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Effect of Acetate on Esterase C Activity During the Growth Cycle of *Paramecium**

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SYNOPSIS. Enhanced esterase C activity could be demonstrated by starch gel electrophoresis in various stocks of *Paramecium* spp. (*P. primaurelia* stocks 90 and 540, *P. biaurelia* stock 93, *P. tetraurelia* stock 29, *P. pentaurelia* stock 87, *P. octaurelia* stocks 31 and 300, and *P. multimicronucleatum* species 3, stock 8 MO) grown in Adaptation Medium. This esterase, however, was barely detectable when they were cultivated in Axenic Medium. Addition of trypticase to Adaptation Medium resulted in reduction of esterase C in the ciliates. This effect is ascribable to Na acetate present in trypticase. Since esterase C increased with the decrease in acetate concentration (as estimated by gas-liquid chromatography) during growth of *Paramecium*, acetate appears to be utilized by the cells. Sensitivity of esterase C to acetate occurs in all 6 species of *Paramecium* examined. Different stocks *within* a species may have different levels of sensitivity; in one case this is genetically determined. The results emphasize the importance of controlling and manipulating growth conditions for the assessment of inter- and intraspecies variations in the isozymes of *Paramecium*.

Index Key Words: *Paramecium primaurelia* stocks 90 and 540; *P. biaurelia* stock 93; *P. tetraurelia* stock 29; *P. pentaurelia* stock 87; *P. octaurelia* stocks 31 and 300; *P. multimicronucleatum*, species 3, stock 8 MO; esterase C; inhibition by acetate; Adaptation Medium; Axenic Medium; starch gel electrophoresis; gas liquid chromatography.

THE pattern of isozymes revealed by gel electrophoresis is commonly used as a means for assessing the degree of variation within and between species and can be translated into a measure

of evolutionary distance. The variations observed in gels are limited to differences in mobility, heat stability, or activity. Activity differences may range from complete absence of an enzyme to slight differences, which may, or may not, be determined by genetic factors. The effect of a null allele may be mimicked by an environmental effect which inhibits the expression of an isozyme. In species comparisons where genetic data cannot be obtained it therefore becomes important to determine whether an environmental effect exists, and, if present, to characterize it.
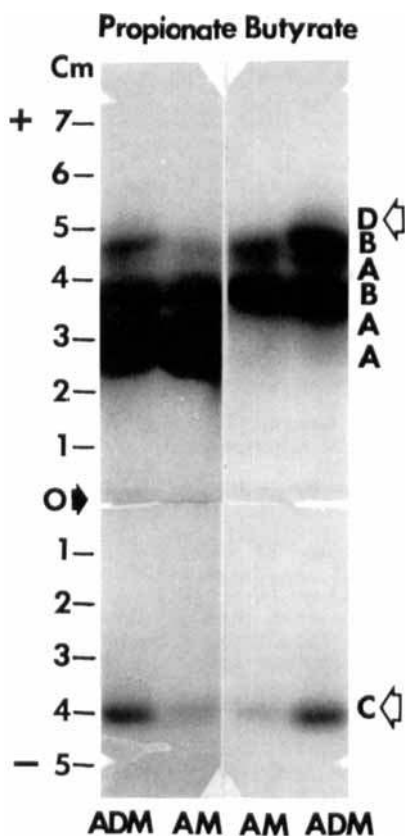
Propionate Butyrate

Fig. 1. Comparison of the esterases of *P. tetraurelia*, stock 29, grown in Adaptation Medium (ADM) and Axenic Medium (AM). The esterases are identified in terms of their class (A,B,D, or D). All classes are demonstrated using α-naphthyl propionate as substrate, but only the B,C and D esterases appear with α-naphthyl butyrate as substrate. The esterases of particular interest (cathodal C and anodal D) are indicated by the open arrows. Distances in migration are marked off in centimeters from the origin (O) on the left margin (Figs. 1-6).

For some time we have been concerned with assessing the intra- and interspecies variation in the isozymes of the 14 species of the *aurelia* complex of *Paramecium* (1, 5-7, 9, 10). Recently we extended our analysis to include 4 (or more) other species complexes. All species of *Paramecium* normally feed on bacteria. Since the presence of living bacteria not only complicates observations on isozymes, but bacterial products can be detected (5, 13, 18), the ciliates are transferred to axenic culture for enzyme analysis. In devising a method for the simultaneous establishment of many axenic cultures, we found that media which contained autoclaved bacteria supported vigorous and indefinite growth of all stocks examined from 22 different species of *Paramecium* (8). Indefinite growth, however, is not supported in a medium lacking autoclaved bacteria. This difference suggests that the autoclaved bacteria contribute additional growth factors. Are there also differences in the expression of enzymes of *Paramecium* grown in different media?

