STUDIES ON THE MAINTENANCE OF ORAL DEVELOPMENT IN *TETRAHYMENA PYRIFORMIS* GL-C

II. The Relationship of Protein Synthesis to Cell Division and Oral Organelle Development

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ABSTRACT

The effects of puromycin on synchronized *Tetrahymena pyriformis* were investigated at two different concentrations, 43 μg per ml and 430 μg per ml. The rate of incorporation of histidine-^{14}C into hot TCA-insoluble material was reduced by 30% at the low concentration and by 80–90% at the high concentration. The rate of oxygen uptake was lowered by only 10–20% at both concentrations. Cell division was prevented at both concentrations, if the drug was added prior to a "transition point" at about 45 min after the end of the synchronizing treatment. Development of "anarchic field" oral primordia was arrested, while primordia in early stages of membranelle differentiation were resorbed. Resorption began shortly after addition of the drug, and proceeded most rapidly at the lower concentration. If the drug was added after the "transition point," cell division and oral primordium formation were completed with only slight delay at the low concentration, and with considerable delay (in some cases complete arrest) at the high concentration. The results thus indicate that protein synthesis is involved in the later as well as the earlier stages of development; what specially characterizes the earlier stages, prior to the "transition point," is a dramatic response to partial inhibition of protein synthesis. It is suggested that this response involves the activation or release of a latent intracellular degradative system which is specific for developing structures.

INTRODUCTION

A considerable amount of evidence has accumulated in support of the generalization that successful consummation of cell division is dependent on preceding protein synthesis. Inhibition of protein synthesis during the G2 period is known to prevent or delay mitosis in cultured mammalian cells (22, 27, 28, 39), in sea urchin eggs (21) and in the slime mold *Physarum polycephalum* (7, 8). At least some of the proteins synthesized prior to mitosis in the sea urchin egg may be structural proteins of the mitotic apparatus (25, 36). In all of the mitotic systems investigated, stages beyond prophase are insensitive to inhibitors of protein synthesis, indicating that once the mitotic figure is established the processes of mitosis and cell cleavage furrowing require little or no further synthesis.

Analogous results, with respect to blockage of cell division, have been obtained with the ciliate *Tetrahymena pyriformis*. Puromycin or *p*-fluorophenylalanine will prevent division if added prior to a "transition point" which occurs a short time before the beginning of division furrowing (30, 52). The agents which affect cell division also appreciably reduce the rate of protein synthesis (3).

These results have been interpreted as indicating that "division proteins" must be produced as a precondition for each cell division (51, 52). Consistent with this idea, Watanabe and Ikeda (44, 45) have isolated a chromatographic fraction of the protein of synchronized *T. pyriformis* which satisfies certain criteria of "division protein."

The fact that specific protein synthesis is apparently required for cell division in both the ciliate and the mitotic cells raises the question as to the degree to which the division systems and "division proteins" of the two types are analogous. When this possibility is considered, two special features of the *Tetrahymena* division system must be taken into account: (1) Cell division is preceded by an amitotic macronuclear division and (in micronucleate strains) an intranuclear micronuclear mitosis. Cell division can take place even under conditions in which macronuclear division is blocked or highly abnormal (15, 17, 26, 31). Therefore, in an amicronucleate strain (such as the widely studied strains GL and W) protein synthesis required for cell division cannot provide *exclusively* protein required for nuclear division; it is, therefore, likely that at least some of the "division protein" is involved in processes directly related to cytoplasmic cleavage. (2) Cell division in most ciliates, including *Tetrahymena*, is preceded by the formation of new oral structures immediately posterior to the site of the prospective division furrow (14, 18). Agents which prevent division bring about arrest in the development of these structures, or resorption of the structures, depending on their stage of differentiation (10, 13, 46, 47). These developmental effects are correlated with "set-back" of progress toward cell division (10, 52). Furthermore, the time prior to division at which "set-back" is no longer inducible (the "physiological transition point" of Rasmussen and Zeuthen, reference 30) corresponds to the time at which new oral primordia are, in general, no longer subject to resorption (the "stabilization point" of Frankel, reference 10).

An explicit comparison of the responses of cell division in *Tetrahymena* and in human cells to inhibition of protein synthesis was attempted by E. W. Taylor (39). He noted that both cell systems were similar in one respect, namely that the next cell division became insensitive to the inhibitor at a time *prior* to the visible onset of division. However, the two systems differed considerably in other respects: first, in *Tetrahymena* an exposure to the inhibitor prior to the transition point elicits a "set-back" of progress toward division, which increases with the age of the cell, while in the human cells there was no evidence of such set-backs, at least at moderate levels of inhibition of protein synthesis; second, the level of inhibition of protein synthesis required to permanently block cell proliferation in *Tetrahymena* was much greater than in the human cells. Taylor suggested that these differences in response might be due to differences in stability of proteins required for division in these two systems.

The present study is an attempt to analyze the relationship of protein synthesis to the processes which lead to cell division in *Tetrahymena pyriformis* GL-C. The method of analysis resembles that employed by E. W. Taylor in his study of the mammalian cell cycle: different concentrations of puromycin were used to elicit different degrees of inhibition of protein synthesis, and these differing degrees of inhibition were then compared with effects on cell development and division. The results obtained by this method of analysis suggest that the relation of protein synthesis to mechanisms controlling the cell cycle in *Tetrahymena* may be quite complex, and different from the relation encountered in classical mitotic systems.

MATERIALS AND METHODS

1. GENERAL PROCEDURES: Stock cultures of *Tetrahymena pyriformis* GL-C (Copenhagen line of strain GL) were maintained axenically at 28°C in slanted test tubes containing 5 ml of medium (0.3% Bacto-Tryptone, 0.5% glucose, vitamins, and salts (12, Table I)). Transfers were made daily. Experiments were performed on cell populations synchronized by a slight modification of the heat-shock method of Scherbaum and Zeuthen (32), as described previously (12, 13). Except where specifically mentioned, cells were synchronized in 500-ml Fernbach flasks (Jena Glaswerk) containing 150 ml of medium, and cell density at the time of synchronization was 10,000–30,000 cells per ml. In some experiments, the cells were concentrated after the end of the standard six-shock synchronizing treatment and were then given two additional heat shocks (30-min periods at 33.8° separated by 30-min intervals at 28.5°), with a total treatment of eight shocks. The time interval between the end of the last shock and the synchronous division was the same with the eight-shock treatment as with the standard six-shock treatment; hence, direct comparison of results obtained with the two regimens is probably justified.

A few experiments were performed on exponentially growing, nonheat-treated cells. These were

simply grown in 500-ml Fernbach flasks maintained, with constant swirling, at 28.5°C.

