyl propionate.

a single cell type and reflect shifts in the internal environment of the cell. The first possibility can probably be discarded since no gross heterogeneity of cell types was suggested in a study of subclones of strain B. Table 1 shows that subclones were alike at each of two stages tested in the growth cycle (two days and five days). Thus, each cell probably produces all of the enzymes of one of the groups of esterases, and, as the cell's internal environment changes during the growth cycle, shifts in activity of these enzymes result. It is possible, of course, that each of these enzymes plays a slightly different biological role in the economy of the cell.

During conjugation a different pattern of variation in activity of these esterases is observed. Using aseptic technique, a large population of cells was collected in the logarithmic phase of the growth cycle and was washed in three changes of glass-distilled water to get rid of the peptone medium. The washed cells were resuspended in distilled water, which was changed after 24 hours. Then, similarly treated cells of a different mating type were added and the mixture was

TABLE 1

Densitometer readings of bands in B subclones at two days and five days during growth cycle

Band:	Two days					Five days				
	1	2	3	4	5	1	2	3	4	5
Subclone										
1	<1	24	24	±1	16	1	25	31	28	20
2	1	26	34	1	19	1	28	32	20	21
3	<1	18	27	1	18	1	20	20	17	16
4	<1	19	27	1	12	1+	35	39	27	32
5	<1	15	26	1	16	1	28	32	23	25
6	<1	19	25	1	10	1+	33	37	32	30
7	<1	13	19	1	13	1	24	35	21	21
8	<1	21	21	1	16	1+	40	48	35	41
Average	<1	19.4	25.4	±1	15.0	1+	29.1	34.2	25.5	25.8

placed in a New Brunswick variable speed shaker overnight. The following day the mixed population was removed from the shaker and dispensed into depression slides where conjugation occurred some 45 minutes later. The procedure of shaking the cells was introduced in the hope of activating the cells for mating so that synchrony in mating might result with the termination of shaking. However, only partial success was achieved: after shaking, more cells mated sooner, but not all of the cells did so. At various times over the next 48 hours samples were collected, pooled, and extracts made of concentrated cells. These extracts were tested by the procedures described above.

Only trends in the data can be pointed out, since the biological aspects of conjugation have not been completely controlled. It was not possible to make all the animals conjugate nor to synchronize them. By the end of the experiment about 80 percent of the population was estimated to have conjugated on the basis of direct cytological examination employing the Feulgen technique.

Some of the observed changes were shown in preliminary tests to be due to starvation, a necessary prerequisite for the elicitation of conjugation. First, the total enzymatic activity of the preparation fell to about 1/32 that of the activity of preparations of feeding cultures. Second, the activity of one of these esterases (C–12), usually the least active in feeding cells, was more active in starved cells (Figure 9: C × C, sample 1).

Other changes, however, were observed during conjugation, which were not observed in starved cultures; these were observed in the intrastrain crosses as well as the interstrain cross (Figures 9 and 10). Certain esterases decreased in activity (B–2; C–9); certain esterases changed little (B–3; C–11); and certain esterases increased in activity (B–4,5) or remained high in activity (C–12,13). Thus, as conjugation proceeded, a shift in activity was observed from the more negatively migrating esterases to the more positively migrating esterases, and the same shift occurred in both the B and C groups of enzymes. This shift in activity might have resulted from a changing intracellular environment accompanying conjugation, and a similar shift in cells of different phenotype might

