REVERSIBLE INHIBITION OF THE DIVISION OF *CRITHIDIA LUCILIAE* BY HYDROXYUREA AND ITS USE FOR OBTAINING SYNCHRONIZED CULTURES

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1. Introduction

Hemoflagellates present an interesting case of coordinate syntheses of nuclear and kinetoplastic DNAs [1,2]. Synchronized cultures of one hemoflagellate would be a very helpful tool in any attempt to investigate the regulatory processes involved in the simultaneous replication of chromosomal and extrachromosomal DNAs.

Although synchronized cultures of some protozoa have frequently been obtained and used in the study of the biochemistry of cell growth and division, only a single case of induced synchronized division of a trypanosomatid has been published so far [3]. Controlled temperature shifts were used, a method first described by Zeuthen and Sherbaum [4] which proved very successful on *Tetrahymena*. Hydroxyurea has been proposed as a cell synchronizing agent by Adams and Lindsay [5] and the present paper deals with its successful use in synchronizing divisions of the trypanosomatid *Crithidia luciliae*. We also report here that the reduplications of the nucleus and the kinetoplast appear to be equally affected by hydroxyurea, as opposed to the inhibitory action of diaminoacridines and ethidium, which exert a selective effect upon the replication of kinetoplast DNA.

2. Material and methods

Crithidia luciliae is grown in Boné's medium [6] at pH 7.4. All the cultures are maintained at 23°C, in test tubes or cylindrical bottles, and sedimentation of the cells is avoided by a very slow rotatory agitation.

Stock solutions of hydroxyurea (Nutritional Biochemicals Corporation, Cleveland 28, Ohio) are sterilised by filtration through 0.45 μ Millipore filters. Growth curves of the cells maintained in calibrated tubes are plotted from optical density readings in a Beckman model C colorimeter. Smears are stained with Giemsa's stain.

Plating efficiency is estimated from colony counts on solid, 1.5% agar, Boné's medium, in standard disposable Petri dishes. 100 to 150 cells, diluted in 0.05 ml of medium, are spread at the surface of the agar plates with a glass rod of suitable shape and thickness. Sizable colonies are built from single cells in about seven days at 23°C.

3. Results

Fig. 1 shows how different concentrations of hydroxyurea affect the growth of *C. luciliae*. Inhibition reaches a maximum 14 to 20 hr after the addition of the drug, depending on the concentration, and a spontaneous release of the inhibition is finally observed. Considering the division index however (fig. 2), it can be seen that the inhibition of cell division occurs much more rapidly and that the growth still observed by optical density readings corresponds to increase of cell size rather than to increase in cell number. This has been confirmed by microscopical observation. A concentration of 200 μg/ml was chosen for experimentally inducing synchronized divisions. As shown in table 1, at this concentration, hydroxyurea does not signifi-

Table 1

	Controls	3 hr	$7\frac{1}{2}$ hr	$11\frac{1}{2}$ hr	$21\frac{1}{2}$ hr
Mean number of cells plated	153	134	145	103	146
Number of colonies	149 ± 15	99 ± 8	126 ± 9	99 ± 7	119 ± 4
Plating efficiency	97%	74%	87%	96%	82%

Plating efficiency of *Crithidia luciliae* treated by 200 μg/ml hydroxyurea during different lengths of time. The colony counts in the second row are mean value for four identically inoculated plates.

cantly affect the viability of *C. luciliae* for treatments up to 20 hr.

Synchronized cultures are obtained as follows. A preculture of the hemoflagellate is first produced and its growth measured by optical density readings. As soon as the stationary phase is reached (10^8 cells/cm^3 approximately), 4 to 10 ml of this preculture are inoculated into a one liter bottle containing 100 ml of medium plus 200 μg/ml hydroxyurea. After 6 hr 30 min the cells are collected by centrifugation in aseptic conditions and resuspended in 100 ml of drug-free medium. Crithidias are sampled and prepared as stained smears at different times after the end of the hydroxyurea treatment. The proportion of dividing cells is estimated from counts covering at least 10^3 cells in each sample. A cell was considered to be in the process of division whenever at least one of the two DNA containing organelles, nucleus or kinetoplast, was found in duplicate.

