

Comparison of the Amount and Demonstrability of Endogeneous Hormones and Bound Insulin After Paraformaldehyde and Edac Fixation in *Tetrahymena*

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Summary. The amount of three endogeneous hormones (insulin, β -endorphin, and triiodothyronine) and FITC-insulin binding were studied in *Tetrahymena* using confocal microscopy and flow cytometry after paraformaldehyde (Pf) or carbodiimide (EDAC) fixation. After EDAC fixation the measurable hormone content was 2.5 \times , 4 \times , 6 \times more, respectively, related to Pf fixation (significance: $p < 0.001$). This means that EDAC, which causes crosslinking between COOH and amino groups inside a molecule and between molecules could inhibit the escape of hormones during the procedure. However, FITC-insulin binding was also threefold after EDAC fixation which means that some other effects must be considered. The results show that 1) EDAC fixation is more suitable to watch the whole hormone content in a cell (in *Tetrahymena*, at present); 2) more vertebrate-like hormone is present in *Tetrahymena*, than it was believed before and 3) triiodothyronine is also present in *Tetrahymena* (first observation).

INTRODUCTION

In the early seventies of the last century was demonstrated at first that protozoa (exactly *Tetrahymena*) can react (sometimes specifically) to the hormones of higher ranked animals and can select between closely related hormone molecules (Csaba and Lantos 1973, 1975; Csaba 1980, 2000). After that, many hormone-

like molecules were found in *Tetrahymena* which were immunologically similar to that of phylogenetically higher animals and could mimic the effects of these hormones (LeRoith *et al.* 1980, 1982, 1983; Lenard 1992). Later the signal transduction mechanisms were studied (Kovács and Csaba 1987, 1990, 1992) as well as the structure of *Tetrahymena* insulin receptor (Christopher and Sundermann 1995, Leick *et al.* 2001, Christensen *et al.* 2003) and both showed similarities to the mammalian ones.

For localization of hormones immunocytochemistry and confocal microscopy are very suitable and up to date methods, while the amount of hormones can be determined by flow cytometry. Nevertheless, the loss of

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hormones during the procedure of preparation seems to be high. For inhibiting the loss, EDAC fixation was recommended by Finnish authors (Panula *et al.* 1988, 1995) in case of the nervous system and biogenic amines, as EDAC causes cross-linking of COOH and amino-groups inside a molecule and between molecules, hindering the escape of small molecules (Handbook 2005). In *Tetrahymena* we also found that more serotonin and histamine can be measured after EDAC fixation related to the traditional paraformaldehyde (Csaba *et al.* 2006). In the present experiments the measurable amount and the localization of three non-biogenic-amine hormone, with different molecular weights, are studied as well as the binding of insulin, comparing the effect of paraformaldehyde and EDAC fixation.

MATERIALS AND METHODS

Cells and culturing. *Tetrahymena pyriformis* GL strain was used in the logarithmic phase of growth. The cells were cultured at 28°C in tryptone medium (Sigma, St.Louis, USA) containing 0.1% yeast extract, for 48 h. The density of *Tetrahymena* cultures studied was 10⁴ cell/ml.

Flow cytometric analysis. Samples of cells were fixed with 4% paraformaldehyde solution [dissolved in pH 7.2 phosphate buffered saline (PBS)] or with freshly prepared 4% N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC, Sigma, St.Louis, USA) solution for 5 min, and then washed twice in wash buffer (0.1% bovin serum albumin [BSA]; 20 mM Tris-HCl; 0.9% NaCl; 0.05% Nonidet NP-40; pH 8.2). To block nonspecific binding of antibodies the cells were treated with blocking buffer (1% BSA in PBS) for 30 min at room temperature. Aliquots from cell suspensions (50 µl) were transferred into tubes, and 50 µl primary antibody [diluted 1:200 in antibody buffer (1% BSA in wash buffer)] was added for 30 min at room temperature. The 1st antibody

were anti-endorphin, anti-T3 and anti insulin purchased from Sigma. FITC-insulin (Sigma) was used for studying insulin binding. Negative controls were carried out with 50 µl PBS containing 10 mg/ml BSA, instead of primary antibody. After washing four times with wash buffer to remove excess primary antibody the 1st antibody treated cells were incubated with FITC-labelled secondary antibody (anti-rabbit IgG for anti-endorphin and anti-T3; or anti-mouse IgG for anti-insulin; both purchased from Sigma; in dilution 1:50 with antibody buffer) for 30 min at room temperature.

