and TrisCl was on the outside. If however under these conditions 2 µg monensin was added, considerable quenching, comparable to figure 2 curve A or to figure 4, was observed. The effect was identical at higher concentrations of monensin, whereas a longer time course was observed at smaller concentrations. Apparently Na^+ ions are largely exchanged for protons by monensin and the pH gradient that was generated previously by the diffusion of HAc is now eliminated and even reversed. Because HAc may still permeate freely through the liposome membranes this final pH gradient subsequently will partly be broken down since HAc will re-enter the liposomes. This last phenomenon can also be seen in figure 3. The opposite events can be observed when NaAc is present in the outside medium and TrisCl in the inside medium at the same pH (pH = 7.4). As was shown in figure 4 the fluorescence of AO was considerably quenched under these conditions. If monensin was then added, quenching of AO fluorescence was greatly reduced (fig. 4).

Before discussing these results we shall summarize the several quenching mechanisms that have been introduced and, in principle, may play a role in this study[2]. 1) Protonation may intrinsically affect the fluorescence characteristics of a probe. 2) If a probe enters the internal osmotic volume of liposomes its fluorescence may become screened by the phospholipid membranes. 3) A decreased quantum yield of fluorescence may arise from energy transfer between the probe molecules. This so-called selfquenching can be observed in figure 1 and also may occur if for example a probe is concentrated to a high degree inside liposomes. 4) Quenching due to energy transfer may also arise from the interaction of probe molecules with each other or from the binding of probe molecules to other species such as phospholipid membranes or buffer molecules.

Mechanism 1 can be involved if one observes fluorescence changes when varying the pH in the neighborhood of the pKa of a fluorescent probe, because then considerable amounts of probe molecules may be converted from the protonated into the deprotonated form or v.v. As far as our present experiments with AO are concerned, there is no need to take mechanism 1 into consideration because the pKa of AO is 10.5, which is about two units above the highest actual pH. Mechanisms 2 or 3 can explain the differences in the quenching levels if the situations with opposite pH gradients are compared (see for example curves A and D in fig. 2). But neither of these mechanisms explains the influence of pH in the absence of pH gradients (see for example curves B and C in fig. 1). To explain this last phenomenon mechanism 4 can be invoked. Because in the actual pH range the charge and therefore the binding properties of the dicetylphosphate in the membranes will strongly depend on pH it is most likely that part of the AO will be bound to the membranes to a certain degree depending on the pH. Dell'Antone et al.[4] found an increase in the amount of binding of AO to energized submitochondrial particles if the pH was raised from 6 to 8. From their experiments these authors even conclude that the uptake of cationic dyes in these energized particles is essentially a binding to membrane sites. Also Kraayenhof[5] speculated that the pH dependence of acridine orange does not result from probe distribution across membranes but rather is due to the influence of $\triangle$pH on the interfacial potential and, consequently, acridine binding. However, although our present results do not allow an explanation merely in terms of a $\triangle$pH dependent distribution of AO between the inside and the outside aqueous phase, an explanation exclusively in term of binding to membranes is not possible either. If the quenching of AO fluorescence were due entirely to binding, then we would for example have expected the quenching levels shown in figure 2 to be in a different order. The strongest quenching then should occur in the situation where the pH inside and outside is 8.4 (curve B), whereas the situation where both pH's are 7.4 (curve C) should give rise to the weakest quenching.

Therefore, distribution of acridine orange between aqueous phases as well as membrane binding are involved in the pH-dependent fluorescence quenching of acridine orange in negatively charged liposomes.

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0014-4754/86/080954-03$1.50 + 0.20/0

The antimitotic activities of some benzodiazepines

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Summary. Among 9 benzodiazepines, tested on the proliferation of synchronously dividing flagellate cells, only diazepam and medazepam can induce an accumulation of abnormal mitotic figures after 24 h of treatment. It seems that there is not a direct relation between the activity of benzodiazepines on the central nervous system and their ability to inhibit mitosis.
Key words. Benzodiazepines; mitosis; flagellate cells.

Since the report of Clark and Rian[1] that some benzodiazepines have inhibitory effects on the proliferation of 3T3 cells, there has been increasing interest in this topic. Recently it was reported that diazepam inhibited the proliferation of several types of mammalian cell in culture[2-4], and induced an accumulation of abnormal mitotic figures[2]. These results were confirmed using synchronously dividing cultures of *Dunaliella,* where diazepam had an inhibitory effect on proliferation whenever the drug was added during the cell cycle[5].

