Endogenous recovery of cell divisions in the presence of cycloheximide by the ciliate *Chilodonella cucullulus*: effects of phenobarbital pretreatment[1]

Mauryla Kiersnowska

Institute of Zoology, Warsaw University, P-00-927/1, Warsaw (Poland), 24 November 1980

Summary. Complete endogenous recovery of morphogenesis and rate of cell divisions of *Chilodonella cucullulus* proceeds in the constant presence of low doses of cycloheximide. Phenobarbital pretreatment modifies the temporal pattern of this recovery, owing to at least 2 different cellular pathways monitored by phenobarbital.

Studies on the ability of Ciliates to grow in the permanent presence of low doses of a variety of drugs have provided evidence for 2 types of responses: 1. The mean generation time of the cells increases proportionally with the concentration of the drug and remains at the same level throughout the course of the experiment; such cases are found in Paramecium[2] and in Euplotes[3]. 2. Alternatively, at first cells do not divide (except a few cells very advanced in their cycle at the start of the experiment), but after some lag, which is proportional to the concentration of the inhibitor, they reinitiate their divisions asynchronously. The latter kind of response occurs in Tetrahymena[4-9] and is called a 'recovery'[4], an 'endogenous recovery'[9], or an 'adaptation'[7].

An earlier study with cycloheximide (CHX), wich is believed to be a specific inhibitor of eukaryotic protein synthesis performed by 80S ribosomes[11,12], revealed that the recovery of Tetrahymena from the effects of CHX involved a reinitiation of protein synthesis from DNA and RNA, and an increase of activity of some enzymes[8]. However, the molecular basis of these 2 responses is not yet known. It is assumed only that recovery in Tetrahymena is based on some mechanism(s) increasing the resistance of the cells towards the action of the drug. This acquired resistance might be obtained through 2 kinds of physiological reactions of the cells, either through mechanism(s) lowering the inhibitory action of the drug, such as detoxification by active depletion, or a decreased influx of the drug, or, alternatively through an increase of activity of physiological systems(s) disturbed by the inhibitor, aiming at overcoming its effects. It could include some conformational change in the ribosomes similar to that observed in CHX-resistant mutants of Tetrahymena[14].

The present study examines some questions concerning the mechanism of the physiological response of Ciliates to low doses of CHX. It has been studied whether the response of the cells to the drug can be modified if they were previously 'conditioned' against the inhibitor action of the drug. For example phenobarbital (PB) modifies many cellular responses of hepatic cells to an addition of many classes of inhibitors[13].

In this report the ciliate *Chilodonella cucullulus* was chosen because of the clearly recognizable stages of cortical development during its divisional morphogenesis. 5 problems were investigated:

1. Does a permanent increase of the mean generation time occur in *Chilodonella* in the presence of CHX, or is *Chilodonella* an adaptative system able to recover its normal fission rate in the presence of the drug? 2. If a recovery of the fission rate occurs, which was in fact observed, is this recovery based on a selection of more resistant cells, or on a gradual phenotypic adaptation of all the cells? 3. Does the sensitivity of cells to the inhibitor vary at different stages of their cell cycle? 4. Is there an increase of total DNA content during the recovery? 5. Does pretreatment of *Chilodonella* cells with PB modify the course of events compared with untreated controls in presence of CHX?

Material and methods. *Chilodonella cucullulus,* a ciliate from the class Kinetophragmaphora was used. The mode of cultivation has been described previously[5]. The experiments were performed with the cells transferred every 2 or 3 days to fresh medium: 2 ml aliquots were supplemented with 3 ml of fresh sterilized and filtered pond water with yeasts. Cycloheximide - CHX (Koch Light Ltd) and phenobarbital - PB (Polfa) were dissolved in sterilized and filtered pond water to make a stock solution of 100 µg/ml. The stock solution of CHX was used during 1 month, that of PB during 1 week.

Morphological studies: Randomly isolated cells were fixed and impregnated with silver nitrate following the procedure of Chatton-Lwoff[17].

