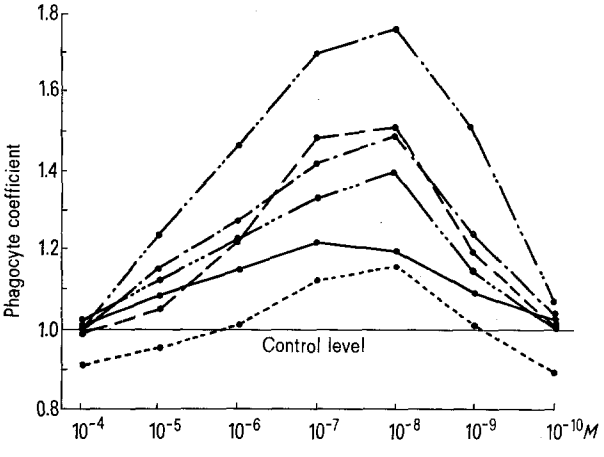
insulin[5] enhanced its glucose uptake. The receptors of *Tetrahymena* are specific enough to differentiate a given hormone from close chemical derivatives[4,6].

Peptide hormones operate mainly via the cyclic AMP-adenylcyclase system, which seems to be present in all living beings[7]. Evidence of the role of cAMP in *Tetrahymena* has been presented by WOLFE[8], ROTHSTEIN and BLUM[9], as well as by CSABA et al.[10], but while the latter authors[9,10] believe it to act as a second messenger, WOLFE[8] does not. Another matter of controversy has been the precise role of cyclic 3′,5′-adenosine monophosphatase diesterase (cPDE) at the lower levels of phylogenesis, which is not well documented[9–13].

In the present experiments, the phagocytic response of *Tetrahymena* to agents that affect the intracellular level of cAMP was tested. Dibutyril cAMP is known to be a highly active cAMP derivative, particularly suited for the experimental study[7] of cAMP action; theophylline is the most active methylxantine inhibitor of cPDE[7], and histamine acts on *Tetrahymena* through its membrane receptors, without entering into the cell, as shown previously[4] in this laboratory.

A *Tetrahymena pyriformis* GL culture grown in 1% Bacto trypton (Difco, Michigan, USA) and 0.05% yeast extract for 2 days at 25 °C was used. 24 h before the experiment the *Tetrahymenae* were isolated from the medium by centrifugation and rendered vacuole-free by incubation in LOSINA-LOSINSKY's solution[14]. The following treatments were carried out: 3 or 10 min exposure to cAMP (N^6, O^2-dibutyril adenosine 3′,5′-cyclic monophosphate Na; Aldrich, Beerse, Belgium); 3 or 10 min exposure to theophylline (Richter, Budapest); 3 or 10 min theophylline treatment followed by 3-min histamine treatment (Reanal, Budapest); no treatment (control series). The concentration range of the test materials was 10^{-4} to 10^{-10} *M*. After pretreatment, Chinese ink diluted in LOSINA-LOSINSKY solution, was added to the *Tetrahymena* lots; after 3 min smears were prepared and were dried rapidly. Each concentration of the applied test materials was tested in 5 replicas in each series and vacuole counts were always determined in 100 animals. The means calculated from readings on 500 protozoa at each concentration level per group were related to the corresponding control reading as 100 to obtain the phagocyte coefficient.



Effect of different combinations of cAMP and theophylline on the phagocyte coefficient of *Tetrahymena*.
—, cAMP 3 min. — — —, cAMP 10 min. · · · ·, Theophylline 3 min. — · · · —, Theophylline 10 min. - · - ·, Theophylline 3 min + histamine 3 min. - · · - · · -, Theophylline 10 min + histamine 10 min.

The experimental results are shown in the Figure. Although the 3-min dibutyril cAMP treatment had little effect, 10-min treatment was sufficient for the development of an action of similar degree to hormonal influence[4]. Theophylline increased the phagocytotic capacity of *Tetrahymena*, but in a lesser degree than cAMP. Its effect greatly depended on the time of treatment, as did the action of cAMP. The phagocyte coefficient obtained on subsequent 3-min exposures to theophylline and histamine did not exceed the value obtained on treatment with histamine alone in earlier studies[4,6], whereas 3-min exposure to histamine following upon 10-min treatment with theophylline enhanced the phagocytotic activity of *Tetrahymena* to a greater degree than any other treatment.

