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ANALYSIS OF THE TIME OF UTILIZATION OF PROTEINS FOR THE INITIATION AND COMPLETION OF DIVISION IN SYNCHRONIZED *TETRAHYMENA PYRIFORMIS*

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SUMMARY

We exposed division-synchronized *Tetrahymena pyriformis* to pulse treatments with the structural amino acid analogue *p*-fluorophenylalanine. We related the timing of the treatments to the delays in onset or completion of division, or both. The results enable us to distinguish between early and late phases in cell division in which the cells bring into action proteins which contribute to initiate and complete division, respectively. Normally, these events are separated by 25–30 min.

INTRODUCTION

Many organisms incorporate *p*-fluorophenylalanine (*p*-FPhe), a structural analogue of phenylalanine, into their proteins. Such 'analogue proteins' have in general reduced biological activity (see Wheatley (1978) for a recent review). Rasmussen & Zeuthen (1962) reported that *Tetrahymena* passes from a *p*-FPhe-sensitive to a *p*-FPhe-insensitive phase at a specific time in its cell cycle. Later, Hoffmann, Rasmussen & Zeuthen (1970) found that *Tetrahymena* incorporates *p*-FPhe at a substantial rate. Together these results suggest that cell division in *Tetrahymena* depends on the prior synthesis of proteins and that *p*-FPhe can prevent the proper functioning of these compounds (Zeuthen & Rasmussen, 1972).

Short exposures of cells to *p*-FPhe have proved excellent in resolving events in the progression of cells into and through division. They have made it possible to differentiate between early and late requirements for accomplishments of cellular and nuclear divisions and it is upon these analyses that we now report.

METHODS

Tetrahymena pyriformis (Nanney & McCoy, 1976), previously *T. pyriformis*, phenoset A, GL-8 (Borden, Whitt & Nanney, 1973), was grown under axenic conditions in 2 % proteose peptone (Difco Laboratories) supplemented with liver extract and salts (Plesner, Rasmussen & Zeuthen, 1964). Synchronous cell divisions were induced by 7 heat shocks, each lasting 30 min and spaced with intervals of 30 min (Scherbaum & Zeuthen, 1954). The cells were

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transferred to an inorganic starvation fluid (Hamburger & Zeuthen, 1957) at the end of the sixth shock by 3 washings in a hand centrifuge (Plesner *et al.* 1964). The cells were kept under starvation conditions at an initial population density of either 2000 or 20000 cells/ml for single cell and mass culture experiments, respectively. The time point of the end of the last heat shock is designated EH. See Plesner *et al.* (1964) for a technical description.

Mass culture experiments

Sub-populations were established by removing samples – usually 15 ml – from a main culture at preselected times after EH. Treatment with 0·4 mM DL-*p*-fluorophenylalanine was terminated by addition of 3 mmol L-phenylalanine/l. Control experiments had shown that this latter addition did not delay cell division. *p*-FPhe and Phe were therefore added from 10× stock solutions to subcultures at preselected times to give the required pulse duration.

Cells were kept under observation in a Zeiss stereo-microscope and the division index, i.e. the fraction of cells in division, was estimated and noted at short intervals. In addition, 0·5-ml samples were removed from each of the sub-populations at preselected intervals, fixed immediately in 4% formaldehyde and counted in a calibrated electronic cell counter.

Single-cell experiments

Both untreated control cells and cells treated with *p*-FPhe in a short pulse were set out in single drop cultures. Isolations of cells were performed immediately after Phe was added to the culture. Samples of a cell suspension containing 2000 cells/ml were quickly distributed with a braking pipette (Holter, 1943) in 2-μl drops into sterile plastic Petri dishes. We covered the drops with a thin layer of sterile paraffin oil in order to protect them against evaporation. Drops which initially contained only 1 or 2 cells were kept under constant observation at 28 °C with a Zeiss stereo-microscope, and the time when cells were observed in the process of division and the time point of completion of cell division (cell separation) were both noted.