Since the benzodiazepines all have a similar chemical structure, we have investigated the antimitotic activity of nine of these drugs, which are used in therapy because of their activity on the central nervous system (CNS).

Material and methods. Dunaliella bioculata, a unicellular green alga, was grown on synthetic sea water[5]. Cells were induced to divide synchronously by being subjected to a 12 h light – 12 h dark cycle. Diazepam, Medazepam, Flunitrazepam, Nitrazepam and Clonazepam were gifts from Lab. Roche (Paris), Tema-

Table 1. Effects of some benzodiazepines on cell proliferation

Drugs tested	I.D. 50	Log P
Diazepam	$1.1 \cdot 10^{-4}$ M	2.82
Medazepam	$1.85 \cdot 10^{-5}$ M	4.05
Clonazepam	$2.2 \cdot 10^{-4}$ M	2.41
Temazepam	$3.3 \cdot 10^{-4}$ M	2.19
Nitrazepam	$3.6 \cdot 10^{-4}$ M	2.12
Oxazepam	$3.5 \cdot 10^{-4}$ M	2.17
Clorazepate	$2.1 \cdot 10^{-4}$ M	3.91
Lorazepam	$3.1 \cdot 10^{-4}$ M	2.38
Flunitrazepam	$9.6 \cdot 10^{-5}$ M	2.11

ID 50 was determined after five days of culture. The Log P were taken from the 'Pomona College Medicinal Chemistry Project 1982' (communicated by the Laboratoire de Pharmacochimie moléculaire, Univ. Paris VII, France). Log P is a physicochemical parameter which describes hydrophobicity of a given molecule.

Table 2

	Cells in mitosis	Interphasic cells
Diazepam $1.8 \cdot 10^{-4}$ M	80%	20%
Medazepam $1.85 \cdot 10^{-5}$ M	51%	49%
Control: 2‰ DMSO		100%

Synchronously dividing cells of *Dunaliella* were treated with diazepam $1.8 \cdot 10^{-4}$ M and medazepam $1.85 \cdot 10^{-5}$ M at the beginning of light (in the G_1 phase) and fixed for electron microscopy after 24 h of treatment (the end of the light-dark cycle). Counting was performed on about 200 randomly observed sections of cells.

zepam from Lab. Carlo-Herba (Paris), Oxazepam and Lorazepam from Lab. Wyeth-Byla (Paris) and Clorazepate from Lab. Clin-Midy (Paris). Each drug was previously dissolved in DMSO. Varying amounts of stock solutions were added to the cultures to obtain final concentrations in the range of 10^{-5} to 10^{-4} M. Final concentration of DMSO in the cultures was 2‰, which has no influence on the proliferation of the cells.

Determination of the cell number and volume in order to establish a cell size distribution was performed on a electronic cell counter (Coultronic model ZF) with an orifice of 100 µm. Drugs were added to the synchronously-dividing cultures at the beginning of a new light-dark cycle. Cells were counted and fixed for electron microscopy after 24 h of drug treatment (one full cell cycle).

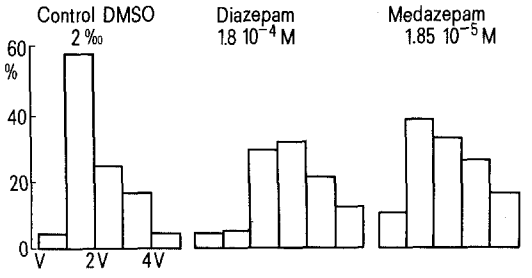

Figure 1. Histograms of cell volume: Comparison between control and cells treated by diazepam $1.8 \cdot 10^{-4}$ M and medazepam $1.85 \cdot 10^{-5}$ M after 24 h of treatment. V = 115 µm³.

Results. Amongst the tested benzodiazepines, only flunitrazepam and medazepam (table 1) inhibited the proliferation of the cells at concentrations equal to or lower than that required for diazepam.

When the drugs were added at the beginning of a light-dark cycle, only diazepam ($1.8 \cdot 10^{-4}$ M) and medazepam ($1.85 \cdot 10^{-5}$ M) induced an increase of the cell volume after 24 h of treatment (one complete cell cycle), as is shown in figure 1.