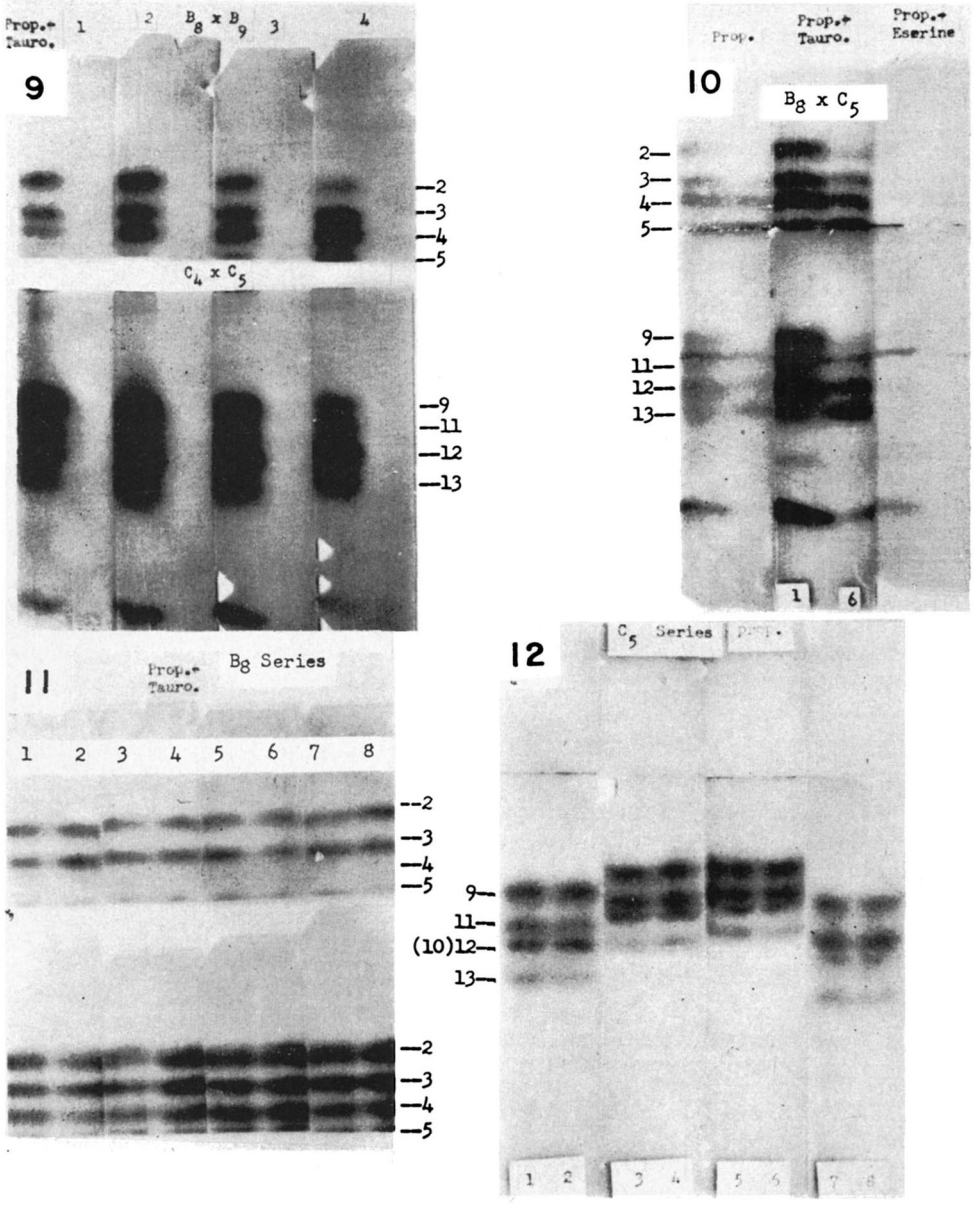


FIGURE 9.—*Top:* B bands during successive stages in conjugation of B × B. *Bottom:* C bands during successive stages in conjugation of C × C. (1) Before conjugation started; (2) 12 hours after conjugation began; (3) 24 hours after; (4) 36 hours after. Substrate: alpha naphthyl propionate, with sodium taurocholate. FIGURE 10.—B and C bands during successive stages in conjugation of B × C. (1) Before conjugation; (6) 48 hours after the beginning of conjugation. Substrate: alpha naphthyl propionate—alone, with sodium taurocholate, and with eserine sulfate. FIGURE 11.—Eight B subclones sampled at two days (top) and at five days (bottom). Substrate: alpha naphthyl propionate with sodium taurocholate. FIGURE 12.—Eight C subclones sampled at five days. Substrate: alpha naphthyl propionate.

reflect similar environmental changes. On the other hand, if each enzyme served a slightly different biological function in the metabolism of the cell, this might argue for enzymes with homologous function in the two groups of esterases.

A systematic investigation of the variations in activity of the class I esterases has shown that a sequence of changes occurs during the growth cycle and during conjugation. These variations were not random fluctuations in amount of enzymatic activity but were ordered and depended upon specific growth conditions. In general, these observations are similar to those of MARKERT and MØLLER (1959) on the lactate dehydrogenases of the pig and other species. They refer to the electrophoretically distinct forms of an enzyme as *isozymes*. Using eight different substrates, they could detect no differences in specificity between these isozymes. Yet, they could show characteristic variations in the amount of enzymatic activity of each isozyme from several different tissues and in the embryonic stages of the same tissue. Their data did not permit genetic interpretations, but they suggested that each isozyme might be controlled by a separate gene or, alternatively, that all the isozymes might represent modifications of a single gene product. As will become apparent in a succeeding section of this paper, a definitive answer to this question has not, as yet, been obtained in Tetrahymena, but the results to date are more compatible with their second hypothesis.