2. EXPERIMENTS ON CELL DIVISION: Synchronization was obtained by the standard six-shock procedure. The original inoculum of the synchronizing flask was very small, so that cell density at the time of synchronization was only 1000–2000 cells per ml. Shortly before the time at which the drug was to be added, two 10–12 ml portions were removed from the flask culture and transferred to sterile petri dishes which were kept in a constant temperature room at 28° ± 1°C. The inhibitor was added at a designated time to one of the petri dish cultures, while the other served as a control. Medium with cells was then removed with a micropipette from each of these cultures, and deposited as separate small drops on the bottom surface of another petri dish which had been covered with a thin layer of paraffin oil; the oil protects the drops from evaporation while permitting gas exchange (cf. ref. 24). The tip of the micropipette was observed under the dissecting microscope as the drop was being formed, and medium was expelled until a single cell (or occasionally two) appeared in the drop. Drops were laid down in quick succession adjacent to numbers painted on the bottom of the petri dish. Each drop thus formed a separate microculture which could be individually identified. In most experiments, 72 drops (48 treated and 24 controls) were deposited in a 10-min interval. These were maintained at 28°C and examined at appropriate times for cell division. Times of the completion of division were recorded with an accuracy of ±5 min or better. Comparison of micro-cultures with the petri-dish cultures from which they were derived indicated that the procedure of culturing individual cells under oil had no effect on the time of cell division; it is unlikely, therefore, that there was appreciable diffusion of puromycin from the aqueous into the oil phase, since if this happened the cells in the drops would have divided earlier than the cells in the petri dish (as occurred in similar experiments conducted with dinitrophenol).

3. EXPERIMENTS ON MORPHOGENESIS: Cells were synchronized in the standard manner. After the end of the synchronizing treatment (EST), the culture was split into a 30–40-ml portion (control) and a 110–120-ml portion (experimental). Both cultures were swirled continuously throughout the experiment. The inhibitor was added to the experimental culture at the appropriate time after EST. At various times thereafter, samples were removed from both cultures, fixed for silver impregnation, and slides made, with the employment of the Chatton-Lwoff technique (modified after Corliss, reference 4). 100 cells from each slide were randomly selected and classified according to stage of development or resorption (cf. references 10, 12, 13).

4. EXPERIMENTS ON AMINO ACID INCORPORATION: Procedures were modeled closely after those employed by Crockett et al (6). Cells were synchronized in 1500-ml Fernbach flasks containing 500 ml. of medium. Cell density at the time of synchronization was 30,000–60,000 cells per ml. Shortly after the end of the sixth synchronizing shock, the culture was concentrated approximately tenfold to a density of 300,000–600,000 cells per ml. The concentrated cells were divided into two 20-ml portions maintained in sterile cotton-stoppered 125-ml Ehrlenmeyer flasks. These two flasks were given two additional shocks. This procedure allows recovery from the effects of centrifugation and assures reasonably synchronous division of the concentrated cells. Shortly after the end of the eighth shock, triplicate 1-ml samples were removed from each flask for counting in a Coulter counter. At 40 min after the end of the last (eighth) shock, puromycin was added to one flask, and an equal volume of glass-distilled water was added to the other. 15 min after addition of puromycin, 5 μc of L-histidine-2-(ring)-^{14}C (Calbiochem, Los Angeles, specific activity 21.7 mc per mmole) were added to each of the two 20-ml cultures (yielding a final activity of 0.25 μc per ml). Immediately after addition of the isotope, and at intervals thereafter, duplicate 1-ml samples were removed from both puromycin-treated and control cultures with a wide-bore pipette, transferred to conical 12-ml centrifuge tubes, and rapidly frozen by the method of agitating the tubes in a mixture of dry ice and acetone. All samples representing a single time point were frozen simultaneously. The samples were stored at −15°C until analyzed. At the time of analysis, samples were thawed, extracted with hot trichloroacetic acid (TCA), and plated. The procedures of Crockett et al (6) were used, with only slight modifications. The samples were counted in a Nuclear-Chicago thin-end-window counter.

The above procedure was modified somewhat in experiments on the effect of puromycin on uptake as well as incorporation of amino acids. Synchronization, concentration of cells, and addition of puromycin were performed in the manner described above. However, shortly after the addition of puromycin, experimental and control cultures were each divided into two 10-ml portions. One portion received 5 μc of histidine-^{14}C 15 min after addition of puromycin, while isotope was added to the other portion much later, 105 min after addition of puromycin. In both cases, the final concentration of isotope was 0.5 μc per ml. Quadruplicate 1-ml samples were removed from each culture at 5 and 10 min after the addition of the isotope, and pipetted into conical centrifuge tubes containing 20 ml of cold culture medium to which a hundredfold excess of unlabeled histidine had been added. The tubes were maintained in an ice bath to keep the temperature below 1°C at all times. These samples were immediately centrifuged for 5

min at 1500 *g* in a refrigerated centrifuge set at 0–1 °C. The samples were washed twice in cold medium, and then frozen until analysis.

At the time of analysis, the samples were thawed; two of the quadruplicate 1-ml samples were plated directly, while the other two were extracted in hot TCA, washed, and plated. The first two samples thus provide a measure of uptake of amino acids (minus possible losses due to water extraction and amino acid exchange—cf. Results); while the latter two provide a measure of incorporation into protein.

5. EXPERIMENTS ON OXYGEN UPTAKE: Cells were synchronized by the standard procedure. After the end of the six-shock synchronizing treatment, the culture was concentrated by centrifugation to attain a final cell density of about 100,000 cells per ml; in general, this involved reducing the original 150-ml volume to 30–40 ml. The concentrated cells were returned to the original flask and given two additional synchronizing shocks. After the end of the last shock, triplicate samples were removed for cell counting. Then, shortly before the time scheduled for addition of puromycin, 1-ml samples were pipetted into small Warburg flasks (gas volume of 4–5 ml) containing filter paper soaked with 0.1 ml of 20% KOH in the center well. Eight flasks (exclusive of thermobarometers) were prepared in each experiment. Puromycin was added, at the designated time, to the cultures in four of the flasks, and an equal volume of glass-distilled water was added to the other four. The flasks were then quickly attached to manometers and placed in a shaking bath regulated at 28.5 ± 0.1 °C. Readings were begun after a brief equilibration period and continued for approximately 2 hr. General procedures and calculations were carried out following standard methods of manometry (43). At the end of each experiment, the number of cells in each Warburg vessel were counted in the Coulter counter.

RESULTS

1. Effect of Puromycin on Protein Synthesis

The effects of two concentrations of puromycin on incorporation of histidine-^{14}C into protein by synchronized *T. pyriformis* GL-C are presented in Table I and Fig. 1. In these experiments, puromycin was added at 40 min after EST and histidine-^{14}C was added 15 min later (the time was chosen to correspond roughly to the time of the most dramatic morphogenetic effect of puromycin—cf. section 4 of Results). The rate of incorporation of the isotope into TCA-insoluble material was constant in the control cultures, while it declined slightly with time at both concentrations of puromycin. Because of the steady decline in treated cultures, relative incorporation rates could not be exactly computed for the whole period of the experiment; however, the *initial* incorporation rate (that is, 0–30 min after addition of the isotope, or 15–45 min after addition of puromycin) at 42 μg per ml was roughly 70% of the control rate, while at 420 μg per ml it was approximately 15–20% of the control rate (Table I).