Accordingly, the present findings support the conclusion that the cAMP-adenylcyclase-cPDE system functions in *Tetrahymena*. Another important information emerging from this study is the decisive role of the time factor, the disregard of which can well account for the contradictory results of earlier experimental studies[9,11–13] on hormonal regulation in unicellular animals.

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Cellular Control of the Tick-Borne Virus Antigen Production in Persistently Infected Cell Culture

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Summary. The influence of inhibition or stimulation of cellular DNA synthesis on tick-borne virus antigen production in persistently infected cell culture was studied. Either mitomycin C or cytosine-arabinoside caused cessation of antigen-containing cell number increase. Stimulation of cellular DNA synthesis by growth medium change increased the level of antigen-containing cells. When HEp-2-Sof culture was synchronized, a correlation was observed between the entrance of cells into DNA synthesis phase and the increase of proportion of antigen-containing cells.

Persistent infection of HEp-2 cell culture by tick-borne encephalitis virus (TBV), designated as HEp-2-Sof[1], has been under study for about 15 years. Some recent findings strongly suggested the participation of cellular genome in the status of chronic infection in this system, namely, 1. stimulation of viral antigen production by 5-bromodeoxyuridine[2], 2. infectious properties of cellular DNA and detection of virus-specific sequences in nuclear DNA by molecular hybridization experiments[3], 3. the lack of DNA excision repair in HEp-2-Sof cells[4].

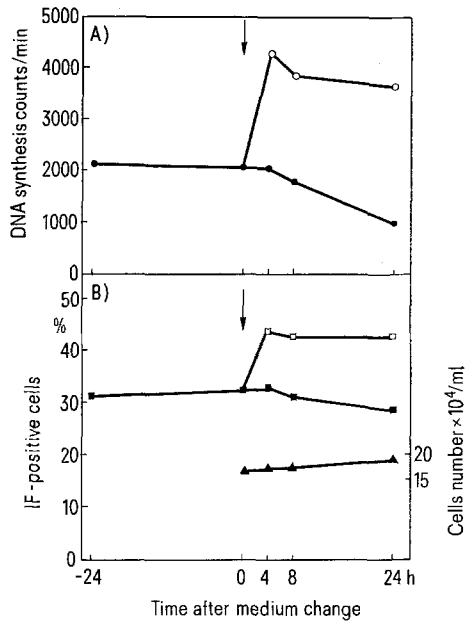

Fig. 1. Growth medium change-stimulated increase of DNA synthesis rate (A) and IF-positive cell number increase (B) in HEp-2-Sof culture. ●—●, DNA synthesis rate without stimulation; ○—○, DNA synthesis rate after stimulation; ■—■, % IF-positive cells without stimulation; □—□, % IF-positive cells after stimulation; ▲—▲, total cell number before and after stimulation. Each point represents the mean of 5 parallel measurements. An arrow indicates the moment of growth medium change.

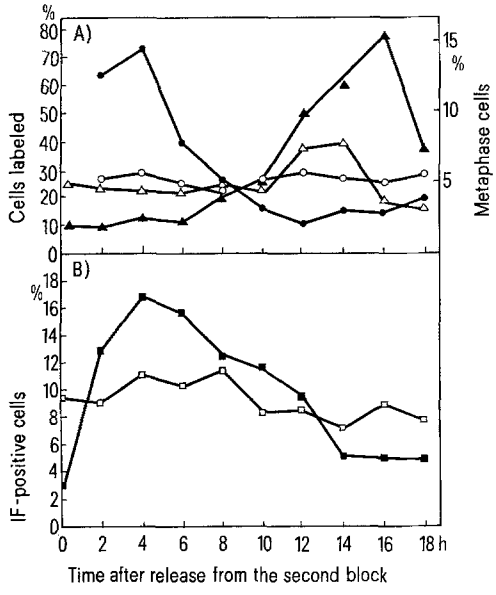

Fig. 2. The pattern of growth of Hep-2-Sof cells, synchronized by double thymidine block (A) and the influence of cell synchrony on the proportion of IF-positive cells (B). ●—●, % cells labeled in synchronized culture; ○—○, % cells labeled in asynchronous culture; ▲—▲, metaphase index in synchronized culture; △—△, metaphase index in asynchronous culture; ■—■, % IF-positive cells in synchronized culture; □—□, % IF-positive cells in asynchronous culture. Each point represents the mean of 3 parallel measurements.

