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CATALASE pI-ISOZYMES IN TETRAHYMENA PYRIFORMIS (GL) AND THEIR CHANGES DURING THE CELL MULTIPLICATION AND MATURATION

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A number of investigations hitherto reported have demonstrated the presence of multiple molecular forms of various enzymes in animal cells. Furthermore, the changes in this heterogeneic pattern of several enzymes were reported to possibly have some correlations with ontogenetic differentiation and also cancerous dedifferentiation. Catalase is an enzyme predominantly located in peroxisomes which are regarded as phylogenetically fossil cytoplasmic organelles (1, 2), and hence may be changed along the evolutionary course of life together with cell differentiation and dedifferentiation. In these respects, the heterogeneic pattern of catalase in the liver have definitely reported to be altered during the course of postnatal growth of rats (3, 8).

Multiple forms of catalase in Tetrahymena pyriformis have not been demonstrated, although the presence of catalase isozymes in liver (4-11), erythrocytes (12-15) and in maize endosperms (16-18) have been reported. On the other hand, several enzymes other than catalase in Tetrahymena

were reported to show multiple forms which were greatly influenced by the environmental conditions during the cultivation (19). As in the case of these enzymes, the heterogeneic pattern of catalase might be expected to be altered by such factors as may control multiplcation and maturation of the cells.

The present study was undertaken to demonstrate the existence of multiple forms of catalase in GL-strain of Tetrahymena pyriformis by isoelectric fractionation and further to clarify the changes in activities and the heterogeneic pattern of the catalase in various growth stages of the cells cultivated at different temperatures. In addition, fructose-1, 6-diphosphate (FDP)/fructose-1-phosphate (FIP) activity ratios in aldolase were also determined, since the ratios were reported to be changed in ontogenetic differentiation and cancerous dedifferentiation (20-23).

Materials and Methods

Organisms. A Gl-strain of Tetrahymena pyriformis was donated by Dr. T. Mita, National Cancer Center Research Institute, Tokyo. 4×10^5 cells were grown in a 200 ml Erlenmeyer flask containing 50 ml of a medium consisting of 2% proteose peptone No. 3 (Difco), 1% yeast extract (Difco), and 0.6% glucose, at 28°C under continuous shaking at approx. 90 oscillations/min. and with aeration for 60 hours. In another case, after the shaking cultivation at 28°C for 15 hours, the flasks were placed at 22°C and the cultivation was continued thereafter for 48 hours without shaking.

Preparation of Enzyme Solution for Isoelectric Fractionation. The cells were chilled in ice, collected by low-speed centrifugation, and then washed twice with cold PBS. The cells were disrupted in the cold by 3-4 passes through a teflon homogenizer and the crude homogenate was then treated with ultra sound for 5 min. at 200 W using a Kubota KMS-250 ultrasonic generator. The preparations were centrifuged at 105,000 x g for 60 min. at 0°C using a Beckman Model L2-65B ultracentrifuge. The clear supernatant was concentrated and dialyzed against cold distilled water using collodion bags (Sartorius Membrane Filter) for about 2 hours at 0°C. A portion of this enzyme solution containing 15 mg of protein was submitted to isoelectric fractionation. The protein was measured according to the method of Lowry et al. (24).

Isoelectric Fractionation with Ampholine Carrier Ampholite. Ampholine carrier ampholite (pH-Range 5-8) was purchased from L. K. B. Produkter AB Bromma, Sweden. Examinations were made according to the method of Vesterberg and Svensson (25), using an electrofocusing column (L. K. B. 8100-10) with a capacity of 110 ml. The enzyme solution containing 15 mg protein was applied to the column which was filled with a 1% Ampholine/sucrose gradient system. To obtain a linear gradient of Ampholine and sucrose, L. K. B. gradient mixer was used. Potentials were raised stepwise up to 700 volts using an electric power supply (L. K. B. 3371 D) during the first 3 hours. Cold water (0-1°C) was circulated through the cooling jackets during the processing. After electrolysis for 45 hours (700 volts, 0.5mA at final stage), elution was carried out in 15

drops at a flow rate of 1.0 ml/min. by the use of an automatic fraction collector and the absorbance of the effluents was measured at 280 mu automatically with an L. K. B. Uvicord. Catalase activity of each fraction was determined by the method of Adams et al. (26), and pH of the fractions was measured at 0-1°C using a Hitachi-Horiba pH-meter, M-5, equipped with a Horiba 6028-10T combined electrode.