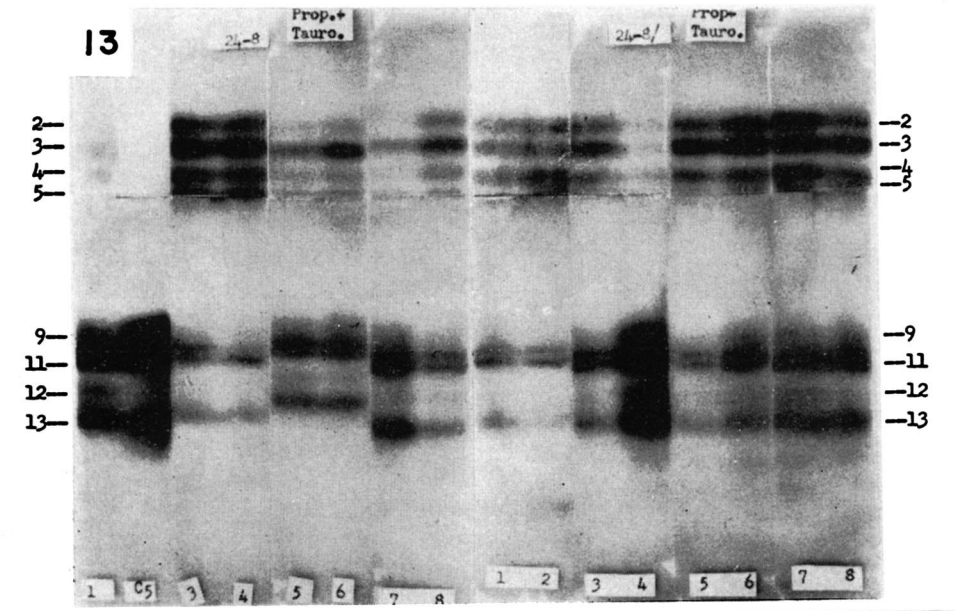
Inheritance during clonal multiplication

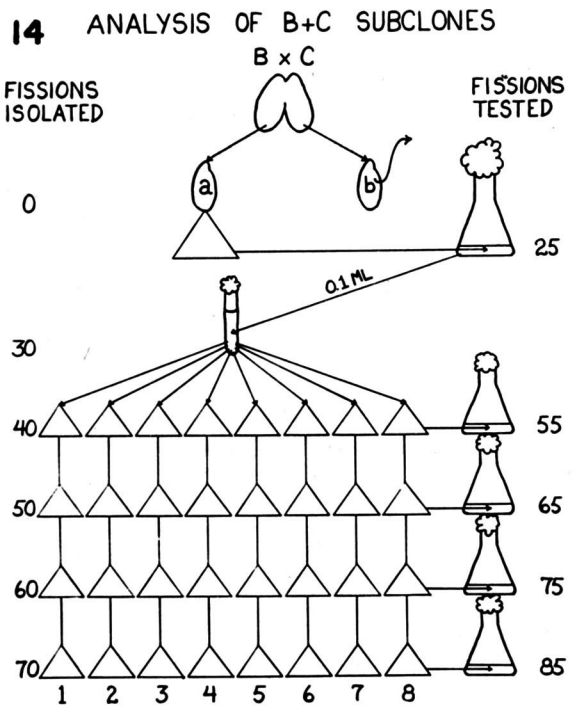
As a clone is propagated by successive fissions, three patterns of inheritance of the class I esterases can be demonstrated. Two of these patterns are stable and show only the quantitative variability associated with growth cycle changes. These are the B and C patterns, as exemplified by strains B and C. Either the B or the C group was perpetuated in the clone since subclones of strains B and C showed only the isozymes characteristic of each strain (Figures 11, 12).

The third pattern of inheritance, on the other hand, exhibited clonal variability and was found in certain hybrids between strains B and C. All of the B isozymes and all of the C isozymes were present in these clones, but variations in the amount of activity of the one or the other *group* of isozymes was observed in different subclones. This variability is illustrated in Figure 13 by two sets of eight subclones. It is apparent that some of the subclones have more B-group than C-group activity, others have more C-group than B-group activity, and others are equally active for both groups of isozymes.

The perpetuation of the pattern of each B+C subclone was followed over the course of 40 fissions by the technique of serial transfer of single cells at 10-fission intervals. The "leftover" populations were inoculated into flasks to provide large populations for making extracts. The design of this experiment is illustrated in Figure 14.