We observed that, at the end of the drug treatment,

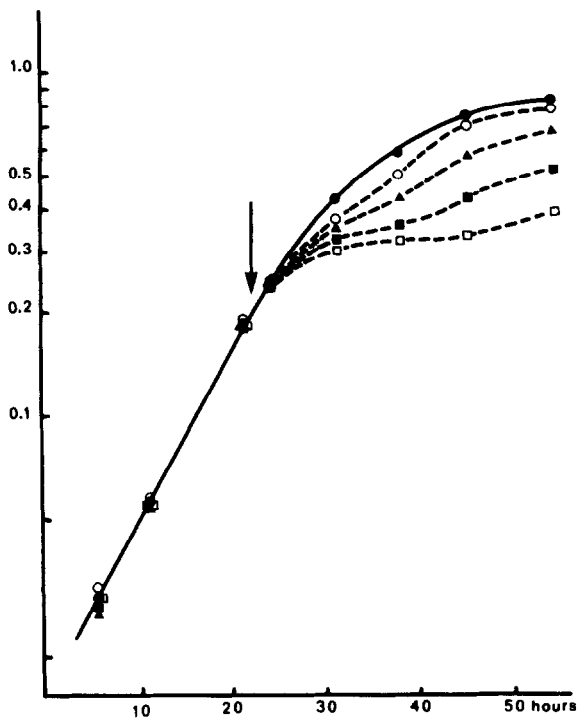

Fig. 1. Growth of *Crithidia luciliae* treated with hydroxyurea. Optical density is plotted against time after inoculation, each experimental point representing the mean value for six separate but identical cultures. Hydroxyurea is added at the time indicated by the arrow. Final concentrations of the inhibitor are 46 μg/ml (-○-), 116 μg/ml (-▲-), 232 μg/ml (-■-) and 465 μg/ml (-□-). Control (-●-).

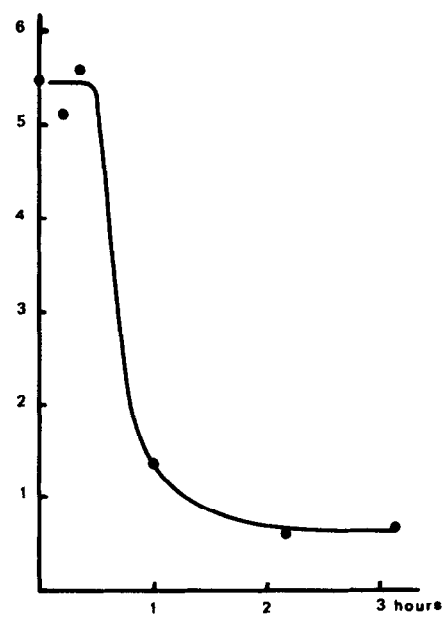

Fig. 2. Inhibition of cell division in a culture of *C. luciliae* treated with 200 μg/ml hydroxyurea. The percentage of dividing cells is plotted against time after the addition of the drug.

the crithidias are approximately twice their regular size, but are otherwise quite normal, particularly in respect of size and microscopical aspect of the kinetoplast and the nucleus. After release from inhibition, two well defined waves of synchronized divisions clearly occur, as illustrated in fig. 3. The first peak is found 2 hr to 2 hr 40 min after transfer of the crithidias from the hydroxyurea medium into drug-free medium, there being a slight variation from one experiment to another. The second peak is observed approximately 4 hr 30 min after the first. 25 to 45% of the cells are found to be dividing simultaneous at the peak of the first division wave. Between the first two waves of mitoses, the proportion of dividing cells decreases to less than one percent. The normal percentage of divisions observed in a non-synchronized log-phase culture, in identical medium and temperature conditions, is approximately 5% (fig. 2).