When such cells were observed by means of electron microscopy, it appeared that most of them were in mitosis, whereas all the control cells had divided (table 2). As we showed previously for diazepam[5], medazepam induced defective mitosis with abnormal metaphases. The chromosomes were well-condensed and the microtubules well-polymerized (we have found that diazepam did not affect the polymerization of tubulin in vitro; results not shown), but the spindle was unipolar. This can be observed in connection with the inhibition of the separation of basal bodies at the end of prophase (fig. 2). During the normal process, this separation is strongly correlated with the bipolarization and the elongation of the metaphasic spindle.

Discussion. We have tested nine benzodiazepines, all of which are active on the CNS, with regard to their potential activity on cell proliferation. We have found that they could inhibit the proliferation of *Dunaliella,* a flagellate unicellular green alga, which we have used as a model. The ID 50 of the drugs were at concentrations between $1.85 \cdot 10^{-5}$ M and $3.6 \cdot 10^{-4}$ M. The most active were diazepam, medazepam and flunitrazepam, but only diazepam and medazepam could induce an accumulation of

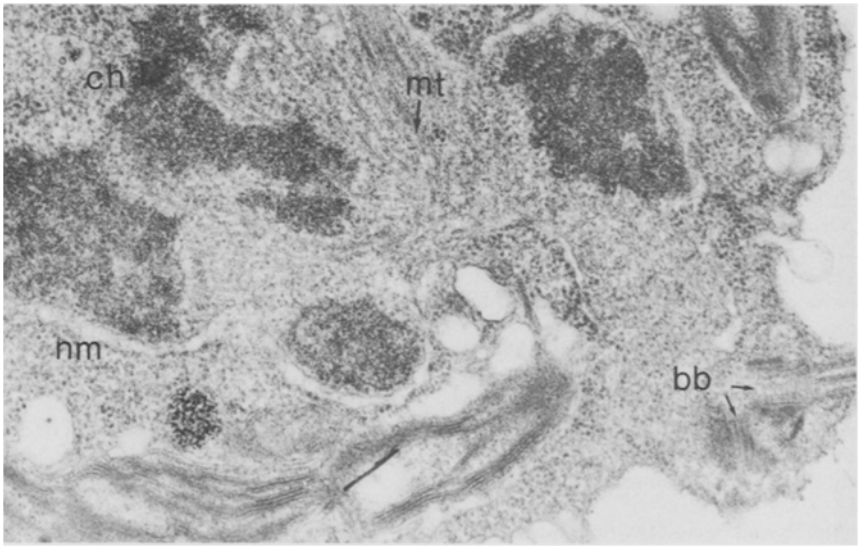

Figure 2. Electron micrography of cell treated 24 h by Medazepam $1.85 \cdot 10^{-5}$ M. The nucleus is blocked in metaphase with distinct chromosomes. The spindle fibers appear normal but are oriented toward a single pole (arrow head). The two basal bodies which are duplicated and largely separated at metaphase in the control cells, are here still joined and unduplicated. bb, basal body; nm, nuclear membrane; ch, chromosome; mt, spindle microtubule. G = 44,000.

abnormal mitoses with unipolar spindles. This effect on mitosis appears with $1.85 \cdot 10^{-5}$ medazepam and $1.8 \cdot 10^{-4}$ M diazepam. This discrepancy could be linked to the stronger liposolubility of medazepam, as shown by the Log. P. (table 1). It is interesting to note that diazepam inhibits culture growth of *Dunaliella* in the same range of concentrations at which it prevents proliferation of several mammalian cells lines in culture[1,2,4,6]. In addition to that, Anderson et al.[2] have shown, on human fibroblasts in vitro, as we have on *Dunaliella,* that diazepam blocks centriole separation at metaphase. So it seems that the effect of diazepam on mitosis is relatively general, since it appears whatever the culture species, and that the target is the duplication and the separation of centrioles or basal bodies. Here, we have pointed out that not all the benzodiazepines have this effect, and therefore there is no direct relation between the activity of benzodiazepines on the CNS and their ability to inhibit the processing of mitosis. It is not possible at present to define the target of diazepam and medazepam in flagellate unicellular cells in relation to the formation of a unipolar spindle.