RESULTS

Following the synchronizing heat shocks, *Tetrahymena* resuspended in inorganic medium show 2 synchronous division peaks (Fig. 1, frame I). These occur about 80 and 190 min after the end of the seventh heat shock (EH_7), the second being broader and lower than the first. Fifteen-minute pulses with 0·4 mM *p*-FPhe given to cultures on a staggered basis between EH + 10 min and EH + 60 min resulted in the series of curves shown in Fig. 1, frames II–VII, frame I being the untreated control. The pulses of *p*-FPhe were reversed in each case by the addition of 3·0 mmol phenylalanine/l. The curves include both cell multiplication and division index parameters. Frame I, the control, shows that the cell number increases to 180% of the initial value when the first division peak has been accomplished. Frames II–IV show that an increasingly larger fraction of the cells delays entering into cytokinesis to a greater extent the later the pulse begins. The first division peak now appears divided into 2 distinct attempts at cytokinesis, as exemplified by frame IV in which the completion of the *first round of division* covers more than the time required for the control culture to go through 2 complete rounds of division. In addition, the ascending and descending limbs of the first division peak become progressively wider apart, suggesting a more protracted period of division. Frames V–VII indicate that when the pulse treatment is given at EH + 40 min or later 2 peaks of cytokinetic activity are observed *before* the second peak appeared in the control. The first division