The control cells divided synchronously during the course of the experiment (without, however, any increase in the incorporation rate as measured

TABLE I

Effect of Puromycin On Incorporation of Histidine-^{14}C Into Hot TCA-Insoluble Material

In all experiments, puromycin (420 μg per ml) was added to the experimental cultures at 40 min after EST, and histidine-^{14}C was added to both control and experimental cultures 15 min later, at 55 min after EST. The times indicated in the table are times after the addition of histidine-^{14}C. For details of the experiments, see section 4 of the Methods.

Exp. no.	Cells per ml (× 10^{-5})			CPM per 10^5 cells					
				0-30 min			30–60 min*		
	Control	Exp.	Conc. of puromycin	Control	Exp.	Exp./ Control	Control	Exp.	Exp./ Control
			μg/ml						
1	3.33	3.23	42	438	321	0.73	413	188	0.47
2‡	5.86	5.87	42	325	226	0.70	355	191	0.54
3	3.76	3.20	420	540	104	0.19	447	99	0.22
4‡	4.13	3.95	420	340	62	0.18	332	49	0.15

* Cumulative cpm at 60 min minus cpm at 30 min.

‡ Results of these experiments presented graphically in Fig. 1.

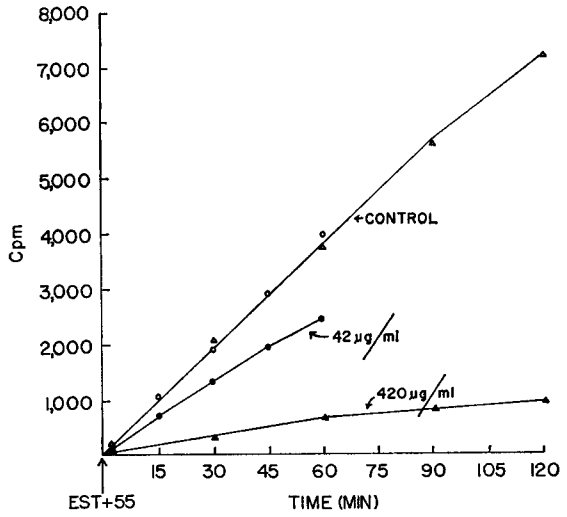

FIGURE 1 The effect of puromycin on incorporation of histidine-^{14}C into hot TCA-insoluble material in synchronized *T. pyriformis* GL-C. The ordinate indicates total cpm of extracted 1-ml samples (see Methods); the abscissa indicates time after addition of the labeled histidine. Puromycin was added at 40 min after EST and the histidine was added (to both experimental and control cultures) at 55 min after EST ("zero time" on the abscissa). The circles and triangles indicate two separate experiments; the filled symbols represent counts from samples of puromycin-treated cultures; the open symbols represent counts from samples of the control cultures.

per ml of culture), while all cells at the high concentration of puromycin and the great majority of the cells at the lower concentration failed to divide.

Two experiments were performed to ascertain whether the greatly reduced incorporation rate at 420 µg per ml was due to a primary effect on synthesis as such, or to a secondary effect resulting from an effect on amino acid transport. Puromycin was added at 40 min after EST, and a 10-min histidine-^{14}C pulse was administered to separate culture aliquots at 15–25 and 105–115 min after the puromycin was added. Parallel samples were plated either directly after washing (to measure uptake) or after TCA extraction (to measure incorporation). The results are presented in Table II. Incorporation of the amino acid was reduced to 6–15% of control values (these figures are somewhat unreliable since the activity of the puromycin-treated samples was only several times background), while uptake was reduced to about 50% of control values at 15–25 min after puromycin addition, and to about 15% at 105–115 min. The amount of amino acid taken into the amino acid "pool" (measured as uptake minus incorporation) is only moderately reduced, compared to the control, when estimated during the time interval shortly after the addition of puromycin. Hence at this early time, synthesis is affected by puromycin to a much greater extent than is uptake, and, therefore, the reduction in rate of synthesis cannot be a secondary consequence of an effect on uptake. The drastic effect on uptake at the later time period, nearly 2 hr after addition of puromycin, might be explained as a consequence of inhibition by puromycin of synthesis of proteins involved in uptake of amino acids.

Before proceeding further, a brief technical comment is necessary. Histidine uptake was measured on cells which were washed, after the original 5- or 10-min exposure to labeled histidine, in ice-cold culture medium containing an excess of unlabeled histidine. This procedure was adopted in order to exchange labeled histidine adsorbed onto the cell surface, and thus avoid the risk of overestimating pool size. This washing method, however, probably causes an underestimation of true pool size, since intracellular amino acids are probably also capable of exchange. Exchange takes place, in *E. coli*, at an appreciable rate, even at 0°C (2). However, not all amino acids of the pool are equally exchangeable; in the yeast *Candida utilis*, the "internal pool" which is directly utilized for protein synthesis is not readily exchangeable, while a water-soluble "expandable pool" is (5). If these findings can be extrapolated to *Tetrahymena*, then the "pool" activities indicated in Table IV represent all (or nearly all) of the histidine-^{14}C taken into the "internal pool," and only a part of the isotope originally present in the "expandable pool." In any case, the "experimental" (puromycin-treated) and "control" columns within an experiment should be strictly comparable, since they represent samples handled in an identical fashion during the washing and plating procedures.

TABLE II

Effect of Puromycin On Uptake And Incorporation Of Histidine-^{14}C

In all experiments, puromycin was added to the experimental cultures at 40 min after EST. Histidine-^{14}C was added either at 55 min after EST (section *A* of the table) or at 145 min after EST (section *B* of the table). Exposure to the isotope lasted 5 or 10 min. For further experimental details, see Methods, section 4.

Exp. no.	Incorp. time	Uptake CPM			Protein CPM			"Pool" CPM (uptake — protein)		
		Control	Exp.	Exp./ Control	Control	Exp.	Exp./ Control	Control	Exp.	Exp./ Control
		A. 15–25 min after addition of puromycin								
	min									
5	5	738	409	0.55	288	27	0.09	450	382	0.85
	10	1246	559	0.45	616	87	0.14	630	472	0.75
6	5	642	379	0.59	214	18	0.08	428	361	0.84
	10	1554	565	0.36	534	43	0.08	1020	522	0.51
		B. 105–115 min after addition of puromycin								
5	5	995	167	0.17	418	25	0.06	577	142	0.25
	10	1870	240	0.13	892	54	0.06	978	186	0.19
6	5	461	64	0.14	141	22	0.15	320	42	0.13
	10	1092	155	0.14	323	38	0.12	769	117	0.15

2. *Effect of Puromycin on Oxygen Uptake*

In preliminary experiments, it was found that the oxygen uptake of synchronized cells maintained in the tryptone-vitamins-salts medium remained constant between EST and synchronous division, and then increased after the end of the synchronous division (100–110 min after EST) to a new constant rate which was 10–30% higher than the predivision rate. The initial rate of oxygen consumption generally varied between 6 and 9 μl per min per million cells (see Table III). Higher and lower values were occasionally observed; these extreme values tended to be inversely correlated with cell density. (A comparable, though less marked, inverse correlation was also observed in the experiments on protein synthesis—see Table I). These variations are probably related to the fact that cells synchronized at higher densities tend to be smaller at EST (i.e., have grown less during synchronization) than cells synchronized at lower densities. The mean initial rate of oxygen consumption of untreated cells, in a total of 34 experiments, was 6.62 ± 0.26 (SE) μl per 10^6 cells per min.[1]