For controlling the specificity, autofluorescence of the cells and aspecificity of the secondary antibodies were detected. This latter means that the fluorescence of cells treated only with the secondary antibody (without the specific first antibody) was also measured in each series. The measurement was done in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA), using 5.000 cells for each measurement. In the cell populations the hormone content (concentration) had been compared. For the measurement and analysis CellQuest Pro program was used. The numerical comparison of detected values was done by the comparison of percentual changes of geometric mean channel values (Geo-mean) to the appropriate control groups by using Origin program and Student t-test. The experiments were done thrice with similar results and Table 1 demonstrates one of these experiments.

Confocal microscopic analysis. After the flow cytometric analysis the cells were subjected to confocal microscopic analysis in a BioRad MRC 1024 confocal laser scanning microscope, equipped with krypton-argon mixed gas-laser as a light source, at an excitation wavelength of 480 nm line.

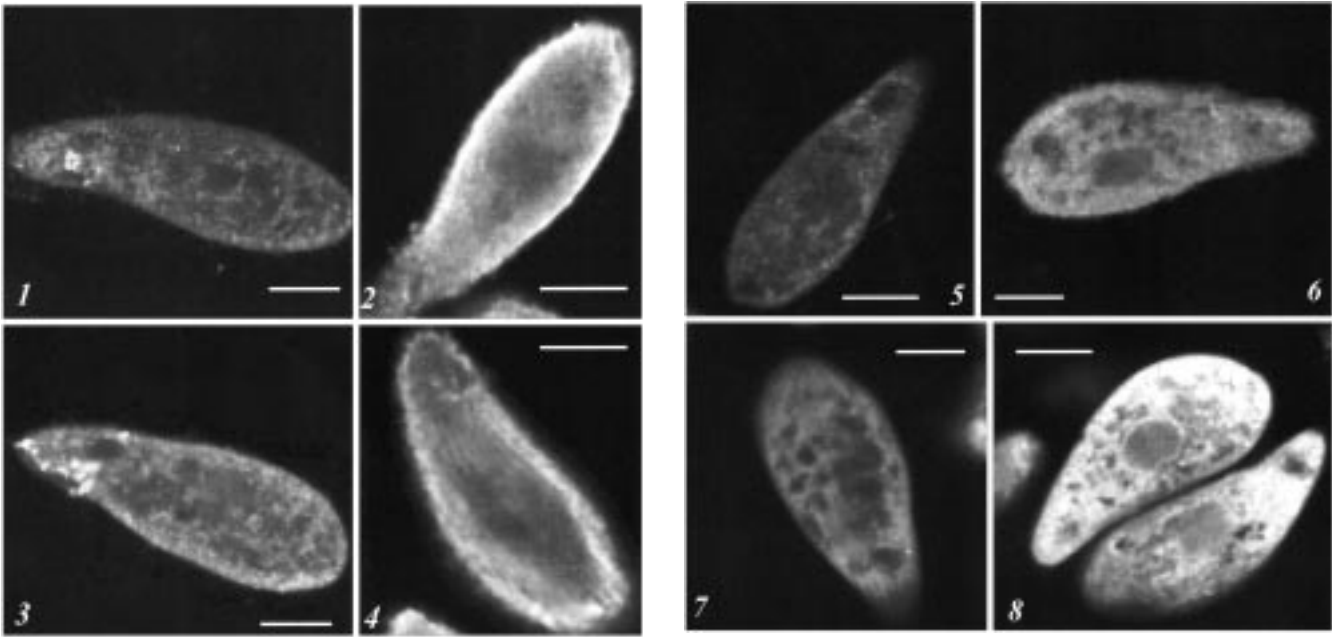
RESULTS

Considering the data of the flow cytometric analysis each hormone was measured at a higher level after EDAC fixation. The significance between the values of the two different fixation was very high ($p < 0.001$). The difference between the values in the case of endorphin was almost fourfold, in case of insulin 2 and a half-fold,

Table 1. Measurable hormone concentrations after different fixations.

Fixative	Hormone	Geo-mean +/- s.d.	Significance to Pf*
Paraformaldehyde	endorphin	16.85 +/- 2.21	
	insulin	6.17 +/- 0.37	
	triiodothyronine	15.27 +/- 1.0	
	FITC-insulin	386.58 +/- 36.8	
EDAC	endorphin	59.39 +/- 4.51	p < 0.001
	insulin	15.15 +/- 0.66	p < 0.001
	triiodothyronine	93.79 +/- 6.48	p < 0.001
	FITC-insulin	1201.1 +/- 31.78	p < 0.001

* Pf - paraformaldehyde



Figs 1-8. 1 - Formaldehyde fixed *Tetrahymena*. Anti-endorphin treatment. 2 - EDAC-fixed *Tetrahymena*. Anti-endorphin treatment. 3 - Formaldehyde fixed *Tetrahymena*. Anti- T_3 treatment. 4 - EDAC-fixed *Tetrahymena*. Anti- T_3 treatment.