The results of this experiment showed: first, that each pattern is perpetuated rather closely in successive fissions; and second, that changes in pattern do occur, but that the rate of change is slow. Quantitative data on all the subclones appear in tabular form (Table 2), based on Densitometer readings. Here, in order to equate the total activities of different samples, the activities of the B group iso-





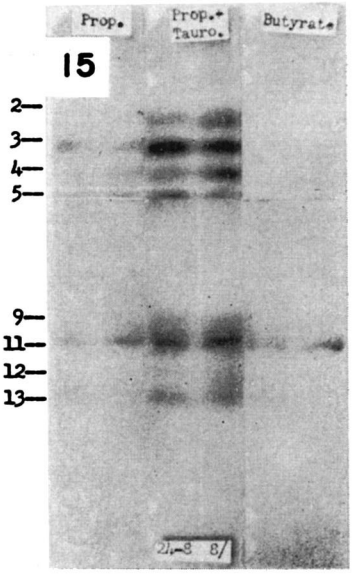


FIGURE 13.—Two sets of eight B+C subclones, sampled at five days—one set from 24–8, the other from 24–8/. Substrate: alpha naphthyl propionate, with sodium taurocholate. Note that 24–8–2 died and was replaced by a C clone. FIGURE 14.—Design of experiment testing B+C subclones as a function of fission. FIGURE 15.—B+C clones (a) 24–8 and (b) 24–8/; exconjugants from the same pair, derived from a cross of B × C. Substrates: alpha naphthyl propionate—alone, with sodium taurocholate; and alpha naphthyl butyrate.

TABLE 2

Percentage of C-group activity in B + C subclones at ten fission intervals

Subclone:	1	2	3	4	5	6	7	8	Mean
No. fissions after conjugation	Exconjugant a (24–8)								
55	74	..	24	18	63	53	66	43	49
65	..	..	28	25	..	72	..	36	40
75	100	..	25	26 22	..	67	..	25	44
85	100	42	11	22 16	..	60 86	..	27	46
	Exconjugant b (24–8/)								
55	25	16	35	87	26	36	33	36	37
65	20	14	41	74	22	34	38	58	38
75	23	12	49	61	22	43	39	56	38
85	20	13	31	60	22	45	37	52	35

zymes and the C group isozymes were totaled and the percentage of C-group activity calculated. Thus, 100 percent signifies absence of detectable B-group activity, and zero percent signifies absence of detectable C-group activity. The percentage of C-group activity is presented for each sampling of each subclone. Although the activities of different subclones are markedly dissimilar (compare 1b *vs.* 2b *vs.* 3b *vs.* 4b, etc.), successive samples of any one particular subclone have very similar activities (compare successive samples of 1b, 2b, or 7b). Yet, shifts in the C direction occur in subclones 6a and 8b, and shifts in the B direction occur in subclones 8a and 4b. Nevertheless, in spite of the initial variability, the rate of change with fissions apparently is not very great, and the mean activity of each clone changes very little. This suggests that small differences in the groups of esterases are maintained in different cell lineages. Whether segregation of cells phenotypically "pure" for B or pure for C could occur with additional fissions was not determined, although in the last sampling of one of the subclones (1a) only C isozymes were present.

This study suggested that all three patterns of inheritance of class I esterases—B, C and B+C—can be maintained in different cell lineages for many cell generations. The first two patterns, which are found in different strains, seem to be entirely stable, while the third pattern, which is found in certain hybrids, is "metastable"—and persists with small changes at each cell division. Therefore, each of these types of clones can be used in breeding experiments with a fair assurance of reproducibility of the results.

Breeding behavior

In order to determine how the class I esterases are inherited during sexual reproduction, a series of crosses was made within strains and between strains. Within a strain each group of esterases bred true; thus, in a cross of B × B, only the B isozymes appeared in the offspring, and in a cross of C × C only the C iso-

zymes appeared in the offspring. Crosses between the two strains indicated that the class I esterases are under genetic control but that the control is modified in some manner so that it appears complex. The results of the initial crosses are summarized in Table 3.

When strains B and C were crossed, only the B isozymes appeared in the progeny (for the present the exceptional clone obtained with a repetition of this cross will be ignored). This was also the result obtained from crosses of other strain combinations (A × C, C × D, A × B, A × D, B × D). Exconjugants from the same pair were found to have identical phenotypes, i.e., both had B group isozymes. This result would be expected if the trait were controlled by nuclear genes and B were dominant to C, since the exconjugants from the same pair are alike genetically. However, it would also follow if a cytoplasmic element were involved and if B cytoplasm "dominated" C cytoplasm in a mixture of cytoplasms brought about by cytoplasmic exchange.

To test for a simple genetic basis, a backcross of the F_1 (B × C) hybrid to the C parent was made. If the trait were inherited as a difference at one or two loci, simple 1:1 or 3:1 ratios would be expected. Instead, 19 out of the 20 progeny were B in phenotype, and the remaining one had all of the B and all of the C isozymes. Here exconjugants from the same pair were identical in phenotype—including the exceptional pair. Not only were simple genetic ratios not obtained in the backcross of (B × C) × C, but this result was also observed in an F_2 from a cross of D × C; all 18 of the progeny had B isozymes only.