The effects of puromycin on oxygen consumption are presented in Table III and Fig. 2. Puromycin was added to the experimental flasks at either 20 or 40 min after EST, and manometer readings were begun 12–15 min after addition of the drug. The rate of respiration in experimental and control flasks was constant either from the start of the readings (Fig. 2 *A*), or else became constant after a short initial lag (Figure 2 *B*), probably resulting from a delay in equilibration of the flasks. In the puromycin-treated flasks, the rate of oxygen uptake remained constant for the entire 2-hr duration of the experiment. This linearity is equally apparent in plots of the oxygen uptake of each individual flask, or of the average of all the flasks in an experiment (Fig. 2). The lack of any increase in rate of respiration in the puromycin-treated flasks was probably related to the

[1] The average rate of oxygen consumption in the present experiments can be compared to the average rates observed in synchronized cells of the same strain by Hamburger and Zeuthen (reference 16). These were 4.6 μl/10^6 cells/min in inorganic medium, and 14.4 μl/10^6 cells/min in a proteose peptone-liver medium. The average we obtained is intermediate between these, which is not surprising in view of the fact that our experiments were conducted in an organic medium which is probably much less rich in nutrients than is the proteose peptone-liver medium.

TABLE III

Effect Of Puromycin On Oxygen Uptake

Oxygen uptake was measured by standard Warburg manometry. For details, see Methods, section 5.

Exp. no.	Cell no. per ml (× 10^{-4})	Conc. of puromycin	Time of puromycin addition*	Interval of comparison‡	Oxygen uptake (μl/10^6 cells/min)			
					Controls	Exp.	Exp./ Control	
		$\mu g/ml$	*min*					
1	10.41	42	20	12–52	6.55	5.66	0.86	
2	6.76	42	20	27–65	8.70	7.73	0.89	Avg.
3§	9.44	42	40	12–52	8.52	7.18	0.84	0.88
4	7.88	42	40	15–55	7.40	6.88	0.93	
5	9.68	420	20	23–63	6.23	6.13	0.98	
6	4.46	420	20	13–53	10.20	8.72	0.85	Avg.
7	7.33	420	40	24–64	7.42	5.83	0.79	0.86
8§	13.08	420	40	32–72	4.62	3.76	0.82	

* Time expressed in minutes after EST.

‡ Time expressed in minutes after puromycin addition.

§ Results presented graphically in Fig. 2.

suppression of cell division by the drug. The most meaningful estimate of the effect of puromycin on cell respiration can be made by comparing rates of respiration of controls and experimentals during the period of constant respiratory rate *prior* to the postdivision increase in respiration in the controls. Such a comparison, made over a 40-min interval (Table III) reveals that puromycin at both 42 μg per ml and 420 μg per ml depresses the rate of oxygen uptake only very slightly. There is no difference in effect at the two concentrations. The profound inhibitory effect of the higher concentration on protein synthesis is thus *not* paralleled by a comparable effect on cell respiration. Hence puromycin appears not to act to any significant degree as a respiratory inhibitor.[2]

[2] It may be worthwhile to note that comparable experiments were also performed with actinomycin D (20 μg per ml) added at 20 min after EST. The relative respiratory rate in these experiments (actinomycin-treated flasks compared to control flasks in the same experiment) was 0.85, 0.65, 0.88, and 0.74 in four experiments (average, 0.78). The respiratory rate of the actinomycin-treated flasks remained constant during the 2-hr experimental period in one experiment (the one in which the relative respiratory rate was 0.88), and declined slightly with time in the other three. Hence in this system, actinomycin acts as a very mild respiratory inhibitor (cf. 23).

3. Effect of Puromycin on Cell Division

Rasmussen and Zeuthen (30) noted that synchronous division in *T. pyriformis* GL was inhibited if puromycin (0.1 mM, or 54 μg per ml) was added at 35 min after EST or earlier, while the division took place with only slight delay if the inhibitor was added at 45 min. These findings were essentially confirmed in our experiments with synchronized cells exposed to puromycin (43 μg per ml) and observed individually in small drops under paraffin oil (Fig. 3). Extremely few of the cells exposed at 30 min after EST divided in puromycin, while 80% of the cells exposed at 60 min divided. The average "transition point," defined as the time at which the next cell division can no longer be prevented in 50% of the cells by immersion in the drug, can be extrapolated to about 45 min after EST, midway between EST and the average time of cell separation. The cells which divided in puromycin did so synchronously, and at about the same time regardless of when the drug was added (the differences among the 40-, 50-, and 60-min groups are small and probably not meaningful). Supplementary observations carried out with phase-contrast microscopy showed that effects on macronuclear division were correlated with effects on cell division: at the end of an experiment virtually all cells, regardless of whether division was blocked or not, had a single compact macronucleus.

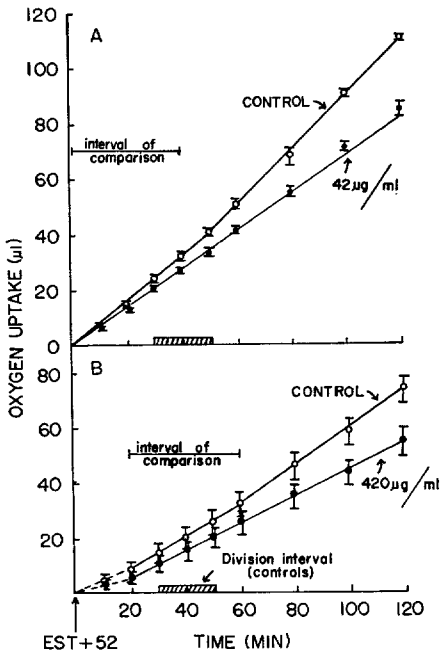

FIGURE 2 The effect of puromycin on oxygen uptake in synchronized *T. pyriformis* GL-C. The ordinates (in both graphs) indicate microliters of oxygen uptake per 1-ml sample (cf. Methods); the abscissa indicates time after the beginning of Warburg readings. Puromycin was added at 40 min after EST, and readings were begun in both experimental and control flasks 12 min later ("zero time" on the abscissa). Each symbol (filled and open circles) indicates the *average* reading for each group of three or four flasks; the extreme readings within each group are indicated by the horizontal bars above and below the symbols. The filled circles indicate the average readings in puromycin-treated flasks, while the open circles indicate the average readings of the untreated control flasks. Graphs *A* (upper) and *B* (lower) each represent a separate experiment. The approximate time at which synchronous division took place in the control cells is indicated as a shaded bar on the abscissa. The "interval of comparison," as utilized in Table III, is indicated for each of the two graphs.