Preliminary tests: *Chilodonella cucullulus* does not swim freely in the culture medium but only over the bottom. Thus the cell counter is of little use for estimating the number of cells in a given dish. The aim of the preliminary tests was to find a practicable method for cell number estimation in a given dish, and for checking the influence of density of cells in an inoculum on the daily fission rate. Additional sets of experiments were performed to test the error in estimation of cell numbers, and to test the proper feeding diet. The experiments were carried out in 5 cm

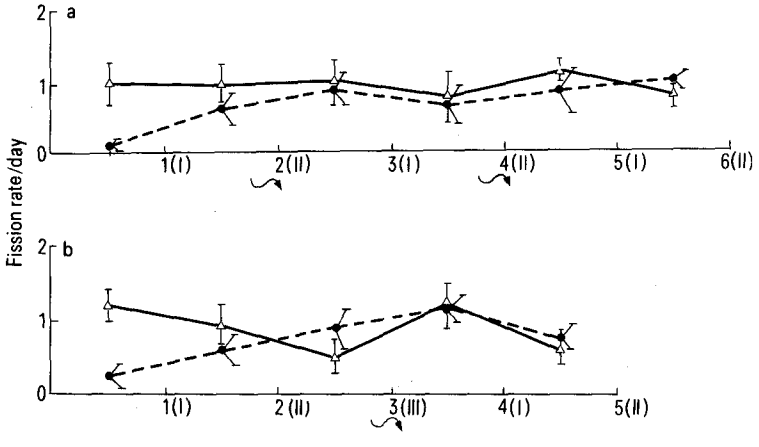

Figure 1. Effects of cycloheximide (CHX) on daily fission rate of *Chilodonella cucullulus.* Logarithmically growing cells are introduced into: control medium without CHX (——) and into medium with 1 µg/ml CHX (– – – –). After either 2 days (fig. 1a) or after 3 days (fig. 1b) cells are replaced (↷) in newly prepared fresh medium, without or with 1 µg/ml CHX. Every point on the diagram represents a mean ± SD of 7 experiments in figure 1a and of 5 experiments in figure 1b. 1, 2, 3.... - sequential days of a given experiment, I, II, III.... - sequential days of maintenance of cells in the same medium.

Petri dishes. It was found that the mean number of cells counted 3 times on 20 randomly chosen fields of the bottom of the initial dish seen at low magnification (1.6 × 2.5) of a dissection microscope was a reliable estimate of the initial cell number. During subsequent days of the experiment, cell counting was performed in the same way. These data were used to calculate the fission rate. Even doubling of the amount of yeast does not modify the mean fission rate of control samples throughout the experiment.

The fission rate of control samples remains comparable throughout the experiments within the limits of 30–70 cells in the initial inoculum. This intitial density was always monitored before the start of experiments.

Statistics: Statistical analysis was carried out according to Sokal and Rohlf[18]. All significance was verified with $p = 0.05$ or less.

Results. 1. *Effects of CHX on daily fission rate of Chilodonella cucullulus.* Samples of logarithmically growing *Chilodonella* immersed in a 1 µg/ml solution of CHX revealed an arrest of their fission followed by a total recovery of the fission rate within 3 days (fig. 1, a and b). The reinitiation of cell divisions began on the 2nd day with an increasing number of asynchronous dividing cells. Regardless of whether the cells were maintained in the same medium or transfered to fresh culture medium supplemented with the same concentration of CHX, the mean fission rate remained the same as in control cells in CHX-free medium.

The recovery of cell division in the permanent presence of CHX is not caused by a decrease of activity of CHX in the medium. Indeed an immersion of fresh cells into medium removed from cultures exposed to CHX for 3 days elicited the same arrest followed by a gradual recovery within 3 days (table 1).

The effect of 2 different concentrations of CHX was tested. It appeared that the phase of inhibition was proportional to the concentration of the drug. It was also found that below 0.1 µg/ml CHX the fission rate was not statistically different from the controls.

2. *Macronuclear DNA content in Chilodonella cells in early G_1 phase.* The macronuclear DNA content in cells dividing after 22–26 h and 46–50 h of CHX treatment was not statistically different (table 2).

3. *The course of recovery of Chilodonella in CHX after preincubation with PB.* PB alone at concentrations of 0.1 µg/mg and 1 µg/ml does not modify the ability of cells to divide. However, cells pretreated with PB (0.1 or 1 µg/ml) for 24 h and then transfered to CHX medium (1 µg/ml) showed a significant modification of their response in comparison with cells not pre-treated and introduced to the same concentration of CHX, or control untreated cells (fig. 2). Cells preincubated with PB divided at the same, or nearly the same rate during the 1st day of their contact with CHX as the controls, but by the 2nd or 3rd day in CHX the division rate dropped and the cells entered a distinct 'lag phase'. The occurence of this 'lag phase' at the 2nd or 3rd day depended upon the concentration of PB used in the preincubation period: After the 'lag phase' a rapid course of recovery was observed and the control level of doubling time was resumed. The recovery from the 'lag phase' to the control fission rate is reached more quickly by cells pretreated with PB than by those exposed to CHX without pretreatment. The 1st doubling time of pretreated cells, after 'the lag phase' in the presence of CHX, is attained about 7 h earlier than in the case of non pretreated cells.