Determination of Aldolase Activity of the Cells. Aldolase solution was prepared by treating with ultra sound, followed by centrifugation at 105,000 x g for 1 hour as mentioned above. The enzyme activity was determined at 37°C spectrophotometrically by the method of Blostein and Rutter (27) with a slight modification in the presence of α-glycerophosphate dehydrogenase and triosephosphate isomerase.

Determination of Cell Number and Sizes of the Cells and Nuclei. The number of the cells was measured using a hemocytometer, and sizes of the cells and their unclei were determined on micrographs of smear preparations with the aid of a Zeiss TGZ-3 particle analyzer. The sizes were expressed in terms of the average of the length of the major and minor axis of the cells.

Results

Growth Characteristics of Tetrahymena pyriformis in Shaking Cultivation at 28°C and Static Cultivation at 22°C.

As shown in Fig. 1, the cells cultivated at 28°C under shaking and

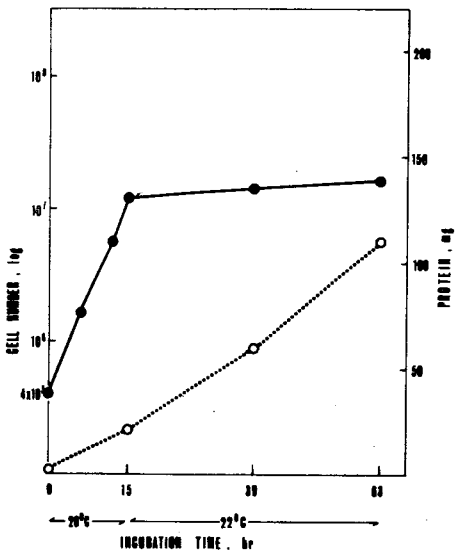


Fig. 1.

Growth curve and total protein content of Tetrahymena pyriformis in shaking cultivation at 28°C and static cultivation at 22°C. Solid circles and line represent the growth curve, and open circles and dotted line are the protein content of the total cells in each culture flask.

Table 1. Changes in Number and Protein Content of Tetrahymena Cells During Shaking Cultivation at 28°C and Static Cultivation at 22°C.

Time of Cultivation, hrs	Number of Cells/Flask	Protein, mg/Flask	Protein, mg/10^5Cells
0	4×10^5	2.5	0.62
15 (15 hrs at 28°C)	120	20.6	0.17
39 (15 hrs at 28°C, then 24 hrs at 22°C)	150	60.0	0.40
63 (15 hrs at 28°C, then 48 hrs at 22°C)	170	110.3	0.65

aeration multiplied in a logarythmic fashion with a generation time of about 3 hours during the 15-hour cultivation. When the culture flasks were thereafter transfered into the bath at 22°C without shaking, multiplication of the cells took place at an outstandingly slow pace showing a mere 40% increase in the cell number during the static cultivation for 48 hours. Total protein content in each culture flask increased almost linearly during these cultivation periods. The protein content per cell basis, however, decreased progressively during the shaking cultivation reaching the smallest amount at the end of the 15-hour cultivation, and then showed an increase up to the end of 48 hours of the static cultivation at 22°C, where the level was found to be approximately the same as that at the starting level (Table 1).

Fig. 2 shows the growth curve of the cells and protein content of each flask in shaking cultivation at 28°C with aeration for 60 hours. The cells showed an exponential growing during the first 15 hours, followed by a deceleration over a period of 15-48 hours, and the cells did not multiply further even when the cultivation period was prolonged for more than 48 hours. The total protein content in each culture flask showed a more rapid increase and was about two-fold at 60 hours as compared against that of the static cultivation at 22°C. The protein content per cell basis, however, maintained a low level without recovering throughout the experiment (Table II).