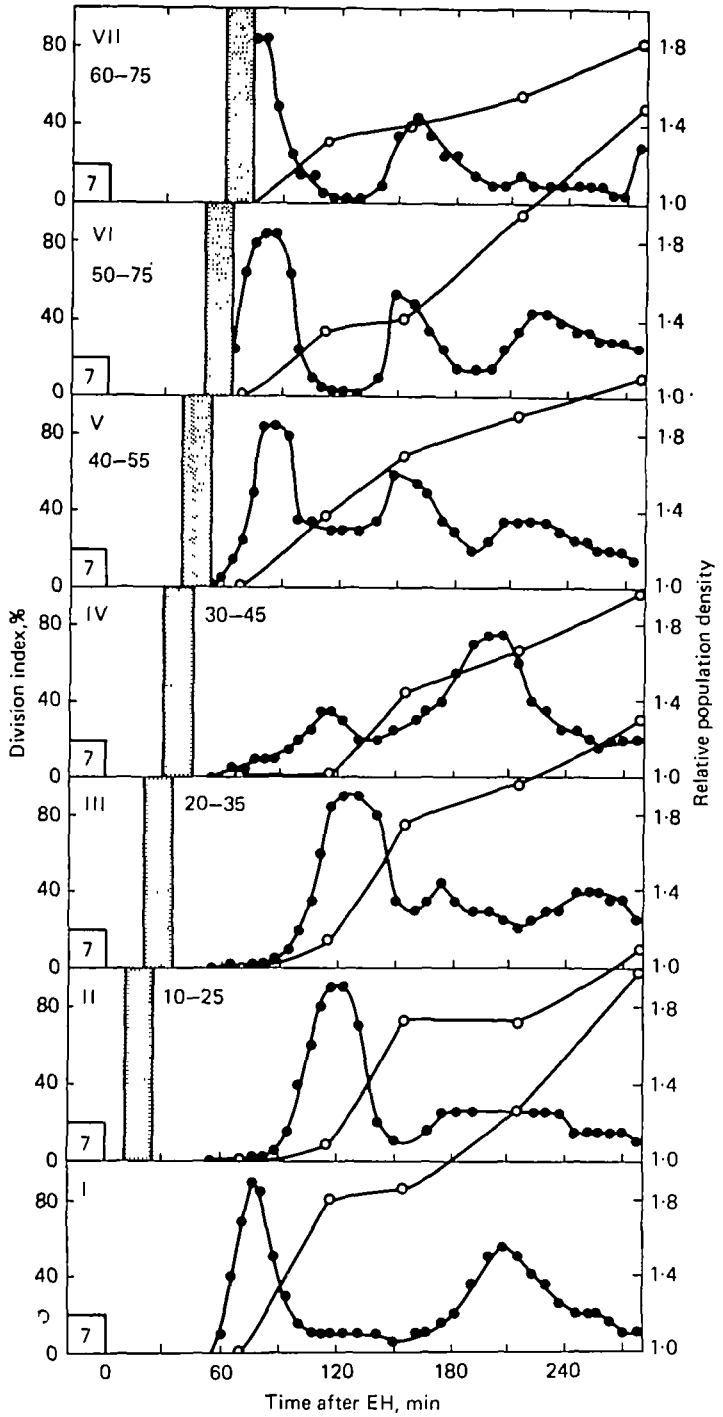

Fig. 1. Division index (left ordinate, ●) and cell multiplication (relative values, right ordinate, ○) in a heat-synchronized mass culture of *Tetrahymena pyriformis* (frame I). Same parameters as a function of pulse treatments with *p*-fluorophenylalanine applied during the shaded intervals (frames II–VII). Note that cell multiplication values establish that the majority of cells divided at their first attempt (I–III); in contrast, only a minority of the cells entering the first division peak completes it without long delays (V–VII).