Temporal patterns of adaptation to CHX, and an influence of PB on this pattern have been verified using the frequency distribution analysis of developmental stages in successive days of the experiments:

4. *Frequency distribution of developmental stages in Chilodonella cucullulus.* In the divisional morphogenesis of *Chilodonella cucullulus* 3 successive developmental stages can be arbitrarily distingushed. The normal sequence involves: a) early dividers i.e. formation of primordia of new cortical organelles such as oral segments of ciliary rows for the future posterior cell, and primordia of new contractile vacuole pores for both daughter cells, b) a stage of morphogenetic movements of the oral segments for the future posterior daughter cell and maturation of contractile vacuole pores for both daughter cells and c) cytokinesis.

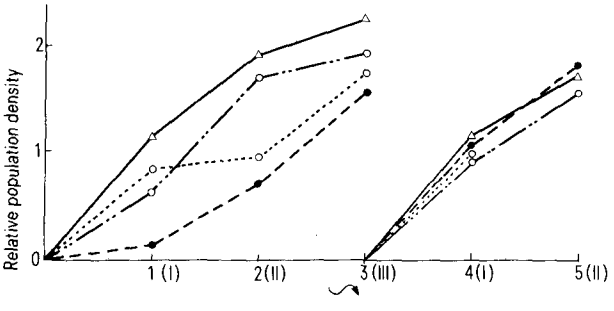

Figure 2. Effects of cycloheximide (CHX) on relative population density of *Chilodonella cucullulus* pretreated with phenobarbital (PB). Logarithmically growing cells are portioned into 4 samples; the 1st is kept in plain fresh control medium without CHX (——); the 2nd is introduced into medium with 1 µg/ml CHX (– – – –); the remaining 2 aliquots are pretreated with PB in concentrations of either 0.1 µg/ml (.....) or 1 µg/ml (– · – · –) and then introduced into medium with 1 µg/ml CHX at time 0 of the experiment. After 3 days all samples are placed in fresh medium with the same initial CHX concentration as before.

Table 1. Effects of 'fresh' and 'used' cycloheximide medium on the fission rate of *Chilodonella cucullulus*

Days / Medium	I (1)	II (2)	I (3)	II (4)	III (5)	Days / Medium	I (1)	II (2)	III (3)	I (4)	II (5)
a) C	0.75	0.52	1.30	0.95	0.80	C+	1.06	0.65	0.32		
b) CHX 1	0.00	0.60	1.07	0.85	0.72	CHX 0.5+	0.30	0.90	0.50		
c) CHX 0.5	0.20	0.70	1.25	0.80	0.70	c) CHX 0.5	0.40	0.88	0.65	1.27	0.80
						d) C	0.73	1.05	0.60	1.40	0.45

Logarithmically growing 'naive cells' are introduced into a) fresh culture medium alone (control), b) into fresh culture medium with 1 µg/ml CHX and c) into fresh culture medium with 0.5 µg/ml CHX. After 2 days cells from every sample a–c) are placed (↷) in new, fresh medium with the same concentration of CHX. During the next 3 days the fission rate is tested. The 3rd day of the 2nd transfer – all cells from samples a and b are eliminated (→). The remaining 'used' media a and b are diluted with the same volume of fresh culture medium with new 'naive' cells (+). Hence in the sample b – the final concentration dropped down to 0.5 µg/ml. The fission rates in continuous samples a and b are also compared with new controls of the same 'naive' cells introduced to fresh culture medium with 0.5 µg/ml CHX (c) and without CHX (d). 1, 2, 3... and I, II, III. as in figure 1.

Abnormal stages, such as a 'resorption stage', can appear. These cells show a bald area at the place of the new oral segments[15]. When protein synthesis is inhibited in cells in which cortical development is proceeding, some cells at the 'early divider' stage may switch into the 'resorption stage'. Cells in the 'resorption stage' are set back in their cell cycle and they may be arrested from further development for a period of many hours[19]. Frequency distribution of developmental stages during permanent exposure to 1 µg/ml CHX revealed (table 3) that on the 1st day of the experiment a fraction of the cells fall into the 'resorption stage'. During the next few days, however, this fraction totally disappears, while the percentage of developmental stages reaches the control level by the 3rd day of the experiment. Cells pretreated with PB (1 µg/ml) did not show any significant change in the frequency distribution pattern. If such cells were immersed into CHX, they maintained their frequency distribution of developmental stages at about the control level during the 1st and 2nd day. During the 3rd day a significant fraction of cells in 'resorption stage' appeared, coinciding well with the 'lag phase' for the increase in number of cells (fig. 2). This fraction totally disappeared by the end of the experiment.

