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AGE OF THE CELL CULTURE: A FACTOR INFLUENCING HORMONAL IMPRINTING OF *TETRAHYMENA*

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Insulin imprinting of *Tetrahymena pyriformis* in different growth phases had been investigated. Cells formed in the early logarithmic phase (18-hour culture) showed enhanced hormone binding at the second encounter with the hormone proving that imprinting had developed. This phenomenon was not observed in cells formed in the late logarithmic phase (42-hour culture) or in the stationary phase (66-hour culture). Lipid transformation processes, alteration of the guanyl cyclase activity and enhanced cell division may be responsible for this effect. Cell-growth phase G_1 was especially favourable for development of imprinting.

Tetrahymena is a wild living ciliated unicellular organism greatly dependent on its environment. The shape of the growth curve of its cell culture is influenced by changes of the environment, some of which being determined by the unicellular itself.

The multiplication curve of *Tetrahymena* can be divided into several phases, similarly to the growth curve of bacteria or other unicellular mass cultures. There is an initial or lag phase showing no increase in cell number and occasionally even a decrease can be observed. Subsequently the acceleration and exponential (logarithmic) phases follow: the increase of the cell number can be delineated logarithmically by a steep straight line that indicates a rather quick multiplication. A moderate cell growth prevails in the next period followed by the stationary phase with a relative constancy of the maximal excessible cell number. In this phase multiplication and cell death are in balance. Finally the culture reaches a declining period where cell lysis exceeds the growth rate of new cells [1].

This process causes alterations in the cell as well in the medium. As the cell number increases, the amount of the nutrient materials decreases and degradation products accumulate parallel with the aging of the culture. The amount of the accumulated "alien" materials is further increased by the degradation material of the lysing cells in course of the life cycle, and a shift in the proportion of the respiratory bases ensues in the logarithmic phase. These changes may also be responsible for the rise of the pH [2] and for the

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growing amount of neutral lipids [1]. The quantity of the free fatty acids deriving from the triglyceride deposits redoubles in the stationary phase compared to that in the logarithmic phase [3]. Subsequently it falls sharply, indicated by the gradually accumulating lipid drops. These changes are accompanied by simultaneous alterations of the intracellular enzyme activity: the acid phosphatase activity decreases in the logarithmic phase and rises to the maximum in the stationary phase [4]. A reversed change is shown in the activity of guanyl cyclase exerting the highest activity in the logarithmic phase and a gradually decreasing activity in the stationary phase [5].

These changes may be accompanied by alterations in the membrane level. An enzyme system is known to transform phospholipids to fatty acids. Its activity is more intense in the stationary phase than in the preceding logarithmic phase [6, 7]. At the same time production of phospholipids undergoes a change, too. A large amount of palmitic acid is incorporated into the phospholipid in the logarithmic phase, while in the stationary phase it is involved in production of glycerides and non-phospholipids [8]. These observations suggest that membrane structure and intracellular parameters are different in each growth phase of these cell cultures.

Hormonal imprinting is a phenomenon developing at the first encounter with the hormone: the cell shows an altered, usually enhanced responsiveness at the next encounter, as a consequence, and is capable of an increased hormone-binding [9–11]. Evolution of an imprinting is a complicated process influenced by several factors, involving momentary structural and functional state of the membrane [12, 13], as well as the activity of associated intracellular systems and enzyme groups [14]. The phases of the growth cycle potentiate the rise of an imprinting differently. Phase G_1, a period after the "birth" of the cell, yields a much easier development of imprinting than phase S or G_2 [15]. This is due to changes of the membrane structure in the different growth phases. In view of these data it may be assumed that since the cells of different growth phases differ in several aspects from each other, they can not be regarded equivalent concerning the development of hormonal imprinting either.

In the present experiment hormonal imprintability was investigated in the different growth phases of *Tetrahymena pyriformis* cultures.

Materials and methods

Cells of *T. pyriformis* GL strain were investigated in three different growth phases in a medium containing 0.1% yeast extract and 1% Bacto Tryptone (Difco) at 28 °C: (i) early logarithmic phase (18-hour culture); (ii) late logarithmic phase (42-hour culture); (iii) stationary phase (66-hour culture).

