ACTIVITY OF RIBONUCLEASE, ACID PHOSPHATASE, AND PHOSPHODIESTERASE IN *TETRAHYMENA PYRIFORMIS* DURING GROWTH

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Studies on the nucleolytic activity of cell homogenates of *Tetrahymena pyriformis* indicated that enzyme activity was, among other factors, a function of the age of the culture (1, 2). It was shown that the specific activities of ribonuclease (RNase) (EC 2.7.7.h) and acid phosphatase (APase) (EC 3.1.3.2) in crude homogenates prepared from stationary-phase cultures were consistently higher than those from log phase by a factor of at least two (2). However, in contrast to these two enzymes, the level of a potassium-activated ribosomal phosphodiesterase (PDase) (EC 3.1.4.1) was reported to be maximal at the end of exponential multiplication (3).

Our results on the level of APase in *T. pyriformis* GL, however, could not be reconciled with the data of Klamer and Fennel (1), who reported increased APase activity in strain W only during the exponential death phase. Therefore, it was of interest to further investigate the activity of these nucleases in more detail. In addition, we wish to report here the effect of puromycin, an inhibitor of protein synthesis, on the change in activity of RNase and APase during growth.

MATERIALS AND METHODS

Tetrahymena pyriformis GL was grown in a proteose–peptone, yeast extract and glucose medium as previously described (2). The number of cells/milliliter was determined either microscopically or by means of a Coulter counter with a 100 μ window. The volumes of the aliquots withdrawn from the culture were decreased with time for the obtaining of approximately the same number of cells per sample. Each aliquot was collected in a flask submersed in a brine slurry at −4°C and centrifuged at 0–2°C. The pellet was washed twice in 20 volumes of ice-cold 0.4% NaCl, frozen in a dry ice–alcohol bath, and stored at −22°C until assayed. Storage at −22°C, had no effect on enzyme activity. All the samples were simultaneously thawed. The volumes were adjusted to 2.0 ml and 0.2 M in Tris-HCl buffer, pH 7.45. Samples were kept on ice, and repeatedly passed through the tip of a micropipet to ensure complete lysis. The homogenates were assayed immediately after lysis because of the instability of PDase activity, although no decrease in RNase and APase activities could be detected for periods up to a week at 4°C.

RNase activity was determined as detailed previously (2), as were the assays for PDase (3) and APase (4). All enzyme assays were carried out at least in duplicate and corrected for blanks without protein or substrate. Protein was determined by the method of Lowry et al. (5) and modified by Lazarus and Scherbaum (2).

Commercial RNA (Boehringer and Soehne, distributed by Calbiochem, Los Angeles, Calif.) was repurified before use (2). Calcium bis-*p*-nitrophenyl phosphate and disodium *p*-nitrophenyl phosphate were purchased from Sigma Chemical Company (St. Louis, Mo.); β,β'-dimethylglutaric acid (C grade) was purchased from Calbiochem, (Los Angeles, Calif.), diethylamine was from Eastman Organic Chemicals, (Rochester, N. Y.) and puromycin–HCl was from Nutritional Biochemicals Corporation (Cleveland, O.).

RESULTS AND DISCUSSION

From the data on cells per ml and enzyme activity per ml, three characteristic features of the change in enzyme activity per cell are illustrated in Fig. 1. First, the activities of both APase and RNase per cell decreased during the initial growth of the culture, reached a minimum in log phase, and increased 5–7-fold, respectively, in stationary phase.[1] Second, the activity of PDase per cell immediately increased after inoculation to a maximum level about 5-fold through late log phase to the beginning of stationary phase and decreased thereafter. Third, the increase in activity per cell occurred in the following order: PDase, RNase, and APase.

It was then of interest to determine whether an intracellular enzyme inhibitor (6,7) and/or

[1] An identical, although less pronounced effect, was observed if the results were plotted in terms of specific activity (enzyme activity/milligram protein).