It was evident from examination of the esterases that there are indeed differences in expression. In paramecia there are 4 different classes of esterases (A, B, C, and D) which are characterized on the basis of substrate specificity and sensitivity to the inhibitor eserine. Of particular interest is a difference in the expression of a C esterase in paramecia grown in different media. This esterase was shown earlier to be "present" in most species
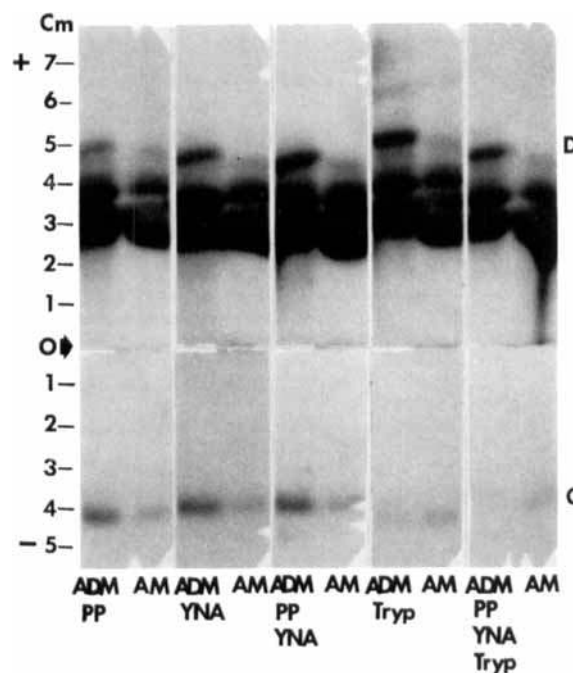

Fig. 2. Comparison of the C (and D) esterases of *P. tetraurelia*, stock 29, grown in Axenic Medium (AM) and Adaptation Medium (ADM) with the addition of components from Axenic Medium. PP, proteose peptone; YNA, yeast nucleic acid; Tryp, trypticase. The substrate is α-naphthyl propionate.

of the *aurelia* complex but either "absent" or "weak in activity" in other species. In addition a difference in activity between stocks of *P. primaurelia* was shown to be due to a single gene difference (11). In this paper we show that this esterase is sensitive to a particular factor present in the growth medium of Soldo et al. (19).

MATERIALS AND METHODS

Stocks

Axenically grown stocks derived from 6 species of *Paramecium* were used: *P. primaurelia* stocks 90 and 540, *P. biaurelia* stock 93, *P. tetraurelia* stock 29, *P. pentaurelia* stock 87, *P. octaurelia* stocks 31 and 300, and *P. multimicronucleatum*, species 3 stock 8 MO. Species names were introduced recently by Sonneborn for each of the species in the *P. aurelia* complex (22) thereby supplanting the numbered species designation introduced previously (21) and the older designation, syngen (20).

Culture Media

Axenic Medium.—The individual components of this medium, proteose-peptone, trypticase, $MgSO_4$, yeast nucleic acid, TEM-4T (tartaric acid esters of beef tallow monoglycerides), stigmasterol, and vitamins, were added at the final concentrations specified by Soldo et al. (19). The method of preparation of this medium was described previously (8).

Maintenance Medium.—This medium consists of the same components as Axenic Medium, and in the same final concentrations; however, it also contains autoclaved bacteria (final concentration: 100 μg dry weight/ml; usually *Enterobacter aerogenes* unless otherwise specified).