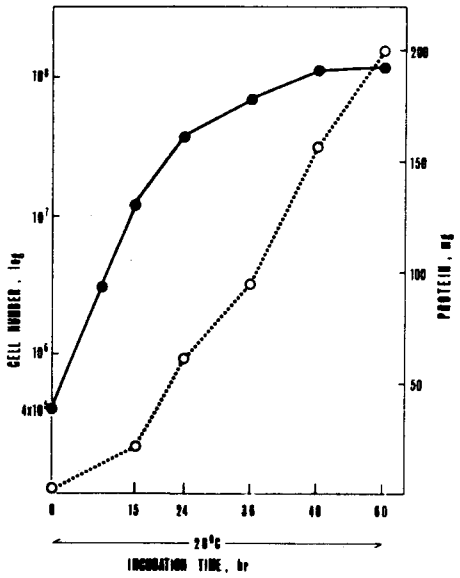


Fig. 2.

Growth curve and total protein content of Tetrahymena pyriformis cultivated under continuous shaking at 28°C for 60 hours. Solid circles and line represent the growth curve, and open circles and dotted line are the protein content of the total cells in each culture flask.

Table 2. Changes in Number and Protein Content of Tetrahymena Cells During Continuous Shaking Cultivation at 28°C.

Time of Cultivation, hrs	Number of Cells/Flask	Protein, mg/Flask	Protein, mg/10^5 Cells
0	4×10^5	2.5	0.62
15	120	20.6	0.17
24	380	60.7	0.16
36	700	95.0	0.14
48	1040	157.0	0.16
60	1060	200.0	0.19

Cultivation was made at 28°C.

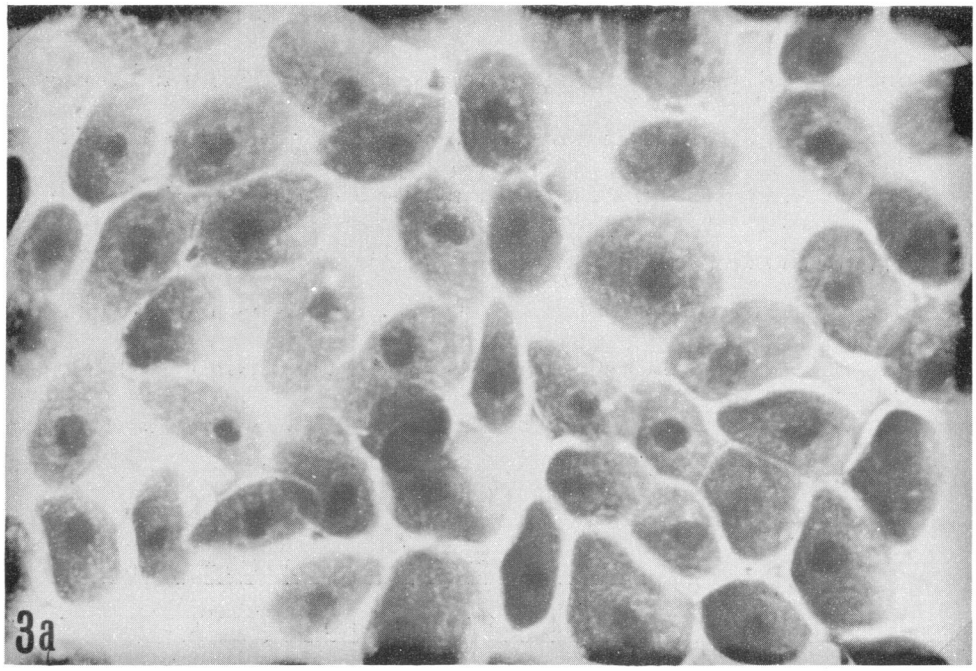
Tetrahymena pyriformis cultivated under shaking for 15 hours at 28°C showed an appearance of homogeneously small in size and round or oval in shape as shown in Figs. 3a and 4a.