4. Discussion

Hydroxyurea appears to be an efficient agent for inducing synchronized division in hemoflagellates. The fact that 100% dividing cells are never observed at any single time could be due either to the fact that synchronization involves only a fraction of the population, the other cells dividing at random or not at all, or (and) to imperfect synchronization. Considering the fact that the two peaks shown in fig. 3 are much wider than would be expected from perfectly synchronized cells, the second hypothesis appears to be more probable. If absolute synchrony had been achieved, the width at midheight of the peak should not exceed the mean duration of the division time, i.e. the time during which the cell is considered to be dividing on the basis of the criteria defined above. This division time has been calculated according to an equation given previously [1] and a value of only 24 min has been found. Although not perfect, the synchronization reported here compares favorably with those commonly obtained on other cells by different methods.

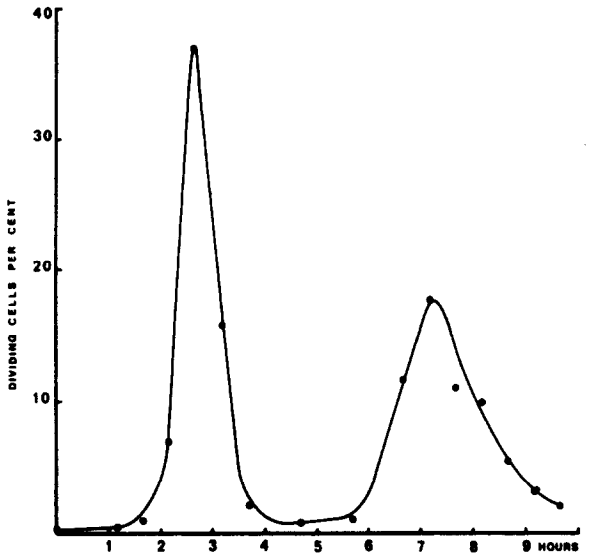

Fig. 3. Synchronized division waves in a population of *C. luciliae*, after release from 200 μg/ml hydroxyurea inhibition.

Synchronized divisions result from release from specific inhibition at some point of the cell cycle, most probably affecting a key step in DNA synthesis. It has indeed been shown, that hydroxyurea inhibits DNA synthesis without apparently affecting either RNA or protein syntheses. This effect has been observed in different eukaryotic cells [7,8,9,10] and also in bacteria [11,12]. The site in the metabolic chain leading to DNA synthesis which is affected by hydroxyurea is not known with certainty, but a number of experimental facts argue in favor of an inhibition at the level of ribonucleotide reduction [7,5,13,14]. An inhibition of the conversion of labelled ribonucleotides into deoxyribonucleotides by hydroxyurea has been described in rat cells and in bacteria [15,16].

Our own observations give no precise information as to the site of action, but it should be emphasized that the effects of hydroxyurea on *C. luciliae* are very different from those of two other inhibitors of DNS synthesis, acriflavine and ethidium. It has been shown recently that these intercalating drugs, which most probably act directly on the replication process, strongly affect the synthesis of kinetoplast DNA at doses which still allow the replication of nuclear DNA to proceed almost normally [17,18,19,20]. This differential inhibition leads, in fast growing hemoflagellates, to the selective loss, by dilution, of kinetoplast DNA, and to the appearance of akinetoplastic individuals. Akinetoplastic trypanosomes have similarly been obtained with berenil, another trypanocide which appears to interact with kinetoplast DNA, specifically blocking its replication [21]. Hydroxyurea has no

such selective effect on the kinetoplast. The size and microscopical aspect of this organelle, cytochemically stained for DNA, remain normal. Nor did we find any evidence for a higher sensitivity of the nuclear system toward hydroxyurea, which could give rise to abnormal cells with supernumerary kinetoplasts. This is of interest if we consider that some similarity exists between kinetoplast DNA and the bacterial chromosome and that DNA synthesis in higher organisms appears to be more sensitive to low concentrations of hydroxyurea than it is in bacteria [11,16].

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