All cultures were divided into two parts: one was left untreated for control, the other was treated with 10^{-6} M insulin (Semilente Novo, Copenhagen Denmark) for 1 h. A representative sample of cells was incubated in normal medium for 24 h. Samples of the six groups were fixed in 4% formalin and washed in PBS. After one-hour incubation with fluorescein-

isothiocyanate (FITC-BHD Chemicals Ltd., Poole, England) labelled insulin (Semilente Novo, Copenhagen, Denmark) the cells were washed in PBS twice and dropped on slide. Binding of labelled insulin was determined cytofluorimetrically in a Zeiss Fluoval cytofluorimeter combined with a HP 41C computer used also for analysis of variance. Insulin-binding of 20 cells from each group was measured. Since the experiment was repeated 5 times the results of each experimental group represented the binding capacity of 100 cells. At the time of sampling the cells were examined with Sudan red staining for the amount of lipid drops accumulated in the cytoplasm [16].

Results and discussion

Hormonal imprinting varied in the different growth phases of *Tetrahymena*. The growth phases were distinguished on the basis of two indices: the cell number of the cultures (Fig. 1) and the accumulation of cytoplasmic lipid drops (Fig. 2). The amount of lipid increased parallel with the aging of the culture [17].

The assay of insulin imprinting revealed a striking difference between the imprintability of the 18-hour and the two older (42- and 66-hour) cultures (Fig. 3). While cells in the early logarithmic phase developed a strong imprinting (120%), those in the later phases showed no significant difference of imprintability as compared to the control.

The development of hormonal imprinting is a complicated process [18]. Reactions on the occasion of the first encounter with a polypeptide hormone capable of binding to the cell membrane, such as insulin, induce the cell in different ways to "remember" the previous encounter at the next one [19], and the cell reacts by an enhanced response, e.g. by hormone binding. The state of the cell membrane plays an important role in the development of this process line. Membrane receptors are the first to meet the hormone and bind it. Subsequent processes may diverge. The membrane itself is capable of preserving such informations by its plasticity [20], but certain enzymes of the membrane may also be active in the development of the membrane-bound memory [14]. An important transferring function is attributed to the phenomenon of mem-

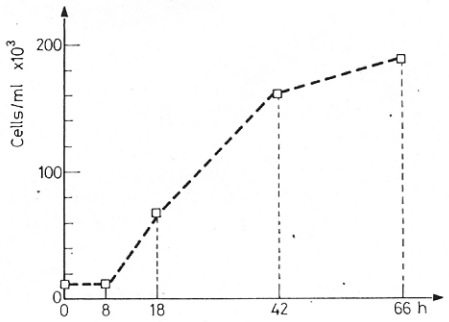

Fig. 1. Numbers of *T. pyriformis* cells in different growth phases

brane-flow, that establishes a continuous connection between the membrane covering the cell surface and the intracellular membrane system [13, 21] It also permits hormone-induced biochemical structural alterations in the outer membrane to be transferred as an information carrier to the nucleus, or facilitates the intracellular, endocytotic processes associated activity of the hormone itself. Thus, state of the cell-covering membrane may be of primary importance