Fig. 3a

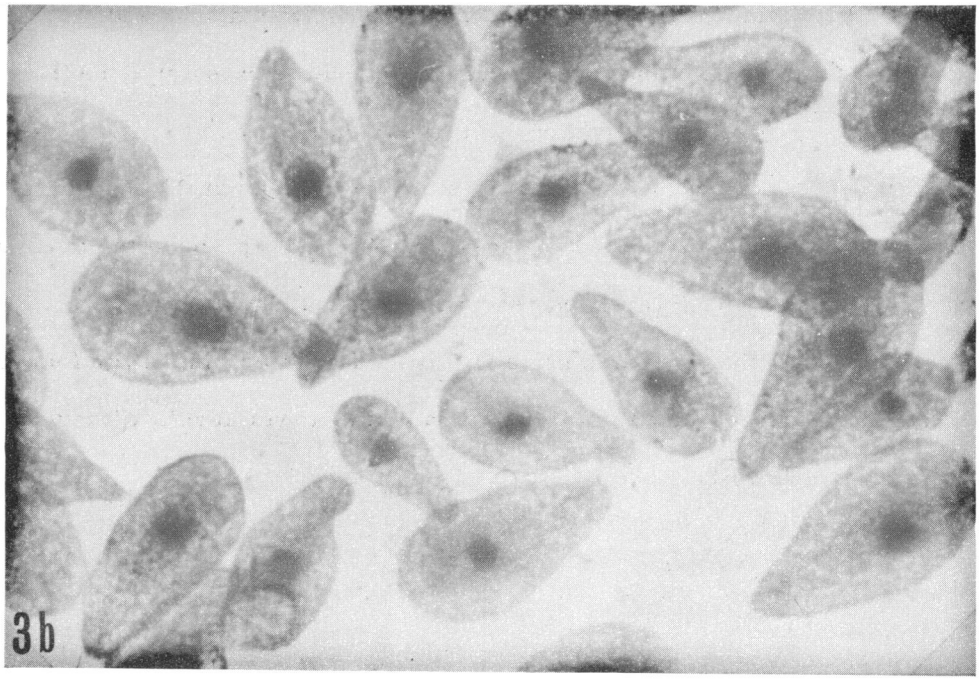
Tetrahymena pyriformis cultivated under shaking for 15 hours at 28°C. H-E staining. x 900.

Fig. 3b

Tetrahymena pyriformis during 48 hours static cultivation at 22°C after exponential growing at 28°C for 15 hours. Note the alteration of the shape of the cells and increase in the cytoplasmic volumes. H-E staining. x 900.



3a



3b

The diameter of the cells was estimated to be about 16.3 u in average. After 48 hours of the static cultivation at 22°C , the cells showed a considerable increase in size and displayed a characteristic form in terms of "pyriformis" (Figs. 3b and 4a). The mean size of the cells increased to 22.3 u in diameter. However, there was no difference in the size of the nuclei between the cells during the maximal cell multiplication in the log phase at 28°C and those during the slow growth in the stationary phase at 22°C (Figs. 3a, 3b and 4b).

Changes in Catalase Activity of Tetrahymena pyriformis during Shaking Cultivation at 28°C and Static Cultivation at 22°C.

Changes in catalase activity of the cells during the log and stationary stages are shown in Fig. 5. The activity decreased progressively in the former stage, reaching a level of nearly one-fifth of the initial values at the end of the 15-hour cultivation at 28°C. After the culture flasks were transfered to static conditions at 22°C, the activity increased almost linearly up to the 48th hour, where the activity showed values approximately the same as those of the starting level. However, when the shaking cultivation at 28°C was continued for longer than 15 hours, the catalase activity remained at lower levels without recovering (Fig. 6).

