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Fluorescence response of acridine orange to changes in pH gradients across liposome membranes

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Summary. The influence that changes in proton distribution have on the fluorescence of acridine orange was examined using negatively charged liposomes. Our results indicate that at least two mechanisms are involved: distribution of the probe between the internal aqueous phase of the liposomes and the outside medium, and binding of the probe to the liposome membranes.
Key words. Acridine orange; fluorescent probe; pH gradient; fluorescence quenching; liposomes.

Fluorescent amines are useful tools to monitor pH gradients across energy transducing and model membranes[1,2]. It is assumed that these compounds, being weak bases, can move freely across membranes in their unprotonated form and, if a pH gradient is established, will accumulate in their protonated form on the side of the membranes where the pH is lower, in accordance with the pH gradient. The distribution of an ideal probe between the inside and the outside aqueous phases of vesicles or liposomes due to a pH gradient can be estimated directly from the quenching of its fluorescence[2]. However the exact mechanism of quenching is not yet fully understood[3].

The question of why acridine orange in the presence of pH gradients does not behave like an ideal probe has been the subject of some discussion in the literature. Some authors have interpreted the fluorescence quenching of acridine orange in the presence of energized membranes largely as a binding phenomenon[4,5]. Lee and Forte however, as a result of their experiments with gastric microsomes, postulated that when acridine orange is used as a fluorescent probe both distribution between aqueous phases and binding may be involved at the same time[6]. Along the same line, we show evidence in the present paper for a mixed mechanism for fluorescence quenching of acridine orange by using a membrane model system, i.e. negatively charged liposomes.

Materials and methods. Dipalmitoyl-phosphatidylcholine (DPPC) and dicetyl-phosphate (DP) were purchased from Sigma Chemical Company. Acridine orange (AO) was obtained from Merck. Monensin was a gift from Eli Lilly Research Laboratories and was added from an ethanolic solution.

Liposomes were prepared according to the method described by Bangham et al.[7]; 20 mg DPPC and 2 mg DP were dissolved in 5 ml chloroform-methanol (2:1). The organic solvents were evaporated in a rotary evaporator. The remaining phospholipid film was suspended either in 1 ml solution containing TrisCl (100 mOsM), MOPS/KOH (10 mOsM) or in 1 ml solution containing NaAcetate (100 mOsM), MOPS/KOH (10 mOsM) at 55°C; mOsM was used instead of mM for the purpose of equal tonicity inside and outside the liposomes. From here NaAcetate will be referred to as NaAc. The liposomes were used after an 1-h period of equilibration.

Fluorescence measurements were performed with a Perkin-Elmer (type 204) fluorimeter. A front surface light cell (angle 45°), fixed in a light cell holder, equipped with a thermostat (temperature 55°C), was used. The light cell was filled either with 1 ml solution containing TrisCl (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM) or with 1 ml solution containing NaAc (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM) or with 1 ml solution containing NaCl (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM). The fluorescence was adjusted to 100% with 490 nm as the excitation wavelength and 530 nm as the emission wavelength. Then 50 µl liposomes were added and the fluorescence quenching was recorded, without corrections for dilution.

Results and discussion. From figure 1 it can be seen that AO fluorescence increases steadily with increasing AO concentration until a concentration of about 0.1 mM is reached. At higher concentrations the fluorescence decreases. The experiments described below were therefore performed using 50 µM AO. Anomalous fluorescence changes, due to concentration quenching, are not to be expected at this concentration. When 50 µl liposomes, which had been prepared in TrisCl buffer (pH 7.4),

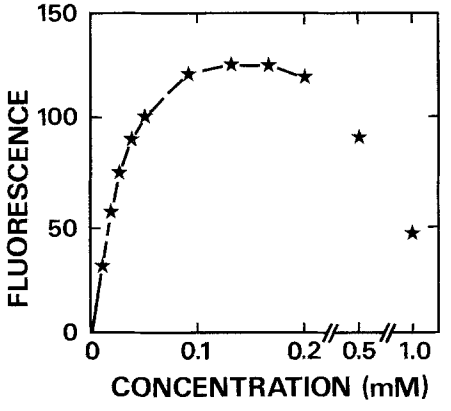

Figure 1. Fluorescence (in arbitrary units) of acridine orange in TrisCl buffer (pH = 7.4) as a function of acridine orange concentration.