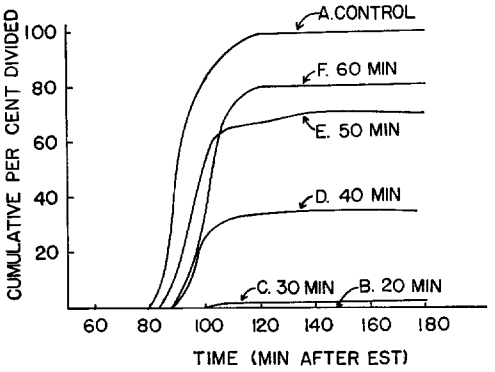

FIGURE 3 The effect of puromycin (43 μg per ml), added at various times after EST, on cell division in synchronized *T. pyriformis* GL-C. All six curves show cumulative percentages of cells which have completed division. The time designation for each curve indicates the time after EST at which the drug was added. The "plateau" reached by every curve indicates the percentage of cells which succeed in dividing (no cells divide after 180 min). Curve *B* (puromycin added at 20 min after EST) follows the abscissa, since under these conditions no cells ever divide.

Cell division does not become absolutely insensitive to puromycin after the "transition point." The effects of two concentrations of this drug, added at 60–65 min after EST[3] are presented in Fig. 4 and Table IV. At 43 μg per ml, 80% of the cells (presumably those which had passed the "transition point") formed furrows and divided. Completion of division (i.e., cell separation) in these cells was delayed only about 10 min relative to untreated control cells. At 430 μg per ml, almost 80% of the cells formed definite furrows, but only 50% divided. In the cells which did divide, the furrow constricted very slowly and completion of division was considerably delayed, with great variation in amount of delay (Fig. 4). In the 30% of the population which formed furrows but did not complete division, the furrows were either permanently arrested at a stage of relatively deep constriction, or else eventually disappeared. Most (about 75%) of the arrested cells had two completely separated macronuclei, while about 15% had single dumbbell-shaped nuclei, and about 10% had single compact nuclei.

Since the period after puromycin addition in

[3] Exposure to the high concentration of puromycin was initiated 5 min later than exposure to the low concentration for the following reason: In experiments on the effects of puromycin on morphogenesis, it was found that development came to a halt about 10 min after addition of 43 μg per ml, and about 5 min after addition of 430 μg per ml. Hence, it was concluded that puromycin at the low concentration required about 5 min longer to take effect than did puromycin at the high concentration. If this conclusion is correct, then the high concentration must be added 5 min later than the low concentration to make the time at which the cells are affected the same.

TABLE IV

Effect Of Puromycin On Division Furrowing

Four categories of cellular response with respect to furrowing are indicated. Cells are considered to have formed a furrow if an indentation is clearly visible on both sides of the equator of the cell. If the furrow has disappeared by the end of the observations (6–9 hr after addition of the drug), it is considered as "aborted"; if it persists but the cell fails to complete division, it is scored as "arrested," while if the cell completes division, with ensuing separation of daughters it is scored as "completed."

Conc. of puromycin	Time of addition (after EST)	No. of cells observed	Furrowing				Average time of division (± SD)
			None	Aborted	Arrested	Completed	
μg/ml	*min*						*min*
None	—	98	0	0	0	98	91.8 (±10.2)
43	60	102	21	0	0	81	102.7 (±7.0)
430	65	102	23	12	16	51	147.6 (±36.3)*

* Three cells which completed division with extreme delay (about 6 hr after addition of the drug) are excluded from this computation.

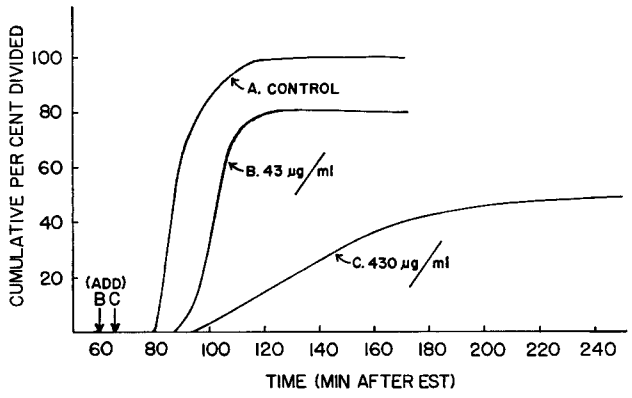

FIGURE 4 The effects of late addition of puromycin on the timing of division in synchronized cells. All three curves show cumulative percentages of cells which have completed division. Curve *A* shows the cumulative percentage of untreated control cells which divide (analogous to curve *A* in Fig. *3*; the two curves however were constructed from different populations). Curve *B* shows the cumulative percentage of cells completing division after addition of puromycin at 43 μg per ml, while curve *C* shows the cumulative percentage completing division after addition of puromycin at 430 μg per ml.

these experiments was roughly coincident with the period of actual cleavage (which, in most cells, begins at about 60–65 min after EST), the delaying effect of the drug is exerted during the cleavage process itself. It is noteworthy that this effect is, in general, correlated with the effect on protein synthesis: delay of cleavage is slight at the low concentration, which has only a moderate effect on protein synthesis (cf. Results, section 1), while the delay is considerable at the high concentration, at which protein synthesis is profoundly depressed.

4. Effect of Puromycin on Oral Development

STAGE—SPECIFIC EFFECTS: Oral development precedes and accompanies cell division in *T. pyriformis* (14, 18, 46). In cells synchronized by the Scherbaum-Zeuthen procedure, the oral primordium is already present at the end of the synchronizing treatment, in the form of an "anarchic field" of nonciliated kinetosomes (19, 48); oral membranelles start to differentiate within this field at about 40 min after EST (46, 48, 49). A sequence of stages used in the description of this oral development (10) is presented and described in Fig. 5.

The morphogenetic effect of puromycin (43 μg per ml) differed according to the time at which it was added. When the drug was added at 20 min after EST, when all oral primordia were still in the "anarchic field" stage (stage 1), development was arrested (Table V). Little change was apparent during the first 2 hr of arrest, but subsequently the fields became gradually attenuated, and some were completely resorbed. The anterior oral area and the remainder of the infraciliature remained normal.

If the drug was added at 50 min after EST,

when most of the cells were in early stages (2 to 4) of membranelle differentiation, development continued for about 10 min at the same rate as in untreated control cultures (Table VI). Subsequently, most of the differentiating primordia underwent resorption, with parallel blockage of cell division (10, 13). However, about 20% of the cells completed development and cell division; these were probably the ones which were in the latter portion of stage 4, or stage 5, at the time when the puromycin took effect.

When puromycin (43 μg per ml) was added at 60 min after EST, most of the cells were initially in stage 4 (Table VII). During the next 10 min, in both treated and untreated cultures, about one-half of the cells initially in stage 4 had advanced to stage 5. Subsequently, in the puromycin-treated culture, some of the cells with stage 4 primordia resorbed their primordia and failed to divide. However, the remainder of the stage 4 cells, and all of those in stage 5, completed oral development and cell division in the presence of the drug.