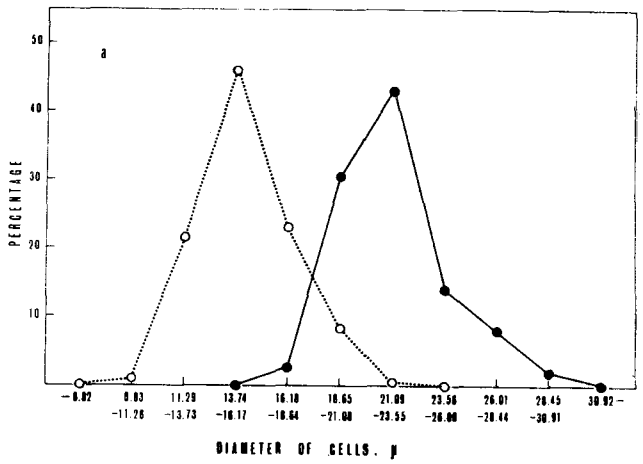


Fig. 4a Distribution of the sizes of Tetrahymena cells in the log and stationary phases of the growth.

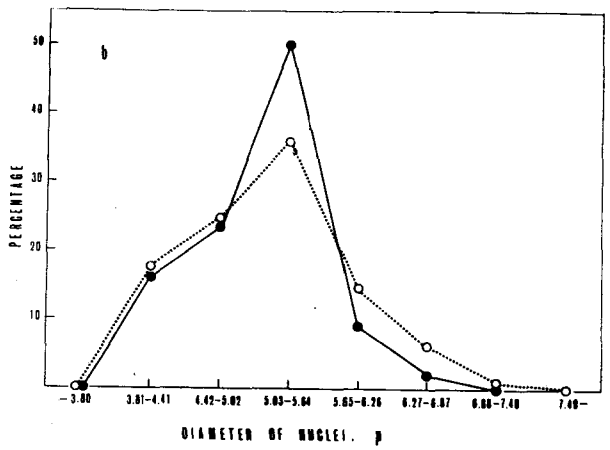


Fig. 4b Distribution of the sizes of the nuclei of Tetrahymena cells in the log and stationary phases of the growth.

---O------O--- 15th hour of cultivation at 28°C with shaking and aeration.

—●———●— 48th hour of static cultivation at 22°C.

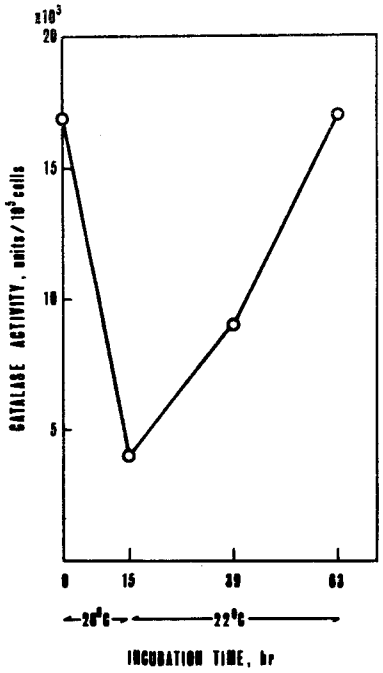


Fig. 5

Changes in catalase activity of Tetrahymena pyriformis during shaking cultivation at 28° C and static cultivation at 22°C. The activity is expressed in terms of catalase coefficient k / min. / 10^5 cells.

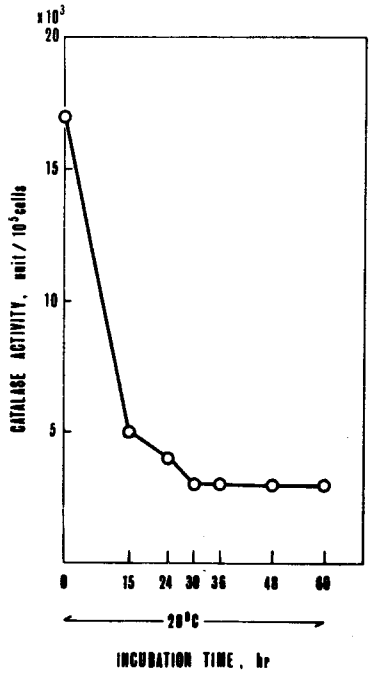


Fig. 6

Changes in catalase activity of Tetrahymena pyriformis cultivated under continuous shaking at 28°C for 60 hours. The activity is expressed in terms of catalase coefficient k / min. / 10^5 cells.