Such behavior might be attributed to the participation of polygenes in the determination of this character. Hence, a second backcross of two "B" hybrids

TABLE 3

Distribution of class I esterases among the progeny of inbred strains and their hybrids

Phenotype	Cross	No. pairs tested	No. pairs with:†		
			B group	C group	B+C groups
B,B	B × B	10	10	0	0
C,C	C × C	22*	0	22	0
B,C	B × C	10*	10	0	0
B,C	B × C	16‡	15	0	1
B,C	A × C	5	5	0	0
B,C	D × C	5	5	0	0
B,B	A × B	5	5	0	0
B,B	A × D	5	5	0	0
B,B	B × D	5	5	0	0
B,B	(D × C)F_2	18	18	0	0
B,C	(B × C) × C	20	19	0	1
B,C	(B × C)C × C	26	25	0	1
B,C	(B × C)C × C	24*	23	0	1

* Two cells selected at random from pair cultures rather than the usual procedure of separating exconjugants.
† Wherever tested, the two exconjugants from the same pair were always identical in phenotype.
‡ Cross performed in peptone medium; exconjugant cultures tested at 25 fissions. All other crosses in this table performed in bacterized medium and exconjugant cultures tested at about 100 fissions after conjugation.

(B × C) C to the C parent was made. If the simultaneous presence of C alleles at several loci were necessary in order to produce the C phenotype, some C-group offspring would be expected to appear in a sample of about 25. This expectation was not realized. Instead, the results were similar to the first backcross generation: most of the offspring were B in phenotype, but in each cross, one clone had all of the B isozymes and all of the C isozymes. Where tested, the exconjugants from the same pair were alike in phenotype. This, of course, does not eliminate the possibility that a large number of polygenes might be involved, but it suggested that a repetition of the original cross (B × C) might be more instructive.

A repetition of this cross was suggested on other grounds. Studies of the B × C cross during the course of conjugation indicated that the C isozymes did not disappear by the end of conjugation (see Figure 10: B × C, sample 6). In repeating this cross, it was carried out in the peptone medium, and the exconjugants were sampled as soon as possible after conjugation—or at about 25 fissions thereafter. Out of 16 pairs examined, 15 were B in phenotype, and one had all of the B and all of the C isozymes. The exconjugants from the same pair were similar in phenotype, including the exceptional pair (see Figure 15). This pair was subjected to an extensive analysis. A previous section documented the behavior of the two exconjugant clones during asexual reproduction. Now, what happens when these clones are bred?

In making crosses with these clones the crosses were carried out in the peptone medium, as described above. The exconjugants were not separated, but the entire exconjugant population was used in making the extracts. Later, from each pair, two randomly selected clones were carried to maturity in the bacterized medium and then retested after being put back on the peptone medium. In every case, each isolate from the same pair tested qualitatively the same as in the previous test of the pair culture. Hence, the kind of medium does not affect the results. Moreover, exconjugants from the same pair were probably alike. In crosses of "recovered" types (see below) the exconjugants were separated and were qualitatively similar in phenotype (with one exception) although quantitative variations were recorded. The results of the crosses made with the F_1 B+C clones are shown in Table 4.

In the first cross they were inbred (two caryonides of different mating type from the same pair were crossed) to produce an F_2, and in two separate crosses, the frequencies of exconjugant clones with the B, B+C and C groups of esterases were 5, 12, and 3, and 2, 11 and 5, respectively. The distribution of these numbers seemed to fit a good 1:2:1 ratio and suggested that a single locus might be involved in the control of the class I esterases. Under such an hypothesis each allele at this locus would determine a protein of different net charge—either B or C in type. Both proteins might be produced by a heterozygote. The isozymes, then, would arise by secondary modifications of these proteins as a result of nongenetic processes.

In the simplest form of this hypothesis, certain predictions were made for the backcross generations. These predictions were not met. Where a 1:1 ratio of B to B+C (or B only) was expected in the backcross to strain B, an 8:9:2 ratio of

TABLE 4

Distribution of class I esterases among the progeny of B+C clones

Cross	No. pairs tested	No. pairs with:		
		B group	C group	B+C groups
(B+C) × (B+C)	20	5	3	12
	18	2	5	11
	38	7	8	23
(B+C) × C	17	4	4	9
	17	6	5	6
	34	10	9	15
(B+C) × B	19	8	2	9

TABLE 5

Results of inbreeding F_2 progeny from a cross of (B+C) × (B+C)

Type of F_2 progeny:	No. pairs tested	B group	C group	B+C groups
B	8	8	0	0
C	8	0	8	0
B+C	12	5	2	5
B+C	8	1	4	3*

* Exconjugants were tested separately from each pair and in all pairs except one (here) the two exconjugants from the same pair were similar in phenotype. Here, the other exconjugant in one of the three pairs was "B" in type.