Adaptation Medium.—We described this medium in a previous report (8). It contains Peter's salts solution, autoclaved bacteria
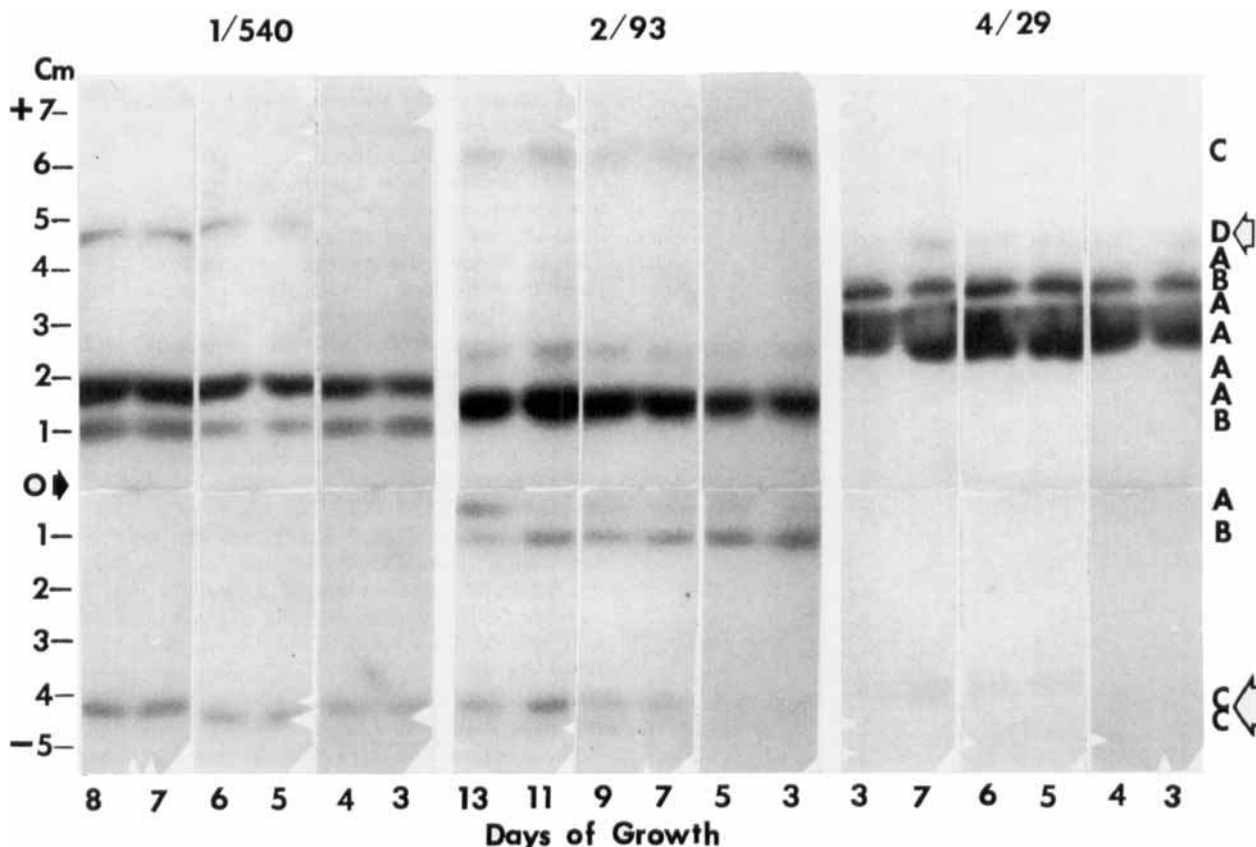
Fig. 3. Comparison of the C (and D) esterases during the growth cycle in Axenic Medium in *P. primaurelia* stock 540, *P. biaurelia* stock 93 and *P. tetraurelia* stock 29. The esterases of the stocks are identified as to class on the right of the photograph, the particular C and D esterases of interest being indicated by the open arrows. The substrate is α-naphthyl propionate. The days of growth of each sample are indicated below the gels.

(final concentration, 100 μg dry weight/ml, and usually *E. aerogenes* unless otherwise specified), and stigmasterol and vitamins at the same final concentration as in Axenic Medium.

Biochemical Analyses

Identification of Esterases.—For enzyme extracts, all cultures were grown at 23 C and harvested by centrifugation; extracts of the cell concentrates were made by freeze-thawing. Electrophoresis of whole-cell extracts was carried out in 12% starch gels using Electrostarch, as described previously (5, 7, 9, 10). Two substrates were used routinely: α-naphthyl propionate and α-naphthyl butyrate. All esterases are observed in a reaction mixture which includes α-naphthyl propionate, while one class is missing (the A esterases) in the reaction mixture containing α-naphthyl butyrate. Details of the histochemical procedures may be found elsewhere (5).

Gas-Liquid Chromatography.—Quantitative analyses of the acetate concentrations in the medium were performed using a Packard Gas Chromatograph, Model 7401, equipped with a flame ionization detector (Packard Instrument Company, Inc., Downers Grove, Illinois). The samples to be analyzed were prepared by passing 5 ml of a *Paramecium* culture through a 0.45 μ Millipore filter. Two-ml of each filtrate were then acidified with 0.3 ml of 3% (w/v) metaphosphoric acid and frozen. On the day of analysis the samples were thawed and centrifuged at 12,000 rpm in a microfuge (Microfuge 152, Beckman Instruments, Southfield, Michigan) for 10 min. Three-μl aliquots of the supernatant fluid were injected into the gas chromatograph. Nitrogen served as the

carrier gas at a flow rate of 30 ml/min. The hydrogen flow rate was maintained at 30 ml/min and the oxygen flow rate at 400 ml/min. The inlet and detector temperatures were 145 C, while the column temperature was maintained at 135 C. The volatile fatty acid column was prepared by coating 1.4 g of FFAP (Varian Aerograph, Walnut Creek, California) on 10 g of Chromosorb W (60/80 mesh; Johns Manville Co., obtained from Anspec Co., Inc., Ann Arbor, Michigan). The column was conditioned overnight at 180 C. The concentration of acetic acid in the sample was computed relative to standards with known concentrations of this compound, according to the method developed by Dr. David P. Chynoweth, School of Public Health, University of Michigan.