Catalase pI-Isozymes in Tetrahymena pyriformis and their Changes in the Log and Stationary Stages of the Growth.

A typical pattern of multiple forms of catalase in Tetrahymena pyriformis is presented in Fig. 7. It was clearly demonstrated that the enzyme was composed of five catalytically active major components, which were fractionated at pH 6.95, 6.65, 6.50, 6.05 and 5.85, respectively. Since the fractionation was made according to the differences in isoelectric points, these were called catalase pI-isozymes (9). Insofar as the pattern of these pI-isozymes is concerned, the present results are considered to be essentially in agreement with that described in rat liver (3, 9).

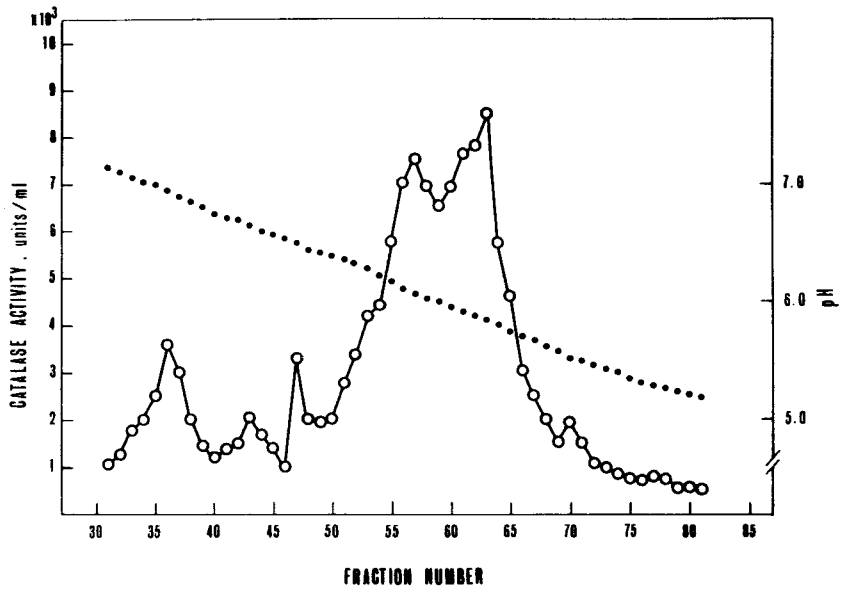


Fig. 7 A typical pI-isozyme pattern of catalase in Tetrahymena pyriformis. Open circles and solid line illustrate catalase activity, which is expressed in terms of catalase coefficient k / min. / ml. Solid circles indicate pH value of each fraction.