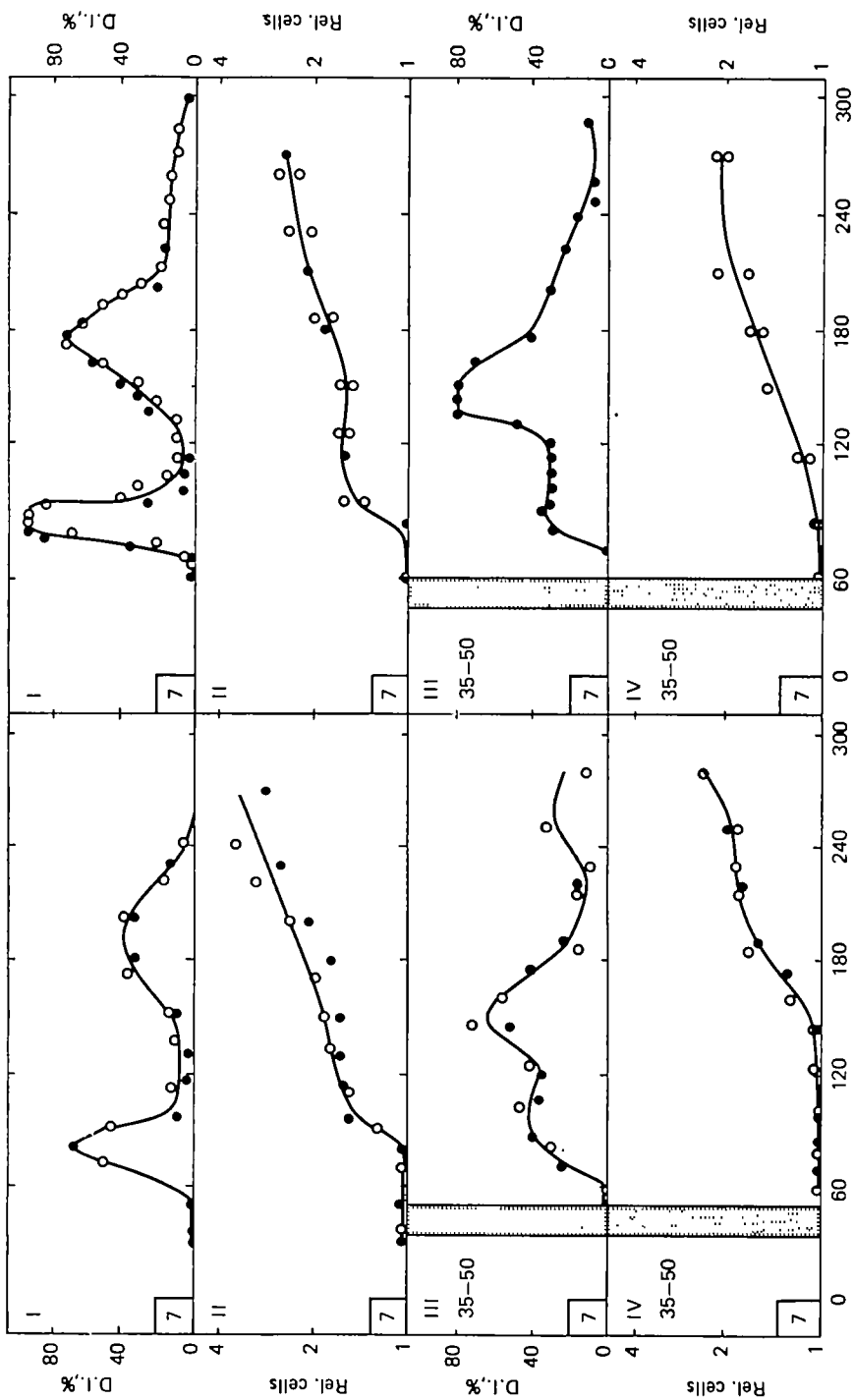

Fig. 2. Left-hand side: division index (frame I) and cell multiplication (relative values, II) based on observation of single cells in small drops of medium. The cells were isolated from a synchronized mass culture of *Tetrahymena pyriformis*. Same parameters for cells pulse treated with *p*-fluorophenylalanine during the shaded time interval (III and IV). Right-hand side: division index and cell multiplication in the mass culture used for isolation of single cells. Note the agreement between results obtained in single cell experiments (left) and mass cultures (right).

peak occurs at the same time as in frame I, but only a fraction of the cells actually complete division (see curves for cell counts). One portion of those cells which fail to divide remains in division (V), but they may also fully revert the division furrow and re-initiate division (second fraction of first peak) after a lag of 1 h (VI). This second attempt at division is successful (VI) or less clearly so (VII). Thus there is a differential response of the cells depending on the time of treatment: both entry and completion are progressively delayed in frames II–IV: entry is not delayed, but completion is in a major fraction of the cells in frames V–VII.

In order to verify that cells remained in division for long periods, synchronized cells were isolated in small drops of inorganic medium. They were constantly monitored microscopically for division stages and multiplication (Fig. 2, left-hand side). Frames I and II in this figure relate to phenylalanine-treated control cells. Division index (frame I) was calculated on the basis of the fraction of cells seen in division at each time point. Frame II shows the proliferation of cells with time. The increase in cell number reflects successful completion of divisions in both the first and second peak. Frames III and IV relate to cells treated with *p*-FPhe from EH + 35 to EH + 50 min. By comparison, no increase in cell number occurs before 2·5 h, i.e. more than 1 h after at least 40% of the cells have entered cytokinesis. Cell number increases as the division index declines in the last portion of the graph. We repeated these experiments with mass cultures under the same treatment (Fig. 2, right hand side). The results were similar and confirm that cells treated with *p*-FPhe during the critical pre-division time of EH + 35–50 min can enter division and may either remain there for an abnormally long time or revert to the interphase state before proceeding at a later time into and through division. In either case these cells spend much longer performing successful cytokinesis than do control cells.

DISCUSSION

Synchronously dividing *Tetrahymena* provide a useful system for study of preparation for cell division. This process requires both the synthesis of proteinaceous compounds and their correct mutual interaction.

Synchronous division in *Tetrahymena* can be studied in several experimental systems (see review by Andersen, Rasmussen & Zeuthen (1975)); here we have chosen the multi-shock system with a medium change between heat shocks nos. 6 and 7, so that cells go through division in a starvation medium (Hamburger & Zeuthen, 1957). Under these conditions respiration is reduced to one third (Hamburger & Zeuthen, 1957) and there is no net increase in DNA (Lowy & Leick, 1969), RNA and protein. However, the cells continue to incorporate amino acids into macromolecules and in this way maintain pools of important proteins. This turnover of protein depends on amino acids supplied by protein degradation (Crockett, Dunham & Rasmussen, 1965; Zeuthen & Rasmussen, 1972). In spite of the overall reduced synthetic activities, the cells prepare for division and divide at the same time as cells maintained in a nutrient medium (Hamburger & Zeuthen, 1957). This preparation obviously can be made exclusively on internal reserves.