B to B+C to C was obtained. Similarly, the backcross to strain C yielded something that approached a 1:2:1 ratio of B to B+C to C where a 1:1 ratio of B+C to C was predicted. In both backcrosses the appearance of the nonparental phenotype was unexpected: C was not expected in the backcross to the B strain or B in the backcross to the C strain. Thus, all classes of offspring appeared in all of the crosses of the B+C hybrids.

A breeding analysis has been carried out on four of the F_2 progeny (from the inbreeding of F_1 B+C clones). Two of these were classified as B+C, one was classified as C and one was classified as B in phenotype. Table 5 shows that, when inbred, each of these classes bred "true". The two recovered "pure" types gave rise to only B or to C in their progeny. The two B+C clones gave rise to all three classes (B, B+C and C) in their progeny, repeating the behavior of their parents. In these crosses the exconjugants were separated, and, with one exception, those from the same pair were alike in phenotype. The exception occurred in one of the B+C crosses, and from one pair one exconjugant was B+C in type, the other being B. Although it is satisfying to be able to recover B's and C's which breed true, from a genetic viewpoint the critical crosses have not, as yet, been made. These are the crosses of clones with the nonparental phenotypes arising in the backcross generations. It is possible that these exceptions are actually B+C in genotype.

In summarizing the results of the breeding experiments it is difficult to put together all of the data into one harmonious scheme. The results of the original cross and the crosses of the "B" hybrids to strain C (Table 3) stand in contrast to the results of the crosses with the B+C clones (Tables 4 and 5). The first series of crosses gave rise to skewed ratios of B to B+C, and the C class was entirely missing from this pedigree. In the second series of crosses all three classes appeared—and in high frequencies.

Curiously enough, a similar pattern of behavior was manifested in these two series of crosses with regard to the presence or absence of mating type I (found in strains A, C and D, but not in strain B). At 30°C the frequency of mating type I in strain C is high (48 percent). At this temperature the frequency falls to about 20 percent in heterozygotes between *some* strain C clones and strain B; however, in heterozygotes between *other* strain C clones and strain B the frequency drops precipitously (zero to two percent). The strain C clone used as a parent in the present series of crosses yielded virtually no mating type I when crossed to the B strain, and, with two exceptions, mating type I was absent from the entire pedigree of the first series of crosses. The two exceptions appeared in subclones of two selfing B+C clones—in the F_1 and in the second backcross generation. In the second series of crosses, on the other hand, mating type I did appear. However, the distribution of mating type I was not random; it was confined to pairs which contained B+C or C class I esterases (Table 6). In no pair that was classed as B did mating type I appear. On the other hand, the mating types characteristic of strain B (IV, VII) were distributed among pairs with all three class I esterase phenotypes. These mating types also appear in high frequency in animals heterozygous for mating type alleles (mt^B/mt^C). It is possible that linkage between the mating type locus and a postulated esterase locus might explain this non random distribution of mating type I. A breeding analysis of the *capacity* of clones, differing in their esterases, to produce the various mating types will be necessary in order to test this possibility. An analysis of the four F_2 clones has been completed. The C clone and one of the two B+C clones gave rise to all mating types in their progeny and were thus heterozygous at the mating type locus (mt^B/mt^C). The B clone and the other B+C clone gave rise to no mating type I and may have been homozygous for the B mating type allele (mt^B/mt^B). It is clear, however, that mating type I and the B group of esterases are not

TABLE 6

Distribution of mating types I and IV (or VII) among pairs differing in class I esterases

Cross:	Pairs: B+C I	B+C IV	C I	C IV	B I	B IV
F_1 (B+C) × F_1 (B+C)	4/23*	12/23	2/8	3/8	0/7	5/7
F_1 (B+C) × C	3/15	6/15	4/9	3/9	0/10	5/10
F_1 (B+C) × B	1/9	4/9	2/2	0/2	0/8	6/8
F_2 (B+C) × F_2 (B+C)	1/5	2/5	1/2	0/2	0/5	4/5

* Of 23 pairs that were B+C, four showed mating type I, etc.

necessarily mutually exclusive since clones of strains A and D, which contain the B group, may manifest mating type I.