5 - Formaldehyde fixed *Tetrahymena*. Anti-insulin treatment. 6 - EDAC-fixed *Tetrahymena*. Anti-insulin treatment. 7 - Formaldehyde fixed *Tetrahymena*. FITC-insulin treatment. 8 - EDAC-fixed *Tetrahymena*. FITC-insulin treatment. Scale bars: 10 μ m.

in case of T_3 -this was the highest- more than six-fold. More than threefold FITC-insulin was bound by the cells after EDAC fixation than after paraformaldehyde fixation (Table 1). This means that EDAC fixation is more advantageous in the study of amino acid and polypeptide hormones, in addition to the previously demonstrated biogenic amines (Csaba *et al.* 2006).

The confocal microscopic analysis supports the flow cytometric data. In each EDAC-fixed cell more hormone is visible, than after paraformaldehyde fixation (Figs 1-8). In addition EDAC-fixation shows clearer the localization of hormones. In case of endorphin it is localized first of all under the surface of the cell membrane (Fig 2) and diffusely inside the cell. There is a similar situation in case of T_3 however, the localization of the hormone on the oral field is better seen after paraformaldehyde fixation (Figs 3, 4). Insulin is localized inside the cell diffusely, in a much higher amount after EDAC-fixation (Figs 5, 6). FITC-insulin is also localized diffusely and the structures on the surface of which insulin is bound are rather visible after EDAC fixation (Figs 7, 8).

DISCUSSION

Four molecules were chosen for studying the differences between the two fixatives. A small amino acid hormone, T_3 , a small and a large polypeptide hormone, endorphin and insulin; and FITC-insulin which can be bound by the insulin receptors present in *Tetrahymena*. The hormones studied can be found in *Tetrahymena*. Insulin was demonstrated at first (LeRoith *et al.* 1980) after its binding sites (Csaba and Lantos 1975), and before the detailed determination of its receptors (Christopher and Sundermann 1995, Christensen *et al.* 2003, Leick *et al.* 2001). Later endorphin and endorphin-binding were also found (LeRoith *et al.* 1980, O'Neill *et al.* 1988, Zipser *et al.* 1988, Chiesa *et al.* 1993, Renaud *et al.* 1995, Csaba and Kovács, 1999, Rodriguez *et al.* 2004). T_3 was not determined previously in *Tetrahymena*, so this is the first exact observation on its presence. In earlier experiments we tried to demonstrate T_3 , but the method was not sensitive enough for proving it (Csaba and Nagy 1987). However the binding sites of T_3 on the cell membrane and in the nucleus were shown

also in earlier experiments (Csaba *et al.* 1977, Csaba and Sudár 1978) The use of the present very sensitive methods allowed the enrichment of our knowledge on the hormone-pool of *Tetrahymena*.

The water-soluble EDAC intermolecularly crosslinks amino- and carboxylic acid- groups and also forms intramolecular crosslinks (Handbook 2005). This crosslinking which can be found not only in hormones but in the surrounding molecules (membranes included) inhibits the free movement of the small hormones out of the cell (Ma *et al.* 2004, Bakos *et al.* 2000). Considering these data it is understandable, why EDAC fixation elevated the measurable level of the hormones. However, further explanation is needed if we observe the size of the hormones studied.

T₃ is a small molecule, an amino-acid-type hormone with very high mobility across the membranes, similar to the biogenic amines, for the fixation of which EDAC was recommended at first (Panula *et al.* 1988, 1995). This explains why was so expressed the difference between the two fixations. Endorphin is a small polypeptide and insulin is a large one. The difference between this two peptides can explain the difference - fourfold and two and a half-fold - in the effect of EDAC. However, it can be supposed that insulin - being a large protein - can not escape from the cell also in case of paraformaldehyde fixation. In addition, significantly more exogenously given FITC-insulin was bound by the cells, which can not be explained by the escape-theory. This means that the cross-linking caused by EDAC develops more changes than simply the condensation of molecules and membranes.

Summarizing the results: the experiments call attention to the priority of EDAC fixation if the amount of some biologically active molecules are studied immunocytochemically. and shows that more amount of hormones can be present than it was believed before. However, support by chemical and/or physiological measurements are needed for the exact statement. As a first observation the T₃ content of *Tetrahymena* can be mentioned.

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