As in other cells, division in *Tetrahymena* requires the creation of a specific structural order within the mother cell. The first sign of this order is the appearance of a cluster of kinetosomes in the mid-ventral region, the oral primordium. Within this primordium morphogenetic influences spread from the anterior and backwards, and also forwards to the right and left following paths which meet dorsally. These patterns in due course define the position of the impending division furrow. This order is elaborated by the subplasmalemmal arrangements of microfilaments, microtubules and other components, and leads to the formation of ciliated membranelles. There is evidence of an early coordination between the macronucleus and the cell surface (Nachtwey, 1965). Firstly, DNA synthesis is not initiated after the oral primordium has been activated (Ikeda & Zeuthen, cited by Zeuthen, 1971). Secondly, the chromatin condenses sufficiently early to invite the suggestion that chromatin condensation and oral morphogenesis are correlated. Following condensation of the chromatin the macronucleus stretches and pinches into two (Nilsson & Zeuthen, 1974). Contractile systems, probably analogous to those in the cell cortex, must presumably be present at that time in and around the macronucleus.

This report shows that *p*-FPhe can interfere with the performance of 'early' and 'late' division-relevant proteins, depending on where in the cell cycle the treatments are applied. Pulses with *p*-FPhe applied from EH+10, EH+20 and EH+30 min (Fig. 1, II–IV) delay onset of cell division, whereas pulses from EH+40 to EH+55 min do not.

This implies that 'early' division proteins (those concerned with *entry* of cells into division) are synthesized before EH+40 min. Whether they continue to be synthesized after EH+40 min is not known. If they are, then the shift in onset of cell division responses, illustrated in frames IV and V, reflects that the 'early division proteins' are removed from a pool before EH+40 min. Addition of *p*-FPhe after this time may therefore contaminate the protein pool, but it does not contaminate structures involved in the early phases of the forthcoming division.

We do not know when the synthesis of 'late division proteins' begins, but the results of frames II and III would be in keeping with the suggestion that it begins early. Frames V–VII show that this synthesis continues for a long time. It can be seen, particularly from frames VI and VII, that the completion of a cell division can be greatly delayed despite the fact that it has been initiated on schedule.

We have observed microscopically cells pulsed with *p*-FPhe and noted the following: (i) cells in which completion is selectively delayed are unable to elongate their macronucleus as would normally be required for cell division, and (ii) the macronucleus does not become re-positioned in the plane of the impending furrow. This results in the appearance of cells having abortive division furrows, pointed anterior and somewhat swollen posterior ends. The nuclei remain posteriorly positioned until they can move, elongate and perhaps supply the signal for the furrow to advance again. In this view the early division proteins give the cells the capacity to form, hold and even re-initiate a division furrow. The late division proteins may have to do with macronuclear functions, such as stretching, and with the emission of signals allowing concerted action of the macronucleus and the cell surface.

The term 'division proteins' (Zeuthen, 1961) has been applied to proteins or proteinaceous compounds used in cell division (Rasmussen & Zeuthen, 1962; Watanabe & Ikeda, 1965*a*; Mitchison, 1971). Isolation of them has been attempted (Watanabe & Ikeda, 1965*b*; Lowe-Jinde & Zimmerman, 1971), but the issue about their nature remains unsettled. Also *Schizosaccharomyces pombe* can be synchronized in division by heat treatments analogous to those which synchronize *Tetrahymena* (Kramhøft & Zeuthen, 1971). Polanshek (1977) discussed in detail the effects of heat shocks and cycloheximide on division of these cells. He pointed out that there is good agreement between the effects of the chemical inhibitor on cell division as compared with heat shocks.

In mammalian cells *p*-FPhe can be incorporated in the second part of G_2 and produce inhibition of mitosis (Wheatley & Henderson, 1974). This is similar to the situation described in *Tetrahymena*, since it involves the synthesis of division-relevant analogue proteins. These proteins enter the division protein pool and can prevent cells entering mitoses. They can also seriously affect the ability of cells which have initiated division from completing it (Sisken & Wilkes, 1967). These findings suggest that controls are exerted by proteins in mammalian cells in a similar fashion to the 'early' and 'late' division proteins described here for *Tetrahymena*.

In conclusion, the analogue protein technique can provide a dissection of some of the separate physiological cycles which must become sufficiently integrated both spatially and temporally to form the overall cell cycle. The time of synthesis of key proteins can be checked with inhibitors of protein synthesis whereas *p*-FPhe allows further information to be obtained about the time when the proteins become used.

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