One can summarize the above-described results as follows: puromycin at moderate concentrations takes effect after a lag of about 10 min, and then brings about arrest of development of stage 1 oral primordia, and prompt resorption of stage 2, 3, and 4 oral primordia. The drug, however, fails to affect development of oral primordia which are near the end of stage 4, or in stage 5. "Stabilization" of the developing system thus takes place, under these conditions, during the latter portion of stage 4. This stage-specific action of puromycin on *T. pyriformis* GL is similar to that exerted by a number of other agents (10, 12, 13). The precise stage of stabilization is known to

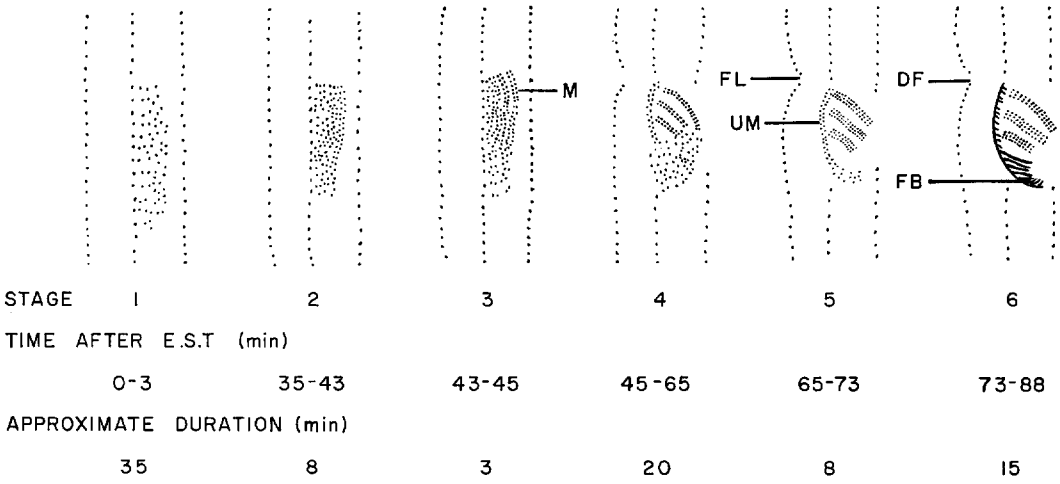

FIGURE 5 Summary of the stages of oral development in *Tetrahymena pyriformis*. In these diagrams, only the developing oral primordium and the region immediately surrounding it are shown. The approximate time at which each of these stages is observed in *synchronized* cells (maintained at 28° C) is indicated beneath the diagrams. These stages may be characterized as follows:

Stage 1, Loose "anarchic field" with indefinite margins.

Stage 2, Compact field, with clearly defined outer margin.

Stage 3, Beginning of membranelle formation; anterior parts of one or two membranelles (*M*) visible, as double files of kinetosomes.

Stage 4, All three membranelles visible; beginning of undulating membrane (*UM*) formation (*UM* less than half complete).

Stage 5, Membranelles complete and now consist of triple files of kinetosomes; undulating membrane more than half complete.

Stage 6, Membranelles sink into shallow buccal cavity; oral ribs appear; deep fiber bundle (*FB*) forms.

The fission line (*FL*) appears as an equatorial zone of discontinuities in the kineties in stage 5; the division furrow (*DF*) constricts along the fission line during stage 6.

depend on the strain and culture conditions (47), but not on the chemical agent utilized (13).

Before proceeding further, a discrepancy between the results presented in this and the preceding section should be pointed out and discussed. In the experiments on morphogenesis, about 20% of the cells were observed to develop and divide after addition of puromycin at 50 min after EST (Table VI), and about 60% divided after addition at 60 min after EST (Table VII). These percentages are considerably lower than in the corresponding experiments on cell division (Fig. 3): 68% divided after treatment at 50 min, 80% after treatment at 60 min. The "transition point" was about 10 min later in the experiments on morphogenesis (Tables V and VI) than in the experiments on cell division (Fig. 3). This difference is partially accounted for by the fact that the cell density of the flask cultures was about twentyfold higher in the experiments on morphogenesis than in the experiments on cell division; in denser cultures, cell division occurred a few minutes later and was somewhat less synchronous than in the extremely sparse cultures used in the experiments on cell division. This, however, is probably not the whole explanation for the difference in response. The experiments on cell division were done more than a year later than most of the experiments on morphogenesis. It is likely that the time required for externally added puromycin to take effect on the cells had increased, owing either to changes in rate of uptake or sensitivity of the cells, or else to variations in the potency of different lots of puromycin (see ref. 38). In any case, recent checks have shown that the basic stage-specific morphogenetic effect of puromycin has remained unchanged.

The morphogenetic effect of the high concentration of puromycin (430 μg per ml) has been compared with that of the lower one. The stage-specific response following addition of puromycin at 50 min after EST was the same at both con-

TABLE V

The Morphogenetic Effect of a Continuous Puromycin Treatment (43 μg/ml) Started 20 Min after EST

At least 100 cells from each of the samples were classified according to stage of development. The per cent distribution of morphogenetic stages at the corresponding time is indicated on each horizontal row of the Table. The distributions observed in the untreated control culture are indicated in parentheses.

Time		Morphogenetic stage			
After EST	After beginning of treatment	R*	0	1	2
min	*min*				
20	0	(1)		(96)	(3)
30	10	1		99	
55	35	3		97	
140	120	1	4	95	
260	240	3	24	73	
500	480	1	41	58	

* R = Oral structures in the process of resorption.

TABLE VI

The Morphogenetic Effect of a Continuous Puromycin Treatment (43 μg/ml) Started 50 Min After EST

For explanation, see Table V.

Time		Morphogenetic stage							
After EST	After beg. of treatment	R*	1	2	3	4	5	6	D/2‡
min	*min*								
50	0	(4)	(16)	(14)	(24)	(42)			
60	10	11	13	5	4	61	6		
		(2)	(18)	(7)	(4)	(68)	(1)		
82	32	60	18			1	10	11	
		(1)	(7)		(1)	(2)	(3)	(66)	(20)
113	63	62	17					8	13

* R = Oral membranelles of stages 3–4 oral primordia in process of resorption.
‡ D/2 = One-half the number of daughter cells, thus the number of parent cells which had recently divided.

TABLE VII

The Morphogenetic Effect of a Continuous Puromycin Treatment (43 μg/ml) Started 60 Min After EST

For explanation, see Table V.

Time		Morphogenetic stage								
After EST	After beg. of shock	R	0	1	2	3	4	5	6	D/2
min	*min*									
60	0	(2)	(1)	(16)	(1)	(2)	(75)	(2)	(1)	
70	10	1	1	17		2	38	38	3	
		(1)	(1)	(12)			(38)	(43)	(5)	
90	30	18*	1	14			3	9	51	4
120	60	20*		19					6	55

* Most of the oral primordia undergoing resorption are clearly derived from stage 4 primordia.

TABLE VIII

The Morphogenetic Effect of a Continuous Puromycin Treatment (430 μg/ml) Started 65 Min After EST

For explanation, see Table V.

Time		Morphognetic stage									
After EST	After beg. of shock	4R*	6R‡	0	1	2	3	4	5	6	D/2
min	*min*										
65	0	(4)		(3)	(14)	(1)		(62)	(16)		
70	5	2		2	14	1		43	35	3	
		(1)		(1)	(12)			(38)	(43)	(5)	
100	35	29		1	17			2	16	34	1
120	55	25			17				7	46	5
180	115	30			17				2	30	21
240	175	35	16		13					10	26

* Oral primordia undergoing resorption in cells lacking division furrows, derived from cells originally in stage 4.