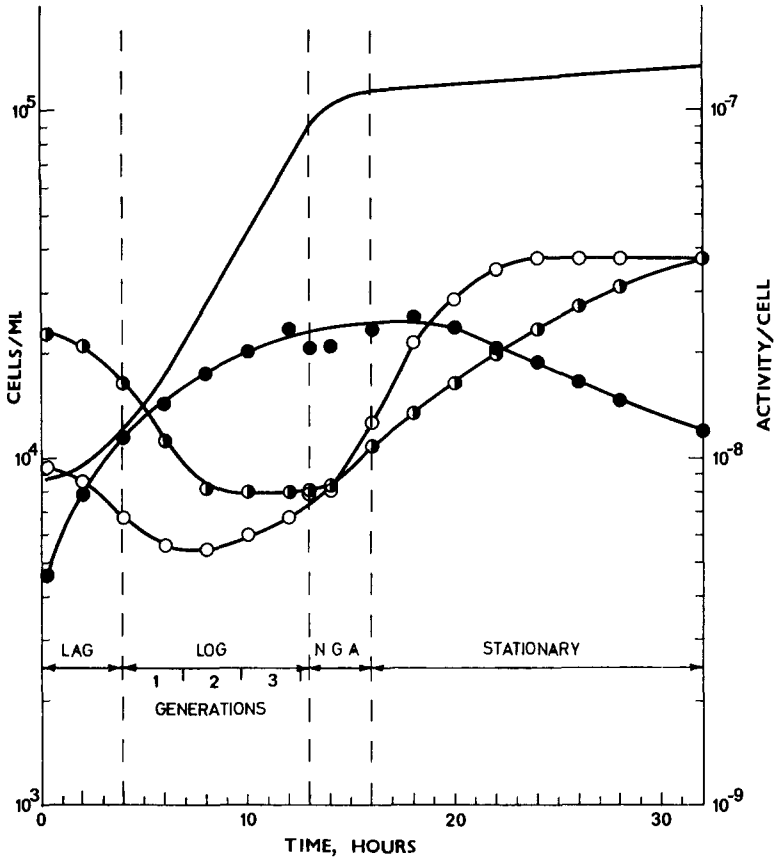

FIGURE 1 Activity of RNase, APase, and PDase per cell during growth of *T. pyriformis*.

A 500 ml, 72 hr *T. pyriformis* inoculum was added to 4 liters of aerated peptone–yeast medium in a 50 liter capacity culture tank at 29°C. After 15 min, samples were taken at intervals, harvested, and washed, and the homogenates were prepared by freeze-thawing and assayed for enzyme activity.

RNase activity was determined (2) in 44 mM β,β'-dimethylglutaric acid buffer, pH 5.0, 8.8 mM EDTA, 2.5 mg RNA and 80–200 μg protein, with incubation at 29°C for 60 min. To the reaction mixture an equal volume (0.5 ml) of cold acidic ethanol (10% HCl) containing 0.01 M $MgCl_2$ was added, the precipitate was removed by centrifugation, and an aliquot of the supernatant was diluted with water to 1.0 ml and read at 260 mμ. The increase in absorbance of 1.0 at 260 mμ/hr is defined as a unit of activity.

The assay for *PDase* (3) was done in 25 μmoles/ml, diethylamine, pH 10.3, 1 μmole/ml $MgCl_2$, 1 μmole/ml calcium bis-*p*-nitrophenyl phosphate, and 0.5–2.0 mg protein, with incubation at 29°C for 60 min. The reaction was terminated by the addition of 100 μl of ice-cold 50% trichloroacetic acid. After centrifugation, an equal volume of 0.5 N NaOH was added to an aliquot of the supernatant and the absorbance was read at 400 mμ. A unit of enzyme activity is defined as the formation of 1 μmole of *p*-nitrophenol/hour.

The reaction mixture for *APase* activity (4) (1.0 ml) contained 125 mM sodium acetate buffer, pH 4.60, 50 mM L-cysteine (prepared fresh before use), 2 mM *p*-nitrophenyl phosphate and 7–28 μg protein. After 15 min at 29°C the reaction was terminated by the addition of an equal vol of ice-cold 1.6 N NaOH and the absorbance was read at 400 mμ. The formation of 1 μmole of *p*-nitrophenol/minute is defined as a unit of enzyme activity.

Growth of the culture is presented by the *solid line*. *NGA* is negative growth-acceleration phase. RNase (○), units/hour/cell; APase (◑), μmoles/minute/cell; and PDase (●), μmoles/hour/cell.