Among the most characteristic features which HEp-2-Sof system preserves during its existence, there is continuous production of virus-specific antigen as revealed by immunofluorescence (IF) technique in a fraction of cells. The level of IF-positive cells is not constant and fluctuates from 5–10% to 30–40%. Occasionally we observed both periods of complete absence of IF-positive cells and periods of IF 'exaltation', when the proportion of IF-positive cells reached 50–60%. Cloning experiments performed in the course of this study have revealed viral antigen producing (IF-positive) and non-producing (IF-negative) clones. IF-positive clones appeared to be resistant to superinfection with homologous virus, whereas IF-negative ones were sensitive to reinfection with TBV.

In this report, we describe the effect of DNA inhibitors, and procedures affecting the rate of DNA synthesis, on viral antigen production in HEp-2-Sof culture.

HEp-2-Sof cells were seeded in tubes with coverslips at a concentration of 1×10^5 cells/ml; 24 h later, the growth medium was replaced by the one containing 5 μg/ml mitomycin C (MC) or 10 μg/ml cytosine-arabinoside (Ara-C). Every 24 h after addition of the chemical, the number of viable (stained with neutral red) cells, incorporation of ^{3}H-thymidine (2 μCi/ml, specific activity 52 mCi/mmol, 30 min) into acid-insoluble material and percentage of IF-positive cells was determined simultaneously in triplicate samples for each time interval.

MC almost completely inhibited DNA synthesis and cell proliferation after 24 h exposure to the drug, and also blocked the increase of IF-positive cell number. Similarly, Ara-C markedly reduced the rate of DNA synthesis, cell number and the proportion of IF-positive cells as compared with control untreated cultures. Neither MC, nor Ara-C have affected the kinetics of IF-positive cell increase when HEp-2 cells were primarily infected with TBV at input multiplicity of 10 $TCID_{50}$/cell.

Another kind of experiment, avoiding the use of chemicals, has been performed in order to determine whether the increase of IF-positive cells in HEp-2-Sof culture was due to proliferation of IF-positive parental cells or, more probably, to the events localized in interphase, presumably in S-period. HEp-2-Sof cells were seeded into tubes containing coverslips of equal size at a concentration $5 \times 10^5/2$ ml. 48 h later, when cells had reached confluency and the level of DNA synthesis became constant, the growth medium was replaced by a fresh one, and, at appropriate time intervals, coverslip cultures were taken for IF test after prelabelling of cells with 2 μCi/ml of ^{3}H-thymidine for 30 min. When the percentage of IF-positive cells was counted, cells from the same coverslips were mechanically scrapped and isotope incorporation into acid-insoluble material was measured. Simultaneously the total number of trypan blue excluded cells were scored in parallel cultures. Figure 1 shows that growth medium change has stimulated HEp-2-Sof DNA synthesis and concomitantly increased the proportion of IF-positive cells, whereas total cell number has not significantly changed. When HEp-2 coverslip confluent monolayers, stimulated to DNA synthesis, were infected

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with TBV, at a multiplicity of 10 $TCID_{50}$ per cell, no shortening of a latent period of IF-positive cell appearance or increase of IF-positive cell number was observed.

Finally, the effect of cell synchronization on the proportion of IF-positive cells in HEp-2-Sof culture was investigated. Preliminary experiments revealed that HEp-2-Sof doubling time did not exceed 48 h. Proliferating pool of the culture was almost the same as previously reported[5]: nearly 95% of cells became labeled after 48 h incubation with 1 μCi/ml ^{3}H-thymidine. These data suggest that HEp-2-Sof cell life cycle has not markedly changed, and enable us to synchronize the cells on the basis of previously reported estimation of HEp-2-Sof cell cycle parameters[5].