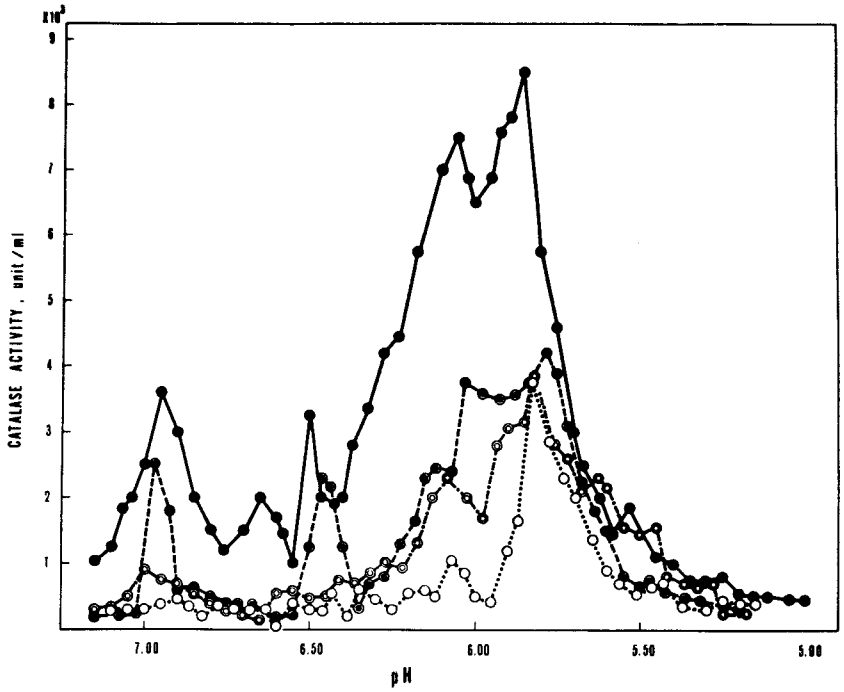


Fig. 8 Developmental changes in catalase pI-isozyme pattern during various stages of growth.

15th hour of cultivation at 28°C with shaking and aeration.

10th hour of static cultivation at 22°C.

24th hour of static cultivation at 22°C.

48th hour of static cultivation at 22°C.

Fig. 8 shows the developmental changes in catalase pI-isozyme pattern in various stages of the cell growth. At the end of the log stages of the cell multiplication, only pI-5.85 enzyme was found to retain its

stability, while other components had disappeared. Furthermore, even if the cultivation was continued for longer than 15 hours and the cell multiplication was decelerated, catalase was resolved into only pI-5.85 enzyme, insofar as the cells were grown at 28°C with shaking and aeration.

At the 10th hour of the stationary cultivation at 22°C, the activity of pI-5.85 catalase first increased slightly, and then pI-6.05 enzyme made its appearance, thereafter this was followed by a restoration of pI-6.95 and pI-6.50 catalase simultaneously at 24th hour. All of the five major components of the enzyme were completely recovered at 48th hour after the initiation of static cultivation.

Changes in FDP/FIP Activity Ratios of Aldolase in Tetrahymena pyriformis in the Log and Stationary Stages of the Growth.

In exponentially multiplying cells, aldolase activity was high with FDP as the substrate, while very low with FIP. The FDP/FIP activity ratios reached approximately 50 at the 15th hour of the log stages of the cell growth. After culture flasks were transfered to static cultivation at 22°C, a decrease in FDP-activity and an increase in FIP-activity were noted to occur concurrently. At the end of the static cultivation for 24 and 48 hours, the ratio showed 6.5 and 6.3, respectively (Table 3).

Table 3. Changes in FDP/FIP Activity Ratios of Aldolase in Tetrahymena pyriformis in the Log and Stationary Stages of the Growth.

Condition of Cultivation	F D P	F I P	F D P / F I P
Shaking Culture, 28°C, 15 hrs	10.2	0.21	50.0
Static Culture, 22°C, 24 hrs	6.8	1.05	6.5
Static Culture, 22°C, 48 hrs	5.3	0.84	6.3

Values are International Units (umole substrate converted per mg protein per minute).

Discussion

Differences in isozyme pattern of certain enzymes particularly those related to glucose metabolism have been reported to exist between tumor cells and their normal counterparts. Furthermore, the deviation in the isozyme pattern in tumor cells are considered to be one of characteristics of their dedifferentiating and/or dysdifferentiating nature, since the isozyme pattern is suggested to be relatively unchangeable in already

differentiated cells even in their actively proliferating state.

In contrast to this, isozyme patterns of various enzymes in Tetrahymena were reported to be influenced by the growth cycle, composition of culture medium, and also temperature during cultivation of the cells. In this respect, Allen (19) reviewed the control mechanism of isozymes in Tetrahymena stating that esterase-1-isozymes and phosphatase-1-isozymes were influenced greatly by the growth cycle of the cells and composition of the culture media, respectively. With regards to this, investigations on an epigenetic control of isozyme pattern in Tetrahymena in multiplying and maturating states might facilitate an understading of dedifferentiating nature of tumor cells in multicellular organisms.