‡ Oral primordia in early to middle stages of resorption, in cells which clearly possess division furrows, therefore derived from cells originally in stage 6.

centrations, although the process of resorption of differentiating oral primordia was completed more slowly at the higher concentration (see below). However, at later times (60–65 min after EST) there was a pronounced difference in morphogenetic response, which paralleled the differences in response of division furrowing activity (Table IV). At 43 μg per ml, cells in late-stage 4 and stage 5 went on to complete development and divide, at a rate only slightly slower than that of the untreated control cells. At 430 μg per ml, the fundamental pattern of response was the same as at the lower concentration, in that many stage 4 primordia underwent prompt resorption, while cells with late-stage 4 and stage 5 primordia continued to develop (Table VIII). The development of these latter cells was, however, exceedingly slow, so that by 3 hr after drug addition, division was completed in only about one-half of the cells which had entered division (stage 6) about 2 hr earlier. Those which did succeed in dividing had apparently normal oral structures; of those which did not, a considerable fraction began to undergo oral primordium resorption 2–3 hr after addition of the puromycin. These cells still possessed clear-cut fission lines, although the constriction between the two components was partly or wholly effaced in some; these cells corresponded to the class with "arrested" or "aborted" division furrows in the experiments on cell division (Table IV).

An experiment was conducted on the effects of

puromycin (43 μg per ml) on cell multiplication and morphogenesis in an exponentially growing (nonsynchronized) culture. Cell number increased slightly (about 15%) during the 1st 2 hr after addition of the drug, and not thereafter. Analysis of the changes in frequency distribution of morphogenetic stages after addition of puromycin revealed that the stage-specific response pattern was the same as that in synchronized cells, with the additional finding that cells initially lacking oral primordia (such cells are not present in synchronized populations) apparently cannot form them in the presence of the drug.

RATE OF RESORPTION OF STAGE 4 ORAL PRIMORDIA: As already mentioned, puromycin at both high and low concentrations brings about resorption of oral primordia in early stages of membranelle differentiation. Data on the time-course of the resorption process is presented in Fig. 6. The cumulative percentage of cells showing initiation (solid lines) and near-completion (broken lines) of the resorption process at the two concentrations is shown. The time of visible onset of resorption is comparable at the two concentrations, being only somewhat later at the high concentration than at the low. Once initiated, the spatial sequence of events during the resorption process is the same at both concentrations (this sequence is illustrated in Figs. 11–16 in reference 13). However, the rate at which cells progress through this sequence is three to four times slower at the high concentration than at the low (compare the distances between the solid and broken curves at the two concentrations). Thus, the severe restriction of protein synthesis obtained at the high concentration of puromycin has little effect on the initiation of resorption, but considerably retards the carrying out of this process once it has been initiated.

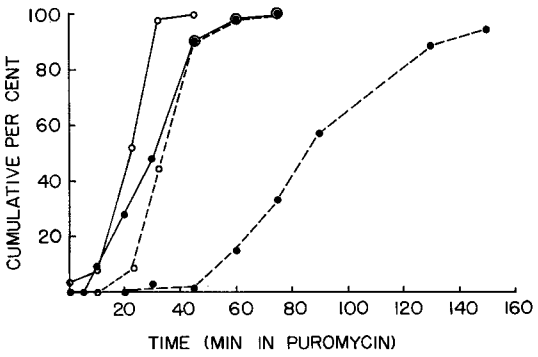

FIGURE 6 The rate of resorption of oral primordia observed during exposure to two concentrations of puromycin. The puromycin was added at 50 min after EST (when most cells were in stage 4). The abscissa indicates time after addition of puromycin. The ordinate gives the cumulative percentage of cells which have either initiated (solid lines) or nearly completed (broken lines) the process of resorption of stage 4 oral primordia. The criterion for initiation of resorption is entry into stage i of the resorption process; the criterion for near-completion is entry into stage iii (stage i is the first microscopically distinguishable phase of resorption of stage 4 oral primordia, while stage iii is a late stage of resorption, which, however, is prior to true completion of the process (for a more detailed explanation, see reference 13). The open circles indicate results obtained with the low concentration of puromycin (43 μg per ml), while the filled circles apply to results obtained with the high concentration of puromycin (430 μg per ml).

DISCUSSION

Chemical Specificity of Puromycin Action

Puromycin affects protein synthesis in *Tetrahymena* as it does in other systems; however, a relatively high concentration of the drug is necessary to obtain near-total inhibition. Even at 430 μg per ml, the rate of histidine-^{14}C incorporation into TCA-insoluble material is 15–20% of that observed in untreated control cells. It is probable, however, that some of the TCA-insoluble material formed in the presence of puromycin may consist of incomplete polypeptide chains, the release of which is brought about by puromycin (1, 29), which acts as an analog of amino acid charged transfer RNA (50). Hence it is possible, particularly at high concentrations, that the inhibition of the synthesis of functional protein is considerably greater than the reduction of the rate of incorporation of labeled amino acids into TCA-insoluble materials (cf. ref. 40).

The concentration of puromycin required to inhibit protein synthesis of *Tetrahymena* is considerably greater than that required for inhibition of cultured mammalian cells. This relatively low sensitivity might be a general protistan characteristic, since it is also found in *Euglena gracilis*, in which puromycin at 130 μg per ml reduced the incorporation rate of a cell-free protein synthesizing system by only 45% (9).

In order to be able to draw any conclusions about the chemical basis of a biological process from studies with an inhibitor such as puromycin,

one must have information about the chemical specificity of the action of the inhibitor. Puromycin is believed to act specifically on protein synthesis (29, 50) and to have a much less pronounced effect on RNA synthesis (20, 34, 39). The drug apparently has no direct effect on cell respiration (23, 37) or on phosphorylation in an in vitro ATP-generating system (50). Our findings indicate that, even at the very high concentration of 430 μg per ml, respiration in *T. pyriformis* is virtually unaffected, and amino acid uptake is reduced very much less than is incorporation into protein. Hence, it is unlikely that puromycin is acting as a metabolic poison; it can, therefore, be reasonably assumed, in line with information from investigations on other systems, that it is acting specifically on protein synthesis.

Effect of Puromycin on Development and Cell Division

If development in *T. pyriformis* GL is considered with respect to the way in which it responds to puromycin, it can be divided into three phases (49): (*a*) During the "anarchic field" stage (and earlier), further development is blocked by exposure to puromycin; the stage 1 primordia eventually undergo slow resorption. (*b*) During early phases of oral membranelle differentiation, exposure to puromycin brings about prompt resorption of developing membranelles and blockage of cell division. (*c*) During the final stages of oral development, and concomitant cell division, puromycin slows but does not block the completion of development and division, except sometimes at the high concentration; prompt resorption of the oral primordium does not occur. These responses do not differ basically from those obtained earlier with *p*-flurophenylalanine, 5-fluorouridine, and actinomycin D (12). In the earlier study, it was concluded that new template RNA synthesis as well as new protein synthesis are required for the initiation and maintenance of development, up to the "stabilization point" at the end of phase (*b*).