DISCUSSION

The application of histochemical techniques to proteins separated by starch gel electrophoresis has shown that variety 1 of *T. pyriformis* contains two classes of esterases: an eserine sensitive class (class I esterases) and an eserine insensitive class (class II esterases). Two groups of class I esterases are found, B and C, and each of these occurs in different inbred strains. Each group of esterases (B or C) contains four to five electrophoretically separated components (isozymes). The isozymes of each group show characteristic levels of activity which are elicited by specific growth conditions. An analysis of the inheritance of the *groups* of class I esterases during asexual and sexual reproduction has revealed the following:

(1) Each group of esterases (B or C) is maintained during asexual reproduction and after intrastrain crosses.

(2) When B is crossed with C, most of the progeny are B in phenotype, and none are C; however, a new class of clones appears in a very few of the hybrids—B+C.

(3) This distribution is repeated in successive backcrosses of "B" hybrids to C.

(4) No dissociation of B isozymes or of C isozymes is observed, although *all* the B and *all* the C isozymes may be produced by a single clone (B+C hybrids).

(5) Exconjugants from the same pair are alike in phenotype.

(6) Clones with both B and C present (B+C) show intraclonal variability, but the rate of change with fissions is low.

(7) B+C clones when inbred or backcrossed to the B or C strains give rise to all three classes (B, B+C and C) in their progeny.

(8) Further inbreeding of a few of the F_2 progeny from a cross of (B+C) × (B+C) has shown that the recovered "pure" clones (B and C) breed true. F_2 B+C clones give rise to all three classes (B, B+C and C) in their offspring.

(9) Mating type I, which is found in strain C but not in strain B, is absent from the B × C pedigree with the exception of two B+C clones. It is present in the offspring of B+C clones but only in progeny which are classed as C or B+C.

The breeding experiments show that the class I esterases are inherited, since the types put into a cross can be recovered in "pure" form in a later generation. Thus, clones differing in phenotype (B and C), when crossed, give rise to offspring which are usually B and occasionally B+C in type. The latter, when crossed, give rise, once more, to the B and C phenotypes, each of which breeds true. The details of these crosses, however, show inconsistencies with one another and rule out a simple genetic explanation.

Several hypotheses can be considered for the control of this system. None of these can be completely rejected at present, and certain aspects of each could be combined into a single hypothesis. The first to be considered is direct genic control of these esterases. The results of the breeding analysis preclude any

simple genetic explanation. However, paramutation (BRINK 1958) could be considered as operating in the crosses of "B" by C clones. Here, the C allele might be inactivated or transformed to B when combined with the B allele. The B+C phenotype might arise in clones in which paramutation had not taken place. The hypothesis runs into difficulty when trying to account for the persistence of effect in the backcross generations unless the ability to cause paramutation is also transmitted.

A second hypothesis which can be considered is particulate in nature. Two types of cytoplasmic particles could be postulated—*b* and *c*. The observation of intraclonal variability in clones containing both groups of esterases lends itself to such an interpretation. An hypothesis similar to that proposed for kappa in *Paramecium aurelia* (SONNEBORN 1959) might be envisioned, although here it might not be necessary to postulate genes in control of the particles. Some of the data support such an hypothesis. Difficulties are encountered when trying to account for the different results of crosses of clones with only *b* particles and of crosses of clones containing *b* and *c* particles.

A synthesis of the particulate hypothesis and a nuclear control system might be found in an hypothesis based upon some type of nucleo-cytoplasmic interaction analogous to that proposed for the mating type and serotype systems of variety 1 of *T. pyriformis* (ALLEN and NANNEY 1958; NANNEY 1959a; NANNEY and ALLEN 1959). In the case of the esterases, a single locus in control of the two groups would be postulated. Superimposed upon this control would be some form of epigenetic mechanism (NANNEY 1958), such as nuclear differentiation, which would be concerned with the expression of these enzymes. Unlike the mating type system in this variety, the exconjugants are alike in their esterase phenotype. Thus, nuclear differentiation must take place very early (ORIAS 1959) or under the influence of a cytoplasmic trigger in order to account for their conformity. If nuclear differentiation occurred in the presence of a cytoplasmic trigger and with cytoplasmic exchange, all the developing new macronuclei would receive the same stimulus, but heterogeneity could arise *within* the macronucleus, and the cytoplasmic trigger could affect the differentiation of the macronuclear subnuclei (ALLEN and NANNEY 1958; NANNEY 1959; NANNEY and ALLEN 1959). Usually the subnuclei would be triggered to differentiate in the B direction in a cross of B × C; but, if some of the subnuclei were differentiated to B and some to C, a heterogeneous macronucleus would result, and the clone would be B+C in phenotype. According to the ALLEN-NANNEY model, segregation of subclones phenotypically pure for B or pure for C should follow with subsequent fissions. A single subclone pure for C was observed. However, the rate of fixation was lower than that predicted on the basis of 45 subnuclei (SCHENSTED 1958).