RESULTS AND CONCLUSIONS

Effect of the Medium on the Expression of the Esterases.— Flasks containing a liter of Adaptation Medium were inoculated with 100 ml of a 4-day flask-culture of *P. tetraurelia* stock 29, growing in Adaptation Medium, and were grown for 4 days. Flasks containing a liter of Axenic Medium were inoculated with 100 ml of a 7-day flask-culture of stock 29, growing in Axenic Medium, and were grown for 7 days. Extracts were prepared, starch gel electrophoresis was carried out, and the gels were incubated in the appropriate histochemical reagents for the esterases. The esterases observed for the 2 substrates, α-naphthyl propionate and α-naphthyl butyrate are compared in Fig. 1. The patterns are similar except for 2 esterases—a cathodally
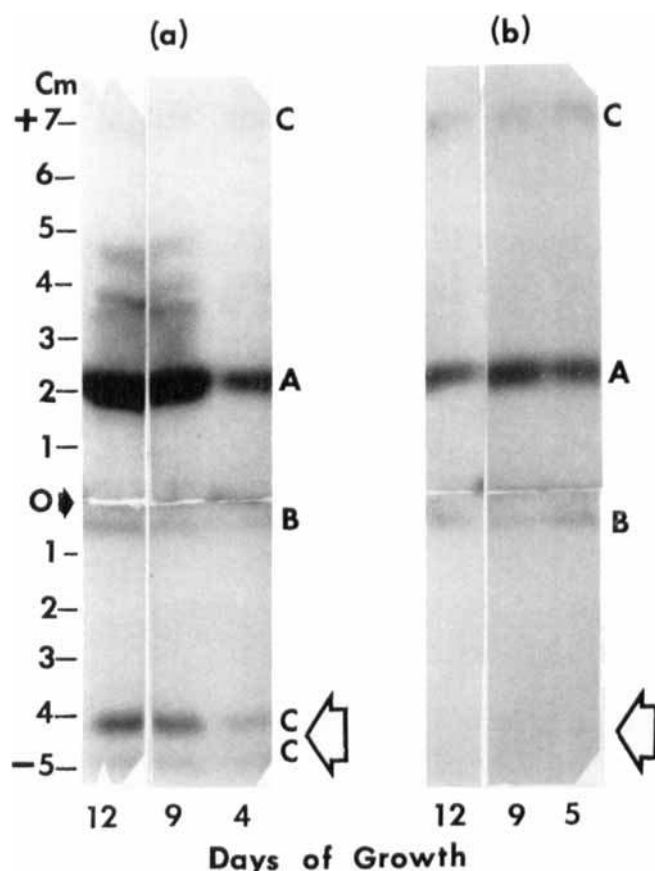
Fig. 4. Esterases during the growth cycle of *P. biaurelia*. stock 93, grown in modified Axenic Media. (a) enhancement of esterase C when trypticase is removed; (b) disappearance of esterase C when 10 mM sodium acetate is substituted for trypticase. Open arrows indicate regions of interest. The days of growth of each sample are indicated below the gels. The substrate is α-naphthyl propionate.

migrating C esterase, present in both media but much more intense in Adaptation Medium, and a rapidly migrating D esterase, barely detectable in Axenic Medium and very intense in Adaptation Medium.

Effect of the Type of Lipids on the Difference in Expression of the Esterases.—To investigate whether substitution of bacterial lipids for TEM-4T (tartaric acid esters of beef tallow monoglycerides), a component of Axenic Medium, might affect the expression of the esterases, 4 different bacterial species. the Gram-positive, *Staphylococcus aureus* and *Streptococcus fecalis*, and the Gram-negative *Enterobacter aerogenes*, and *Salmonella typhimurium*, were used in the following experiments. The bacteria were grown in Tryptic Soy Broth to the mid-log stage of growth. The cells were then washed 2× by centrifugation in sterile Peters' salts solution. and the final pellet was resuspended in Peters' salts solution and autoclaved at 15 lbs, 121 C for 15 min. An autoclaved suspension was then added to a liter of Adaptation Medium to give a final concentration of 100 μg dry weight/ml. A similar amount of bacterial suspension was also added to a liter of Axenic Medium minus TEM-4T. As controls we employed complete Axenic Medium and Maintenance Medium. Experimental and control media were then inoculated with 100 ml of a culture of *P. tetraurelia* stock 29, growing in the same medium. Cultures containing autoclaved bacteria were harvested when the media were observed to be clear (the sus-

pended bacteria were removed). Cultures in Axenic Medium were harvested after 7 days of growth.