The results of the present study are, in general, consistent with the above-mentioned conclusion. However, two findings require that these conclusions be somewhat amended and qualified. These findings are: (1) During phases (*a*) and (*b*) development is blocked (or reversed) even at a concentration of puromycin that reduces the rate of protein synthesis relatively slightly. (2) Cell division furrowing can be retarded or even arrested by puromycin during phase (*c*). The first of these two findings has already been made with *p*-fluorophenyl-alanine (3, 6, 30); however, with this agent it is difficult to disentangle effects due to reduction of rate of protein synthesis and effects caused by incorporation of the analog into proteins which might then be nonfunctional (53). With puromycin, there is no such incorporation except at the end of incomplete polypeptide chains, some of which may be precipitated by TCA (cf. above).

The significance of the first finding can be most clearly appreciated if the results obtained with *Tetrahymena* are compared with those obtained by E. W. Taylor (39) in his study of the effects of puromycin on cell division in human carcinoma cells. Taylor found that puromycin caused a slowing of the rate of cell multiplication and a concomitant lowering of the mitotic index, with a close correlation between the degree of slowing of the division cycle and the degree of reduction of protein synthesis. Only when the incorporation rate was reduced to 20% of the control level did cell multiplication cease altogether. No evidence was obtained to indicate that the mammalian cells responded to partial inhibition of protein synthesis with any substantial "set-back" of cell division. More recent studies of the effects of puromycin and cycloheximide on cell division in Chinese hamster cells (41, 42) clearly demonstrate the absence of set-back, since cell multiplication is resumed at the normal rate shortly after the washing out of either of these inhibitors. Set-back was only observed after very long exposures (3 hr or more) to cycloheximide, which was interpreted as being a result of eventual decay of division-specific messenger RNA (42).

The response of *T. pyriformis* GL-C to puromycin during phases (*a*) and especially (*b*) is radically different from that of the mammalian cells. At a concentration of puromycin (43 μg per ml), which initially reduces protein synthesis only to 70% of the control value, development is permanently blocked, and resorption phenomena, known from earlier studies (10) to be correlated with "set-back" of cell division, are rapidly set into motion (cf. Fig. 6).

It is possible to account for division blockage by assuming that puromycin affects the formation of different proteins differentially, and that "division proteins" are especially sensitive. This

assumption, however, does not fully explain the resorption and associated "set-back" phenomena. These phenomena are elicited by many other agents as well as puromycin. A detailed analysis of the resorption phenomenon (13) demonstrated that agents which bring about resorption do so by triggering an intracellular degradative process which, once initiated, goes to completion regardless of whether or not the initiating stimulus is still present. Thus, the reduction in the rate of protein synthesis brought about by the action of puromycin might, in some manner, trigger this degradative system. The way in which such triggering could be achieved is discussed in detail elsewhere (13).

As mentioned earlier, during phase (*c*), after the "stabilization point," resorption and "set-backs" are no longer readily elicited by treatment with chemical agents. Somewhat unexpectedly, puromycin was found capable of retarding or even arresting the process of division-furrowing in cells affected during this late phase. At 43 μg per ml the effect was slight, while at 430 μg per ml it was very considerable (cf. Fig. 4; Table IV). Comparison of these results with effects on protein synthesis (Fig. 1; Table I) indicates that there is at least a rough correlation between the degree of inhibition of protein synthesis and the degree of retardation of furrowing. This correlation, plus the fact that long delays of cleavage were also brought about by cycloheximide (unpublished observations), suggests that protein synthesis is somehow involved in the process of cleavage furrowing.

When added late (during phase *c*), puromycin (430 μg per ml) delayed the completion of oral development concomitantly with the delay of cleavage. The drug, however, failed to bring about the prompt resorption of these advanced oral primordia.[4] Daughter cells had oral structures which looked normal when silver stained; however, we did not test their functional capabilities. Nachtwey (personal communication) has observed that after a treatment with 2×10^{-3} M (110 μg per ml) puromycin between 60 to 103 min after EST, which was followed by transfer to puromycin-free medium, almost all daughter cells were unable to form food vacuoles. In most of these cells, the capacity to form food vacuoles was not recovered until after the subsequent cell division. This result indicates that new protein synthesis may be involved in the final steps of stomatogenesis (elaboration of the food vacuole–forming region) as well as in cell cleavage. It should be pointed out that the late events of stomatogenesis are carried out during cleavage by *both* proter and opisthe mouths, since the deep fiber bundle of the anterior oral area is resorbed at the beginning of cleavage and then reforms at the same time that the posterior oral primordium is forming its fiber bundle (49). This fact explains why Nachtwey observed failure of food vacuole formation in virtually all daughters, rather than only in the one-half of the daughters derived from opisthes, after puromycin treatment during the cleavage period.

The evidence for protein synthesis directly associated with division furrowing necessitates some reconsideration of earlier interpretations of the "physiological transition point" (30). The "physiological transition point" has been interpreted as the time at which protein syntheses required for cell division (30) and oral organelle development (49) are completed. An alternative interpretation, suggested by the results of this and a previous (13) study, is that the sharp transition from maximal to no "set-back" of division in response to treatments with inhibitors is not necessarily a reflection of completion of relevant protein synthesis, but may rather be a result of the shutting off or removal of a latent degradative system which operates to amplify disturbances which (in some cases) may initially have been quite small.

Effect of Puromycin on Resorption

In the previous paper of this series (13) it was concluded, on the basis of indirect evidence, that the resorption process is enzymatic. If this is so, one can ask whether the relevant enzymes are preformed, and need only to be activated and released, or whether the formation of these enzymes is induced by the stimulus which causes resorption. The results favor the former interpretation, since resorption is initiated nearly as rapidly at 430 μg per ml as it is at 43 μg per ml. While a small amount of true protein synthesis

[4] Delayed onset of resorption was observed in some cells in which division had been blocked for several hours (Table VIII); this is comparable to the delayed resorption observed in heat-induced chains (11) and can be considered a secondary consequence of prolonged division arrest rather than a primary effect of the treatment which caused the arrest.

may still occur at the high concentration of the drug, enzyme induction under these conditions is extremely unlikely; it has been shown in *E. coli* that enzyme induction is far more sensitive to the action of puromycin than is over-all protein synthesis (35). Hence, it is probable that the enzymes involved in resorption are present in the cell prior to the appearance of the stimulus which triggers resorption. However, the fact that the completion of resorption is slowed at the higher concentration of puromycin implies that some protein synthesis is necessary to maintain resorption at a maximal rate. The nature of this synthesis is unknown. It is interesting to compare, in this regard, the *Tetrahymena* resorption system to the system which is responsible for turnover of proteins in nitrogen-starved *E. coli*: the turnover system is believed to involve the action of constitutive enzymes, yet it, too, is affected by inhibitors of protein synthesis (33). Both systems possess the somewhat paradoxical attribute that protein synthesis appears to be required for the optimal function of a degradative system whose action can be initiated by a reduction in the rate of over-all cellular protein synthesis.

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