The chief difficulty with this hypothesis, as with the particulate hypothesis, lies in explaining the difference in outcome of the cytoplasmic triggers in the crosses of "B" × C and in the B+C crosses. If paramutation occurred in the first series of crosses, it would mask the effect of nuclear differentiation, triggered by a cytoplasmic influence, which could operate in the second series of crosses. A combination of events might actually take place. It is hoped that future investi-

gation will reveal the mechanism or mechanisms controlling the inheritance of the esterases.

Whatever mechanism is involved in the control of the class I esterases, the significance of the isozymes should be mentioned. If, indeed, these esterases are under the control of a single locus, each allele must be responsible for several gene products. No dissociation of the isozymes from the group was observed although quantitative variations in their activity could be elicited by particular growth conditions. Whether the isozymes are interconvertible, as was the case with different polymers of bromocresol green (FRANGLEN and GOSSELIN 1958), is not known. If so, it might argue for one of the two possible structural arrangements discussed below.

Multiple gene products have been reported for the haptoglobins of man (SMITHIES and WALKER 1955) and the Beta-globulins of cattle (SMITHIES and HICKMAN 1958). Two interpretations of the observed multiplicity can be made with regard to protein structure (see ANFINSEN 1959, for a review of the 1 gene: 1 polypeptide hypothesis). The polypeptide produced by a gene could form a single protein, perhaps made up of other polypeptides produced by genes at other loci, and this single molecular species might be aggregated to form polymers of various size. Or, the polypepetide produced by a gene could be combined with several different polypeptides to form several different proteins. Recently, SMITHIES (1960) was able to resolve the protein portion of the human haptoglobins into a single molecular species for each allele by thioethanol and urea treatments, which would be expected to break SH-bonds. This result suggested that a single macromolecule is produced by each gene and that polymerization of these in various ways leads to the appearance of multiple gene products.

Similar treatments would be useful in deciding which type of arrangement occurs in Tetrahymena with regard to the esterases. In any event, the active site of the enzyme apparently is not affected by gene substitution, by polymerization, if a single macromolecule is involved, or by its combination with other gene products, if different macromolecules are involved. The ultimate biochemical basis of the class I esterases and the nature of their genetic and epigenetic control remains for the future to decide.

SUMMARY

The application of histochemical techniques to proteins separated by starch gel electrophoresis has shown that variety 1 of *Tetrahymena pyriformis* contains two classes of esterases: an eserine sensitive class (class I esterases) and an eserine insensitive class (class II esterases). Two groups of class I esterases (B and C) are found and each of these occurs in different inbred strains. Each group contains several electrophoretically distinct components or isozymes. The isozymes of each group show characteristic levels of activity which are elicited by specific growth conditions. Each group of class I esterases (B or C) is strictly inherited within a clone during vegetative reproduction and after conjugation within the strain. However, when clones with different groups of esterases are crossed, only the B phenotype is usually observed in the hybrid, although in about

one out of 25 of the hybrids both groups appear. Exconjugants from the same pair are alike in phenotype. This behavior is repeated in successive backcrosses of "B" hybrids to the C parent. In clones containing both groups of esterases (B+C) both groups are maintained with successive fissions, although intraclonal variation is observed in the level of activity of each group. When such clones are crossed, all three classes (B, B+C, and C) appear in the F_2 and in backcrosses to the two parents. The recovered "pure" clones in the F_2, when inbred, breed true, while the B+C F_2 clones, when inbred, give rise to all three classes of progeny. As a working hypothesis, it is suggested that a single locus may be involved in the control of the groups of class I esterases but that the expression of a particular group is influenced by paramutation and/or the interaction of a cytoplasmic trigger and nuclear differentiation.

ADDENDUM

Since submitting this manuscript, the apparent dilemma between the data of Table 3 and Tables 4 and 5 has been resolved. Two phenomena were confused. The C strain clone used as a parent in crosses (Tables 3 and 4) has been found to act as a leaky "genetic dud". Using normal members of the C strain, F_1 hybrids (B × C) are initially B+C in phenotype but with subsequent fissions "differentiate" to B or to C. No carry-over of this differentiation to subsequent generations occurs and good genetic ratios characteristic of a single locus are obtained.

ACKNOWLEDGMENTS

The technical assistance of MRS. ILSE L. MUNYON, MISS KAREN P. SEARS, MR. KEITH A. ARNOLD, and MR. ROBERT A. BERKOFF is gratefully acknowledged.

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