Synchronous cultures were obtained by the double thymidine block technique[6]. HEp-2-Sof cells were seeded in tubes containing coverslips at a concentration 1×10^5 cells/ml. 24 h later exponentially growing cultures were treated with 2 mmol of thymidine for 21 h ($G_2 + m + G_1$), then washed 3 times with warm medium, allowed to grow for 10 h (S) and again treated with 2 mmol of thymidine for 21 h. After release from the second block cells were pulse-labelled every 2 h with ^{3}H-thymidine and prepared for autoradiography. Percentage of labelled cells and mitotic index were determined in the same samples stained with haematoxyline. Proportion of IF-positive cells was scored in parallel coverslip cultures. Figure 2 illustrates the pattern of synchronous growth of HEp-2-Sof cell culture. A good correlation has been observed between the entrance of cells into S period and the increase of IF-positive cell number in culture. The proportion of IF-positive cells gradually decreased as the majority of cells left the S period and proceeded to mitosis. Infection of S-phase synchronized HEp-2 cells by TBV caused only a slight increase in number of IF-positive cells as compared to infected asynchronous HEp-2 cells[7]. The alternative results obtained for HEp-2 cells primarily and chronically infected with TBV may reflect the particular type of cell-virus relations at persistent TBV infection in HEp-2-Sof system.

The reason for S-phase dependence of viral antigen production in HEp-2-Sof culture is so far obscure. It seems feasible that the incorporated state of TBV genome in HEp-2-Sof cells[3] is responsible for the observed phenomenon. Expression of several integrative viruses is known to be increased during cellular DNA replication phase[8,9]. Whether S-phase dependent enhancement of TBV antigen production is of identical origin remains to be determined. Nevertheless it is now apparent that cell cycle activities may be operative in TBV antigen production during growth of persistently infected HEp-2-Sof cells.

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Distribution of Cadmium, Zinc and Copper in the Mussel *Mytilus edulis*. Existence of Cadmium-Binding Proteins Similar to Metallothioneins[1]

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Summary. The study of the distribution of Cd, Zn and Cu in homogenates of mussels has shown that Zn and Cu are principally associated with high mol. wt. proteins. The same distribution is observed for Cd in untreated mussels, but in Cd chronically intoxicated animals, the metal is principally bound to low mol. wt. proteins synthesized in response to the uptake of the cation and similar to metallothioneins of vertebrates.

The existence of metallothioneins, specific Zn-, Cd-, Hg- and Cu-binding proteins, is now quite established in various organs of mammals, birds and recently of fishes[3,4]. These proteins, present in the cytoplasmic soluble fraction, are characterized by their extraordinary metal-binding capacity, their high content in cystein (approximately $^1/_3$ of the total amino acid residues), their low molecular weight (about 10,000) and their inducible nature on administration of heavy metals[4–7]. The role of metallothioneins in the detoxification of Cd and Hg has frequently been implied[8–10]. The very important acccumulation of these two metals in the organisms, as well as their very slow rate of elimination, is likely to be explained by their binding to metallothioneins.

To our knowledge, there is no report on the finding of such proteins in invertebrates. Owing to the enormous cadmium accumulation observed in molluscs[11], the possible existence of metallothioneins in one species of the phylum has been investigated.

Methods. For each essay, the soft parts of 2 mussels are pooled and homogenized in 3 volumes of 0.5 *M* sucrose by means of a Polytron Homogenizer. The supernatants obtained by centrifugation at 100,000 *g* are chromatographied on Sephadex G-75 or LKB Ultrogel AcA 54 columns equilibrated in NH_4HCO_3 0.05 *M*. The fractions are analyzed for Cd, Zn and Cu by atomic absorption spectrophotometry.

Results and discussion. The results presented in Figure 1 have been confirmed by many other chromatographies. Graphs A and B show that the Cd accumulated by the mussels during the chronic intoxications is associated with 3 fractions. Fraction I is situated at the level of high molecular weight (mol. wt.) proteins. Fraction II, when large columns are used, corresponds to 2 unresolved peaks, the second one (IIb) having an elution volume corresponding exactly to that observed for metallothionein in different eel tissues[4]; the position of this peak is characteristic of substances having a mol. wt. close to 10,000. Fraction III has the same elution volume as free Cd. It must be pointed out that a number of authors have already observed heterogeneity at the level of the Cd-binding peak of low mol. wt.[3,9,12,13]. Note that fraction II does not contain high amounts of Zn and Cu, in contrast to metallothionein from eel liver[14].