When the esterases of stock 29 growing in the various media were compared, the patterns observed were very similar regardless of the type of bacterium present or the type of medium, *with 2 exceptions*. The cathodal C esterase was almost absent in Axenic Medium with autoclaved bacteria minus TEM-4T. It was present in complete Axenic Medium or in Maintenance Medium, but was much more active in Adaptation Medium. The D esterase, in contrast, appeared to be very active in Adaptation Medium and in Axenic Medium minus TEM-4T, but much less active in complete Axenic Medium or in Maintenance Medium. The behavior of these esterases is not correlated in the same way with the presence or absence of TEM-4T. For the D esterase, low activity is correlated with the *presence of TEM-4T*, suggesting that some component(s) of TEM-4T affects the expression of this esterase. For the C esterase, low activity is correlated with the *presence of Axenic Medium* (and the absence of TEM-4T). This observation suggested to us that some other component of Axenic Medium not found in Adaptation Medium (proteose-peptone, trypticase, or yeast nucleic acid) was responsible for the observed decrease of this esterase activity. The fact that the activity of esterase C was faint or not detectable in Axenic Medium minus TEM-4T suggested that more of this factor was left at the time of harvesting than in complete Axenic Medium. This inference seemed logical since a lower population density of *Paramecium* was observed at plateau phase in Axenic Medium without TEM-4T than in complete Axenic Medium.

Effect of the components of Axenic Medium on Esterase C Activity.—To determine if proteose-peptone, yeast nucleic acid, or trypticase, present in Axenic, but not in Adaptation Medium, were responsible for reduction of the cathodal C esterase activity, these components were added to the latter medium individually or in combination (proteose-peptone + yeast nucleic acid or proteose-peptone + yeast nucleic acid + trypticase). In addition to the 5 variously supplemented batches of Adaptation Medium, a batch of Axenic Medium was employed. Each of these batches was inoculated with *P. tetraurelia* stock 29, growing in the same medium. The cultures grown on batches of the supplemented Adaptation Medium were harvested when the medium was cleared of bacteria; that grown in Axenic Medium was harvested on postinoculation day 7.

The esterase patterns are shown in Fig. 2. Note that when proteose-peptone, or yeast nucleic acid, or proteose-peptone and yeast nucleic acid were added, the activity of esterase C was high (and similar in activity to Adaptation Medium without any additions). Additions of trypticase, or trypticase in combination with proteose-peptone and yeast nucleic acid, resulted in reduction of esterase C activity, less activity being observed with the combination of components. This observation suggests that a factor found in trypticase, but also present in small amounts in other components of the medium, causes reduction in esterase C activity.