According to Prescott (28), Tetrahymena was found to multiply in a logarythmic fashion at 25°C during 5-50 hours, followed by a growth deceleration during 40-49 hours, and not to multiply further during 71-120 hours of the cultivation. In prolonged cultivation, however, changes in the medium might occur to influence events leading to degenerative processes within the cells, although the cellular changes were not mentioned. In the present experiments, on the other hand, cultivation of the cells was made at two different temperatures, in order to avoid possible alterations in the culture media influencing the cell metabolism and to obtain the cells in different growth cycles. It might be worthy of note that Tetrahymena was found to multiply in an outstandingly slow rate during the static cultivation at 22°C, associated with an increase in cytoplasmic volumes and protein contents. This might be a reflection of cell maturation.

Catalase activity in Tetrahymena cells was found to vary with culture conditions, the specific activity being highest in the cells grown under static conditions at 22°C and lowest in those cultivated under shaking at 28°C. In other words, catalase activity of the cells might be low in their multiplying state, while high in maturating state. This result is considered to be similar to those reported on activities of other peroxisomal enzymes such as isocitrate lyase, malate synthase (29-31), and L-α-hydroxy acid oxidase (32).

pI-isozymes of catalase in Tetrahymena were resolved into five major components as were in the case of rat liver (3). During the exponential multiplication, only pI-5.85 catalase was found, and even if growth deceleration followed, catalase was separated into only pI-5.85 enzyme, insofar as the cultivation was continued at 28°C with shaking and aeration. Under these conditions, it may be surmized that biosynthesis of pI-5.85 enzyme alone took place and it corresponds to the immature state of cell development and repression of the ability to synthesize all the necessary subcomponents of the catalase molecule. On the other hand, in the process of the stationary phase at 22°C, activities of other four pI-isozymes appeared in succession. In other words, during the process of the cell maturation, de-repression of pI-isozymes is considered to occur. In the liver of rats, it was reported that isozymic pattern of catalase was changed around 3rd to 4th week after birth (3, 8). It is emphasized that such a differentiational change in the isozyme pattern may develop the course of maturation of Tetrahymena.

This assumption is supported by another fact that FDP/FIP activity ratios of aldolase in Tetrahymena were about 50 in the cells grown undergoing rapid multiplication at 28°C, whereas these were 6.5-6.3 in the cells in the process of maturation. According to Rutter and Weber (33), aldolase A showed FDP/FIP activity ratios of greater than 50 predominates in fetal liver, while during liver organogenesis, a progressive decrease in the synthesis of aldolase A and an increase in the synthesis of aldolase B was demonstrated, and thus FDP/FIP activity ratios reached about 1 in normal adult liver. It is suggested that de-repression of aldolase B in Tetrahymena might be operated as the cells undergo maturation and both A and B type aldolases were considered to appear. The mechanisms of the change-over might be identical with those found during differentiation in liver cells.

It might be probable that changes in pI-isozyme pattern of catalase during multiplication and maturation of Tetrahymena is a reflection of a sequential "switch on" and "switch off" of gene activities for biosynthesis of catalase isozyme, and furthermore, cellular processes controlling the genetic activities along the course of ontogenic development of higher animals seem to be operated within one cell cycle in Tetrahymena. The identification of pI-isozyme activities of catalase at a molecular level remains to be investigated.

Summary

Catalase extracted from Tetrahymena pyriformis (GL) was separated into five pI-isozymes, each of which exhibited its own isoelectric point at pH 6.95, 6.65, 6.50, 6.05 and 5.85, respectively, at 0-1°C by isoelectric focusing. These multiple forms differed clearly at each stage of growth of the organism. In exponentially multiplying cells, in which the cells were small in size and round or oval in shape and catalase activity was strikingly low, pI-5.85 enzyme alone appeared. As the stationary phase of the growth at 22°C proceded, a significant increase in cytoplasmic volume and catalase activity was found. In this process of the cell maturation, catalase pI-isozymes appeared in the ascending order of pI-5.85, 6.05, 6.95, 6.50 and 6.65. FDP/FIP activity ratios of aldolase were about 50 in exponentially multiplying cells, whereas these were 6.5-6.3 in the cells under maturation.

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