Discussion. It was estimated in the experiments that the entire population of *Chilodonella* doubles 1.5 times during 3 days in the presence of 1 µg/ml CHX. However, it is possible that in the initial samples some cells may be more resistant to CHX than others. In such cases the mean value of 1.5 fissions during 3 days would include some cells which divide 2 or 3 times and others which do not divide at all during the same period. In fact the recovery of the normal fission rate in a low concentration of CHX (fig. 1) is not accompanied by a change in its standard deviation. In other words, this recovery is not a result of a positive selection of more resistant cells, for all samples behave like a homogeneous group. Thus the initial response of the cells to CHX is uniform and the temporary lag phase supports the idea that the mechanism of adaptation involves all cells. This phenomenon can be considered as a phenotypic adaptation or an endogenous recovery. It seems also that asynchronous recovery of cell division does not reveal a diversity of resistance, but a cell cycle stage dependant sensitivity of the cells toward the inhibitory agent. The clearest evidence of recovery of *Chilodonella* in the presence of CHX is visualized in the disappearance of the 'resorption' stage. This means that the critical phase in divisional morphogenesis becomes progressively more and more resistant to CHX. This effect was also reported in *Chilodonella* kept permanently at high temperature[19]. Similar effects have also been seen in *Tetrahymena* kept in the constant presence of CHX[10].

No significant change in the macronuclear DNA content was observed during the recovery related to exposure to CHX. This means that during the 'lag phase' and the 1st cell divisions following it no noticeable uncoupling of DNA replication and the cell cycle occurs. One might think that an increased resistance is related to an increase in the number of copies of some genes in the polyploid macronucleus. But our results rule out the possibility of a regulation on the basis of a simple increase of macronuclear DNA content.

Results of the experiments by PB pretreatment of *Chilodonella* cells prior to their contact with CHX lead to following conclusions:

1. PB pretreatment delays the inhibitory effects of CHX i.e. 'the lag phase' and the appearance of cells in resorption stages. 2. The delay depends on the concentration of PB used in preincubation. 3. Cells pretreated with a low concentration of PB divided about once before falling into the 'lag phase', while those pretreated with a high concentration of PB divided about twice before the appearance of the 'lag phase'. 4. The normal fission rate is reached later in cells pretreated with a high concentration of PB, than in cells pretreated with low doses of PB or unpretreated cells. 5. Cells pretreated with PB manage to recover their normal fission rate from the 'lag phase' more rapidly than non-pretreated cells, i.e. the phase of recovery is shorter. 6. In a separate experiment (not shown here) it was excluded that the appearance of the 'lag phase' at the 3rd day for cells pretreated with PB 1 µg/ml resulted from an entrance of cells of the sample into a stationary phase. On the contrary, this 'lag' is only a temporary arrest of fissions followed by their reinitiation.

It follows from the above results that the protecting action of PB against an inhibitory action of CHX is only transient. It means also that pretreatment by PB delays the occurrence of the 'lag phase', but does not shorten this phase; however, the following recovery is then shortened. In other

Table 2. Effects of 1 µg/ml CHX in the medium on the macronuclear DNA content

Time (h)	DNA contents in arbitrary unit ± SD Control cells	Experimental cells
22-26	6.99 ± 1.6	6.85 ± 1.75
46-50	8.21 ± 1.48	8.35 ± 1.50

Postdividing cells (G_1) were isolated from samples at 22-26 h of incubation (probable 1st division in CHX) and then at 46-50 h of incubation (expected divisions of adapted cells). Number of macronuclei for every tested group averaging 30-40 cells.