Does the Pattern of Esterase C Activity change during the Growth Cycle?—In this experiment 2 different stocks of *Paramecium* (*P. primaurelia* stock 540 and *P. tetraurelia* stock 29) each growing in Adaptation and Axenic Medium were harvested after increasing numbers of days of growth. In addition, samples of *P. biaurelia* stock 93, growing in Axenic Medium, were examined after different postinoculation days. When stocks 540 and 29 were grown in Adaptation Medium, the activity of esterase C remained high throughout the growth cycle. As shown in Fig. 3, however, when the 3 stocks were cultivated in Axenic Medium, esterase C increased in activity as the cultures aged. This pattern of expression might be expected if the factor in trypticase respon-
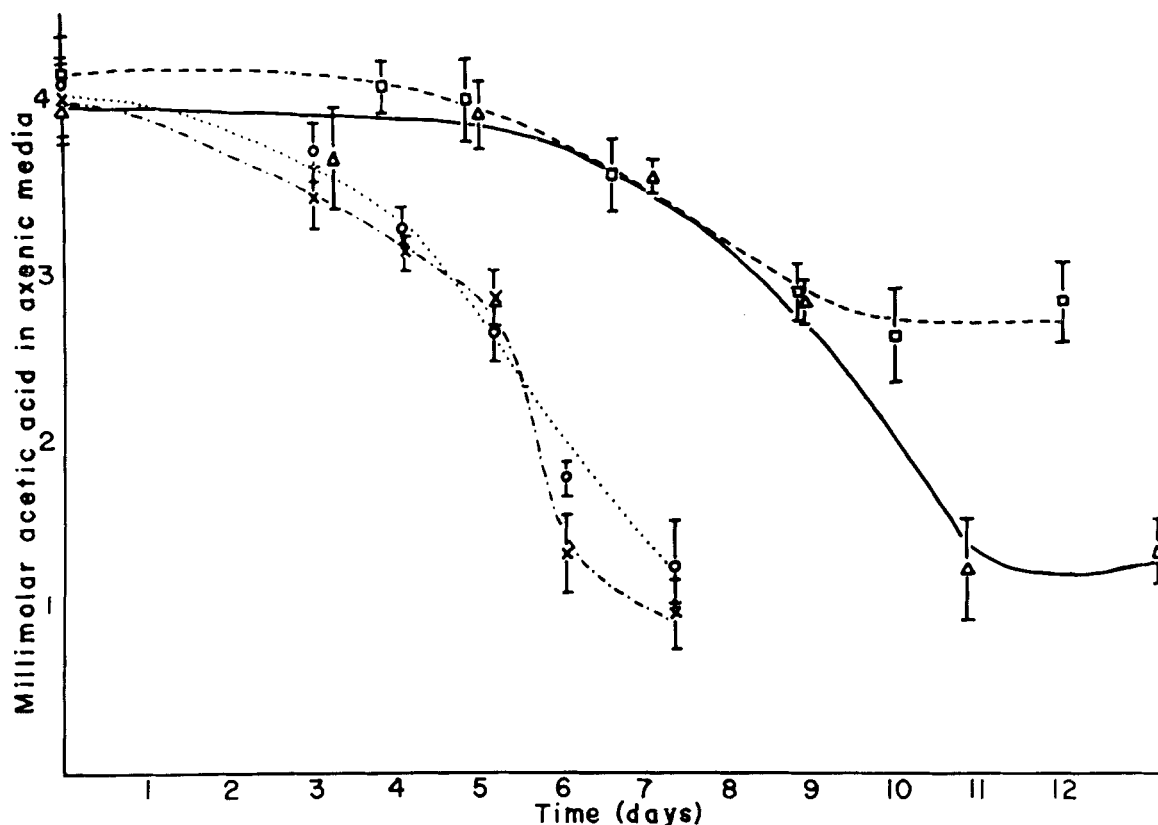
Fig. 5. Acetic acid concentration in Axenic Medium during the growth cycle of *P. primaurelia* stock 90 ($\square$ – – – $\square$); *P. primaurelia* stock 540 ($\times$–·–·$\times$); *P. biaurelia* stock 93 ($\triangle$——$\triangle$); *P. tetraurelia* stock 29 ($\bigcirc$– – –$\bigcirc$). For each sample the mean measurement is given, the bar indicating 95% confidence limits.

sible for reduced esterase C activity, and present in Axenic but not in Adaptation Medium, is slowly being used up during the growth cycle.

Is the component affecting Esterase C Activity used up during growth?—If esterase C activity is enhanced because a factor present in trypticase is used up during growth, we might expect that *without trypticase* in Axenic Medium esterase C should be active throughout the growth cycle. This possibility was tested with *P. biaurelia* stock 93, grown in Axenic Medium without trypticase. Samples, harvested on 4, 9 and 12 postinoculation days, were examined for their esterase activities (Fig. 4a). After 4 days of growth in this medium esterase C was as active as in extracts taken from 7-9 day cultures growing in complete Axenic Medium. By 9 days the intensity of the band was greater than that observed at any stage in the growth cycle in the latter medium.

A clue as to the identity of the factor responsible for reduced esterase C activity came from the statement made by van Wagtendonk (23) that a mixture composed of Na acetate, $CaCl_2$ and the trace elements Fe, Zn, Mn and Cu could be substituted for trypticase in Axenic Medium. We tested this by replacing trypticase with 10 mM Na acetate and growing *P. biaurelia* stock 93 in this medium for 5, 9 and 12 days. Esterase C was not detected in any of the samples taken throughout the growth cycle (Fig. 4b). From these experiments we conclude that the factor in trypticase to which esterase C is sensitive is Na acetate and that this compound is used up during the growth cycle. It was evident from measurements of the amount of acetate in the various components of Axenic Medium by gas-liquid chromatography that 93% of the acetate is contributed by the trypticase com-

ponent. This distribution correlates with the previous finding that most, but not all, of the observed reduction in esterase C activity could be attributed to the presence of trypticase.

Do other Species of Paramecium *have an Acetate-sensitive Esterase C?*—In the 1st experiment, 2 different species of *Paramecium* and 2 different concentrations of acetate were used. *Paramecium primaurelia* stock 540, and *P. tetraurelia* stock 29 were grown in Adaptation Medium, Adaptation Medium with 1 mM Na acetate or in Adaptation Medium with 10 mM Na acetate. In the presence of 1 mM Na acetate the activities of the cathodal C esterases were very weak; they were totally absent in the presence of 10 mM Na acetate. In the 2nd experiment *P. biaurelia* stock 93, *P. pentaurelia* stock 87, *P. octaurelia* stocks 31 and 300, and *P. multimicronucleatum*, species 3, stock 8MO were grown in Adaptation Medium with and without 10 mM Na acetate. Whenever acetate was present, the cathodal C esterases were not detected. These stocks have C esterases of different mobilities; thus, despite the differences in mobility (and the presumed genetic alteration which changes mobility), all these enzymes are sensitive to acetate. We conclude that acetate sensitivity is a general response of esterase C in species of *Paramecium*.