Recent reports show that 15 benzodiazepines, including diazepam and other newly synthesized ones, which inhibit the proliferation of mouse thymoma cells in culture, bind to the cells in a specific, saturable and reversible manner[4]. Scatchard analysis shows a single class of binding sites which appear as peripheral sites and are different from the specific sites of the central nervous system. The authors find a strong correlation between the binding constant of these compounds for the peripheral sites and their ED_{50} in inhibiting the incorporation of ^3H thymidine into the cells. They have concluded that these sites may be involved in the regulation of cell proliferation[4]. Our results on flagellate unicellular alga, which are situated far away from mammalian cells on the evolutionary scale, but which divide by the classical mitotic process[7], seem to indicate that diazepam and medazepam could interact with a very fundamental and widespread cellular mechanism.

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0014-4754/86/080956-03$1.50 + 0.20/0

Septation and fragmentation in *Oedogonium* mitochondria as different and independent effects of dimethyl sulfoxide (DMSO) treatment[1]

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Summary. Oedogonium cardiacum Wittrock, a filamentous green alga, was treated with DMSO. The substance induced active swelling and fragmentation of mitochondria at both 5 and 7.5%. Septa were observed at 7.5% only and were not identical with the fragmentation sites. Thus it is concluded that internal partition by septa is not a prerequisite for mitochondrial fragmentation or division.

Key words. Dimethyl sulfoxide; mitochondria; mitochondrial septation; mitochondrial fragmentation; *Oedogonium cardiacum.*

The theory that new mitochondria arise by division of preexisting ones is generally accepted. Controversy exists about the possible involvement of septation, i.e. the partitioning of the matrix space by the inner membrane, in mitochondrial division or fragmentation (division under unphysiological conditions)[2]. In the present study, *Oedogonium cardiacum* was treated with DMSO, a substance that is widely used in human and veterinary medicine and serves as a solvent and as a cryo- and radioprotective agent in biological experiments[3]. Our experiments show that mitochondrial septation and mitochondrial fragmentation or division are independent from each other.

Material and methods. Oedogonium cardiacum Wittrock was obtained from the Algensammlung Göttingen (catalogue No. B 575-1a). The algae were grown vegetatively in a medium for Cyanophyceae[4]. Cytochemical localization of cytochrome oxidase was performed according to Smith[5].

For electron microscopy the cells were fixed with glutaraldehyde and OsO_4 according to the method of Kiermayer[6]. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 400 T and an AEI Corinth 500 electron microscope. O_2 uptake was measured in a 10 ml stirred cell, using an O_2 electrode (Yellow Spring Instruments, Biological Oxygen Monitor 53).

Chemicals were from Merck and Serva. DMSO and diamide were dissolved in the culture medium. The stock solution of 2×10^{-4} M antimycin A contained 2% DMSO.

Results. The mitochondria of *Oedogonium cardiacum* are up to several µm long and motile. Branches are frequently formed and retracted (fig. 1). Addition of culture medium containing 5% DMSO causes a transient plasmolysis[7], during which most mitochondria fragment into pieces of 1–2 µm length. Their motile behavior suggests that some fragments are linked to each other like a string of beads, parts of which are easily separated from each other during movement. The effects of 7.5% DMSO are similar. Many cells do not survive the deplasmolysis which immediately follows the plasmolysis. In the case of 5% DMSO about 26% and in 7.5% DMSO more than 50% of the cells die. Those which survive this phase are viable in DMSO for several days at least. The rate of fragmentation, i.e. the proportion of surviving cells in which all of the long mitochondria have divided into pieces of maximally 2 µm length, is about 75 (5% DMSO) and 88% (7.5% DMSO) respectively (64 and 96 cells examined). O_2 uptake is 78 and 65% of the control. Thus, it is almost unchanged when the corresponding mortality is taken into consideration.

DMSO up to 7.5% affects only the ultrastructure of mitochondria. Other parts of the cytoplasm appear normal. The fine structure of a control mitochondrion is shown in figure 1. Treatment with 5% DMSO for half an hour leads to a marked swelling of mitochondria which is statistically significant (data not shown, fig. 2). Constrictions are abundant. After 30 min treatment with 7.5% DMSO the matrix space is further enlarged. The cristae appear elongated and flattened with narrow intracristal spaces. Most conspicuous is the appearance of internal septa in about 80% of the mitochondria. The septa are independent of the constrictions which are also abundant (figs 3