Table 3. Frequency distribution (in percent) of developmental stages of *Chilodonella cucullulus* in sequent days of experiments

Days	n	Morpho-static	Early dividers	Resorption res*	res	Advanced dividers	n	Morpho-static	Early dividers	Resorption res*	res	Advanced dividers
	Control medium						Medium + PB 1 µg/ml					
1	842	87.5	6.5	0.2	-	5.8	314	89.2	7.6	-	-	3.2
2	251	79.7	11.0	-	-	9.2	210	90.0	5.2	-	-	4.8
	Medium + CHX 1 µg/ml											
	Cells transfered from the control medium						Cells transfered from PB (1 µg/ml) medium					
1	1116	88.9	5.1	0.4	3.2	2.3	695	86.3	8.1	0.7	0.1	4.6
2	300	93.6	4.4	0.3	-	1.7	395	91.0	6.8	1.0	0.2	1.0
3	150	85.1	8.5	-	-	6.4	188	86.2	4.8	-	4.8	4.2
4	275	86.1	8.8	-	-	5.2	444	87.8	3.6	2.0	0.2	6.3
6	226	84.1	11.9	-	-	4.0	360	85.8	10.0	-	-	4.2

Developmental stages as defined in text; cells in morphogenetic movements and in cytokinesis correspond to class 'advanced divider'; res* cells inhibited at early stage of entry to the divisional morphogenesis; n, total number of cells. After every 2 days all samples are placed in fresh culture medium with the same initial CHX concentration as before.

words PB to some extent still affects the cells by the end of the experiment and so the delay of the CHX-related lag phase is not the result of a simple decay, inactivation or dilution of PB. In this sense PB is not a simple antagonistic factor against CHX, but a factor which modifies the whole temporal sequence of the response to CHX.

Therefore another model is proposed; PB pretreatment of *Chilodonella* cells stimulates some short-lasting and gradually-disappearing effects, preventing temporarily an inhibitory action of CHX, but it also enhances the cellular activity needed for a more rapid recovery from CHX action. If PB pretreatment temporarily induces an action of enzymes to inactivate most of the CHX molecules, or prevents their activity against the cell, then the concentration of the active CHX molecules at the start of the experiment is too low to inhibit the protein synthesis and to stop cell divisions. However, this pretreatment does not prevent a gradual accumulation of active CHX molecules during the next 2-3 days of CHX treatment. When the critical concentration of CHX molecules is reached within the cells, they fall into the 'lag phase'. But there are still other effects of PB pretreatment which make cells more ready to adapt to CHX. Thus the period of recovery from the 'lag phase' to the control level of the fission rate is shortened.

Based upon known caracteristics of barbitals and upon experiments done on liver cells[13,20-25], one can suggest that an activation of some drug-inactivating system or/and an increased level of transcription and translation induced by PB might be involved in the specific temporal pattern of reactions of *Chilodonella* to CHX following PB pretreatment. While this explanation seems plausible, there is no direct evidence regarding the pathways sensitive to PB in ciliates, apart from the finding of cytochrom b_5 in Tetrahymena[26], which is involved in such a pathway in liver cells.

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Enzyme electrophoretic approach to the systematics and evolution of the butterfly *Euchloe ausonia*[1]

H.J. Geiger and A. Scholl

Zoological Institute, Baltzerstrasse 3, CH-3012 Bern (Switzerland), 19 February 1982

Summary. Enzyme electrophoretic studies show that the *Euchloe* taxa *simplonia* and *crameri* share no common gene pool and are genetically as much differentiated from each other as from the North American *E. olympia.* It is concluded that the taxa *simplonia* and *crameri* are specifically distinct; separation of their gene pool must have occurred long before the end of the last glacial period.

Butterflies of the pierid genus *Euchloe* Hbn. (Lepidoptera, Pieridae) are distributed over the holarctic region. The taxa of this genus show a remarkable uniformity in coloration and wing pattern which often makes their identification rather difficult. Among the European members of this genus, *Euchloe ausonia* is one of these problematic taxa: a great variety of subspecies, races or forms has been described whose systematic rank is still unclear. In the most recent revision, Back[2] referred to *E. ausonia* from the European continent and North Africa as a complex which he subdivided into 3 groups: a) '*crameri*-group' (range of distribution: northwest Africa, Spain, France and Liguria eastward to Genoa); b) '*ausonia*-group' (Italy, from Modena south- and eastward, to Asia Minor); c) '*simplonia*-group' (Alps and Pyrenees). However, the systematic relationships among these 3 groups remain unclear, in particular since data on genetic isolation are not yet available.

A convenient approach for investigating genetic relationship among populations is provided by enzyme electrophoretic analysis[3]. Populations sharing a common gene pool will in general be very similar in allelic compositions at individual enzyme loci. However, following an interruption of gene flow, the populations tend to diverge and accumulate different alleles. In general, the degree of genetic differentiation depends mainly on the period of time since the separation from a common gene pool[4]. In fact, bio-