Does the Acetate Concentration deline with Growth in Axenic Medium?—*Paramecium primaurelia* stocks 90 and 540, *P. biaurelia* stock 93, and *P. tetraurelia* stock 29 were cultivated in Axenic Medium and harvested at different stages of growth. For each sample, the concentration of acetate in the culture was determined by gas-liquid chromatography and the esterase patterns by starch gel electrophoresis. The results of the acetate
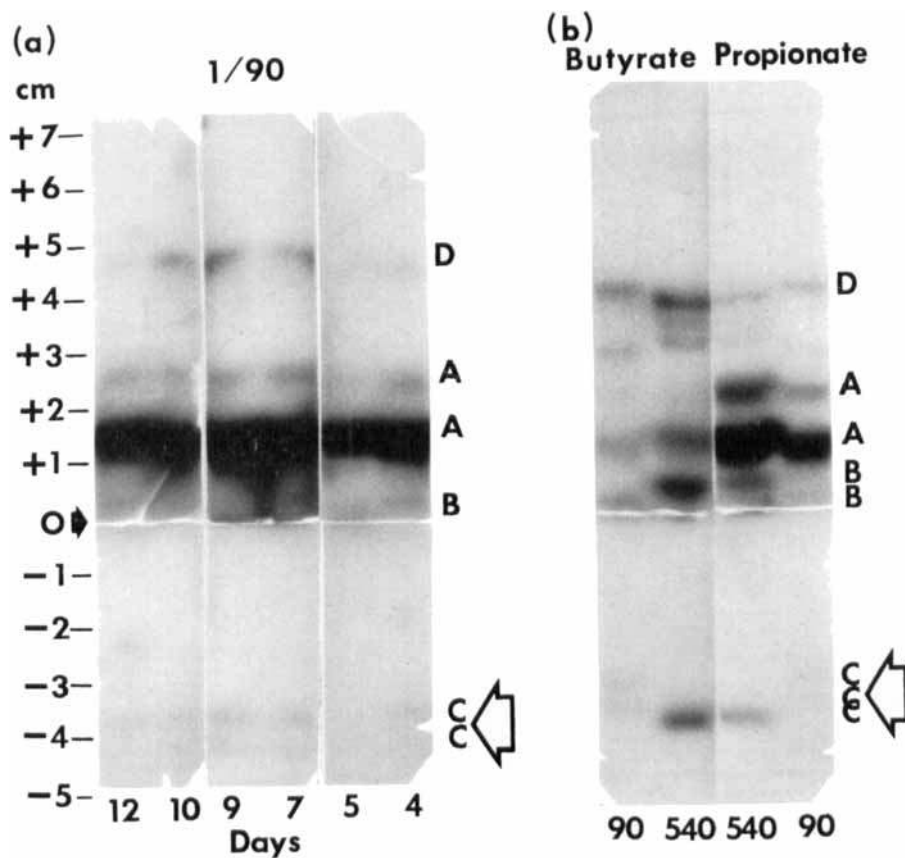
Fig. 6.   Esterase C activity in stock 90 of *P. primaurelia* (a) during the growth cycle in Axenic Medium; (b) compared to stock 540 of *P. primaurelia* in Adaptation Medium. The esterases of the stocks are identified as to class on the right of the photographs. The C esterases of interest are indicated by the open arrows. In (a) the days of growth of each sample are indicated below the gels, and the substrate is α-naphthyl propionate. In (b) the results with both α-naphthyl propionate and α-naphthyl butyrate are shown.

determinations are shown in Fig. 5; those of the gel analysis are shown in Figs. 3 and 6.

In general, we conclude that as the acetate concentration is reduced the activity of the C esterases increases. Differences among stocks, however, are observed. They depend, in part, on differences in growth rate and final population density, and on inherent differences in sensitivity to acetate. Stocks 29 and 540 grow more rapidly and reach higher densities than do stocks 90 and 93. Thus, there is a temporal delay in the acetate concentration decrease in the latter 2 stocks, and this delay is accompanied by a delay in the appearance of the C esterases. Innate differences in sensitivity also occur, the C esterase in stock 540 being more active early than in other stocks. Of particular interest is the difference in esterase C activity between stocks 90 and 540 (Figs. 3, 6), both members of the same species, *P. primaurelia*, in which a single gene has been found to control this difference (11). In old cultures of stock 90 the C esterase appears as a faint doublet (Fig. 6a). In Adaptation Medium, it also appears as a faint doublet, and we can see that it differs from the allozyme in stock 540 in mobility as well as in activity (Fig. 6b).