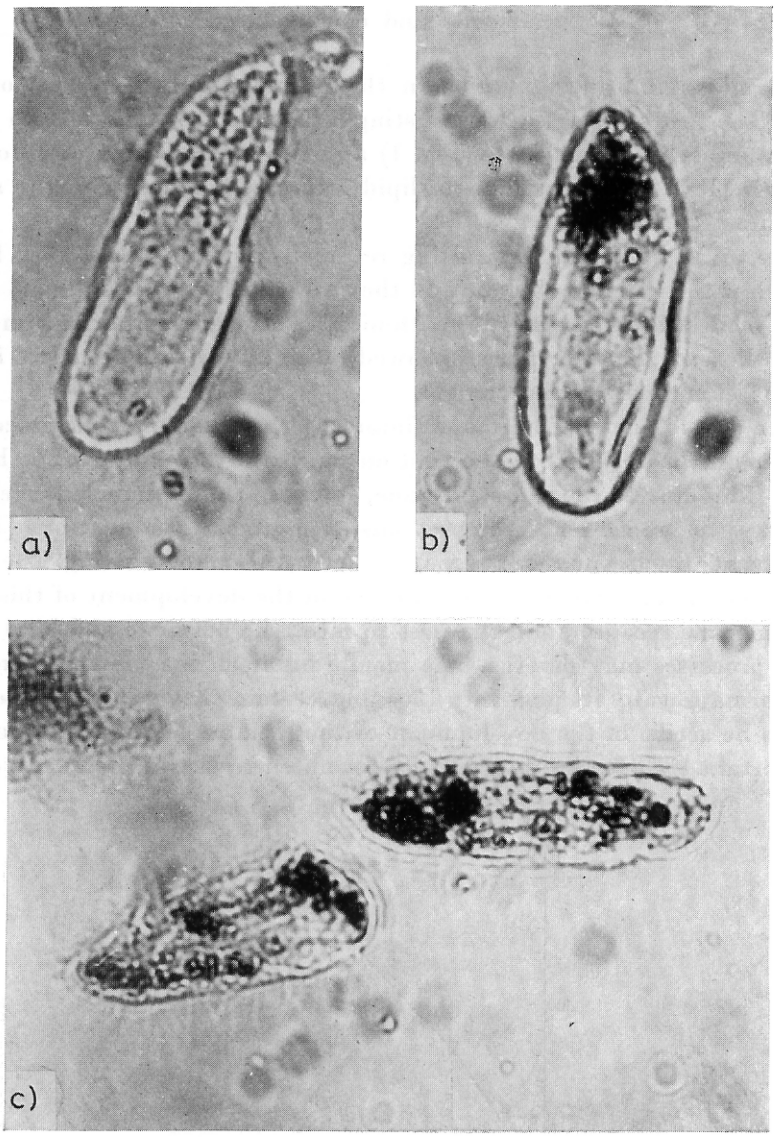

Fig. 2. Change of lipid contents of *T. pyriformis* cells stained with Sudan red. (a) 18-hour, (b) 42-hour, (c) 66-hour culture

for the development and effectiveness of the imprinting. The variable responsiveness of the different growth phases found in this study may also be partly attributed to these changes in the membrane structure.

Changes in the membrane structure may be reflected by the two-fold increase of the amount of the free fatty acids in the stationary phase compared to that of the logarithmic phase [3]. Other data indicate an 8-fold increase of

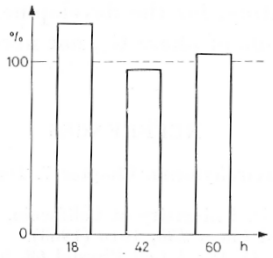

Fig. 3. Imprintability of *T. pyriformis* cells by insulin (control = 100% intensity of fluorescence)

the triglyceride level in 24–72-hour cultures [22]. These changes may be partly due to active transformation processes of the membrane. The enzyme system capable of degradation of phospholipids to fatty acid, functions with a much greater activity in the stationary phase than in the preceding logarithmic phase [6, 7]. These changes may indicate an essential structural transformation of the membrane and, as a conseuqnce, a modification of the hormone binding capacity may ensue.

An important determinant of the membrane's structure and function is its state of fluidity. It has been demonstrated that the amount of saturated fatty acid grows and the lipid composition changes parallel with the aging of the cultures: the consequence on membrane level is the formation of a more rigid membrane structure [1].

After the establishment of the hormone-receptor connection, adenylate-cyclase is the enzyme that plays an essential role in transferring information to the intracellular space. Guanylate cyclase is another, similar enzyme detected in *Tetrahymena* in insoluble and in soluble form [23]. Its membrane associated form together with the regulatory calmodulin-Ca^{++} complex is an important factor in transformation of the membrane associated processes [24]. The activity of the guanyl cyclase varies in the different growth phases of the cultures [5]. It is the greatest in the early logarithmic phase, in a period when the cells seemed to be the most sensitive to insulin. This finding suggests the presumption that this enzyme also plays a role in the development of imprinting.

Finally a type of insulin effect should be mentioned in which insulin is bound to the membrane and permeates into the cell by endocytosis and evokes

there specific alterations. The endocytotic processes in the model cells are at their maximum in the pre-division period [25]. In tissue culture the greatest number of subsequent divisions takes place in the early logarithmic phase. Should insulin have an imprinting effect in this phase, imprintability of these cells is expected to be the greatest. Considering, however, the results of our earlier studies [15] giving evidence for the greatest imprintability of the "newborn", just divided cells, it may be assumed that cell-formation is a favourable state for hormonal imprinting, for the development of receptor memory, but in view of the particular role of phase G_1, not necessarily by endocytosis.

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