TABLE I

Assay for Inhibitors and/or Activators in Homogenates of Tetrahymena pyriformis

Assay material	Protein per assay mixture	RNase activity	APase activity
	μg		
Log phase	17.2	0.84	36.4
Stationary phase	8.2	0.73	37.7
Homogenates mixed	25.4	1.56	72.6

Log phase homogenates were obtained from cells 10 hr after inoculation, whereas stationary phase homogenates were obtained from the 28 hr culture (see Fig. 1). Enzyme activities were determined in duplicate by the methods given in the legend to Fig. 1.

TABLE II

Effect of Puromycin on the Specific Activity of RNase and APase in Tetrahymena pyriformis

Enzyme	Time	Specific activity		Inhibition
		Control	Puromycin	
	hr			%
RNase	0	31	—	0
	1	35	31	11
	2	44	42	5
	4	72	37	49
	6	104	36	65
APase	0	137	—	0
	1	144	120	17
	2	140	107	24
	4	201	140	30
	6	228	136	40

A log phase culture (41,000 cells/milliliter) was divided in half: one part was transferred to a sterile 2 liter, low-form culture flask containing puromycin to give a final concentration of 20 μg/ml, while the other half, under identical conditions (minus the drug), served as the control. Aeration by shaking was continued and 40-ml samples were taken at intervals, washed, freeze-thawed and the homogenates were assayed in duplicate as described in the legend to Fig. 1. Per cent inhibition of the specific activity is relative to the control at that time.

activator were present which might have been responsible for the observed change in the activities of RNase and APase during growth. This question was examined by three independent methods. First aliquots of stationary-phase homogenates were mixed and assyed with a 2-fold excess of log phase homogenates. The results in Table I clearly show that the activities of these enzymes were completely additive. Second, since this assay would detect only a nonbound inhibitor and/or activator, the homogenates were extracted under acidic conditions, a procedure which has proven effective in rupturing the binding between RNase and RNase-inhibitor (6). The samples were extracted with 0.4 N H_2SO_4 for 16 hr at 4°C, neutralized with concentrated NH_4OH, and centrifuged; the supernatants were assayed for enzyme activity. The values for the specific activity of RNase were identical with those of the crude homogenate.[2] Last, a log-phase culture was treated with puromycin (20 μg/ml), which is a potent inhibitor of protein synthesis and which depresses the increase of isocitric lyase (EC 4.1.3.1) during glyconeogenesis in *T. pyriformis* (8). After 1 hr the growth rate decreased to 50% of the control and then ceased altogether. As seen in Table II, the increase in RNase and APase activities which normally occurs after an initial decrease in log phase was inhibited by 65 and 40%, respectively, after 6 hr.[3]

The timing of the synthesis of these enzymes may be either the result of an ordered transcription of the genome or a function of their biological roles. The change in the activity of the ribosome-bound PDase could reflect the variation in ribosome content during growth (see reference 9) or could involve the turnover of messenger RNA (10). The increased levels of RNase and APase in stationary phase suggest either a scavenger role (11) or activity associated with transfer reactions.

From the results presented in this paper, we conclude that an intracellular inhibitor and/or activator do not regulate the activity of RNase and APase and that the increase in activity of these enzymes which had occurred prior to and during stationary phase was due to the synthesis of enzyme protein. Moreover, since the activities of RNase and APase per milliliter of culture remained essentially constant during early growth,

[2] After extraction under these acidic conditions, neither APase nor PDase activity could be detected.

[3] Although cell division was inhibited by puromycin, the cells were still viable after 6 hr, i.e., microscopically the cells were actively motile. Therefore, the relative decrease in enzyme activity is not attributable to cell death.

the initial decline in enzyme activity appears to be a dilution phenomenon, i.e., over-all protein synthesis continues without concomitant synthesis of either RNase or APase. However, excretion of these enzymes during growth cannot be entirely ruled out, since an extracellular RNase has been characterized (12), although it differs in enzymatic properties from the intracellular enzyme (2, 12, 13). Similar differences were reported also between the intra- and extracellular proteinases in *T. pyriformis* (14).

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