DISCUSSION

The cathodally migrating C esterase is widespread in various species of *Paramecium* (5, 10). It can catalyze α-naphthyl acetate, although very poorly compared to its ability to catalyze α-naphthyl propionate or α-naphthyl butyrate. It is insensitive to high concentrations of eserine sulfate (5). We can now add one

more property of this enzyme: during growth of *Paramecium* it is sensitive to acetate; therefore, its expression reflects the level of acetate present in the medium. Axenic Medium contains acetate primarily in trypticase. As acetate is consumed during the growth cycle of *Paramecium*, the acetate concentration in the medium decreases and the esterase C activity increases. We do not know what the real function of this enzyme is in the cell, nor do we know how it is affected by acetate.

Genetic factors as well as the conditions of growth determine the pattern of esterases observed after gel electrophoresis. All 4 classes of esterases are present in all species of *Paramecium* so far examined (Allen & Nerad, in preparation). Single gene differences have been found for mobility differences of the A and B esterases between stocks of the same species (7, 11). Variations among stocks in the thermal stability of A or B esterases with similar mobility have been observed, implying further genetic differences (6). Differences in the activity of certain A esterases in *P. biaurelia* and *P. octaurelia* have also been noted (7, 11), and in the case of *P. octaurelia* they appear to be genetically controlled. The cathodal C esterase also varies in expression which is both genetically (11) and environmentally determined, through its sensitivity to acetate.

The conditions of growth appear to be important for expression of certain esterases (7). We know that the presence of living bacteria can alter the activity of particular esterases as well as contribute new esterases (5, 13, 18). The presence of particular components in the growth medium also influences the activity of particular C and D esterases, as shown in this paper. In both

cases enhancement of activity is due to the removal of a factor present in Axenic Medium. For the C esterase, the factor is acetate present chiefly in trypticase. For the D esterase, a factor is present in TEM-4T (with some effect exerted also by acetate). The observed expression of these esterases depends upon when the cultures growing in Axenic Medium are examined during the growth cycle. "Old" cultures have higher esterase C activity because the concentration of acetate in the medium has been reduced.

The effect of growth conditions on the expression of enzymes is complex and not unique to *Paramecium*. In *Tetrahymena thermophila* (the new name for syngen 1 of *T. pyriformis*; see Ref. 15) the activities of individual esterase isozymes are sensitive to growth cycle changes (2, 3). Some of the acid phosphatases in this species also respond to growth conditions (4). In other eukaryotic microorganisms, such as *Aspergillus, Chlamydomonas*, or *Neurospora*, a complex system of genetic and environmental factors determines the activities of particular phosphatases (12, 14, 16, 17). We do not know the manner of interaction between genetic and environmental factors in *Paramecium* for any of the enzymes which respond to growth conditions, i.e. whether "synthesis" or "activity" is responsible for the changes in band intensity in gels.

The fact that some of the esterases respond to growth conditions and that in some cases they change in expression with the growth cycle is important information for assessing intra- and interspecies variability. It means that in screening large numbers of stocks we must control very carefully the conditions of growth for each stock. Where we know that a particular enzyme is sensitive to particular growth conditions, as in the case of esterase C, we can use this information to identify homologous enzymes in different species.

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BOOK REVIEW . . .

Starr, M. P., Ingraham, J. L. & Balows, A., eds. 1977. *Annual Review of Microbiology*. Vol. 31. Annual Reviews, Inc., 4139 El Camino Way, Palo Alto, CA 94306. 695 pp. $17.00 in USA, $17.50 elsewhere.

This book contains only 2 articles that deal with protozoa, and these are the only ones that I shall deal with. A. G. Fredrickson (University of Minnesota) wrote 25 pages with 116 references on *Behavior of mixed cultures of microorganisms*, and Chandler Fulton (Brandeis University) wrote 33 pages with 127 references on *Cell differentiation in* Naegleria gruberi.

The role of protozoa in mixed cultures is said to be primarily that of predation, mostly on bacteria. There are many unsolved problems in the predator-prey relationship, including the mitigating factors that allow both populations to co-exist. Fredrickson stresses quantitative relationships.

Cell differentiation is defined differently by almost everyone who works on it. Fulton is concerned with the ameba-flagellate transformation of *Naegleria*, and especially with the events that occur during transformation. Most of his discussion deals with the formation of flagellates from amebae, but part of it deals with the reverse. This is an interesting paper about a puzzling phenomenon. What goes on here? Answer this question, and you may have a clue to the evolution process.—NORMAN D. LEVINE, *College of Veterinary Medicine, Univ. of Illinois, Urbana IL 61801, USA*.