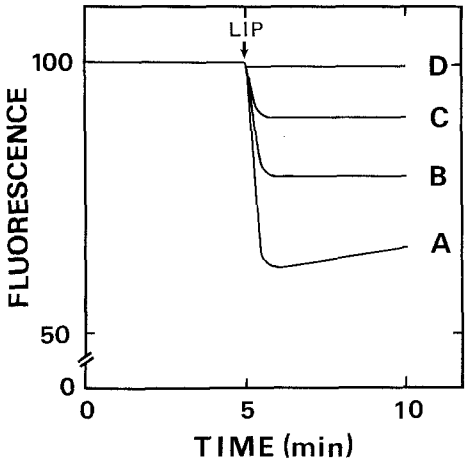

Figure 2. Fluorescence (in arbitrary units) of acridine orange after the addition of liposomes as a function of the pH of the internal and the external TrisCl buffering solutions. A: $pH_{in} = 7.4$, $pH_{out} = 8.4$. B: $pH_{in} = pH_{out} = 8.4$. C: $pH_{in} = pH_{out} = 7.4$. D: $pH_{in} = 8.4$, $pH_{out} = 7.4$.

were added to 1 ml of the same buffer containing 50 µM AO, the fluorescence was quenched by about 10% (fig. 2, curve C). The level of quenching turned out to be exactly the same (not shown) when 50 µl liposomes, which had been prepared in TrisCl buffer (pH 7.4) containing 1 mM AO, were added to 1 ml of the same buffer without AO, so as to have the same total amount of AO as before. Clearly the former positioning of the probe, i.e. whether it is inside or outside the liposomes, makes no difference to its final distribution. In the experiments described below AO was always added to the outside medium.

Figure 2 shows the fluorescence quenching of AO after the addition of liposomes as a function of the pH of the internal and external TrisCl buffering solutions. Four different situations are outlined. Curve A represents the quenching when the pH of the internal aqueous phase of the liposomes was 7.4 and the pH of the external medium was 8.4. In the reverse situation ($pH_{in} > pH_{out}$) no quenching was observed, as appears from curve D. Curve B represents the quenching in the absence of a pH gradient when the pH of both the internal aqueous phase of the liposomes and the external medium was 8.4. Finally curve C represents the quenching in the absence of a pH gradient, but this time the pH was 7.4 both inside and outside the liposomes. The quenching represented in curve B and curve C is intermediate between the two levels that appeared when a pH gradient was present. Quenching was less at pH 7.4 (curve C) than it was at pH 8.4 (curve B). Similar results were obtained when pH gradients were generated by the diffusion of undissociated acetic acid (HAc) through liposome membranes in one or the other direction. If in the initial situation the pH both inside and outside the liposomes was 7.4 but the inside buffering solution consisted of NaAc instead of TrisCl, no quenching of AO fluorescence was observed after the addition of the liposomes, as is shown in figure 3. Because undissociated HAc can easily permeate phospholipid membranes, it will, under the present conditions, diffuse out of the liposomes down its concentration gradient. Thus a pH gradient ($pH_{in} > pH_{out}$) is generated. Due to this pH gradient the result this time (fig. 3) is comparable to the one outlined in figure 2 curve D. No quenching was observed in that case either. If the other way round the outside buffering solution was made up of NaAc and the buffer inside the liposomes was TrisCl, then substantial quenching of AO fluorescence did occur after the addition of the liposomes, as appears from figure 4. The amount of quenching is comparable to the quenching shown in figure 2 curve A. Quenching under these conditions must be attributed to the generation of a pH gradient ($pH_{in} < pH_{out}$) due to the influx of HAc, down its concentration gradient, into the liposomes.

This line of evidence is substantiated further by the fact that NaAc could not be replaced by NaCl: with TrisCl inside and NaCl outside the liposomes the same quenching was observed after the addition of liposomes (not shown) as in the case where TrisCl was both inside and outside the liposomes (fig. 2, curves B and C). If both the inside and the outside buffer contained NaAc the same amount of quenching was observed (not shown) as in the case where TrisCl was used (fig. 2, curves B and C). This is of course to be expected since no pH gradient will be generated if HAc is present in equal amounts on both sides of the liposome membranes.

Subsequently we noticed that if a pH gradient was present, as in the situation outlined in figure 2 curve D, but the outside medium now contained NaAc instead of TrisCl, some quenching did occur. Actually the amount of quenching this time is comparable to the quenching shown in figure 2 curve C, where no pH gradient was present. Apparently under the present conditions the pH gradient is eliminated because of the diffusion of HAc into the internal osmotic volume of the liposomes.

Quenching of AO fluorescence also could be affected by pH changes induced by monensin. This ionophore is known to exchange Na^+ ions for protons[8]. As was shown in figure 3 no quenching was observed if the pH on either side of the liposome membrane was equal (pH = 7.4) and if NaAc was on the inside

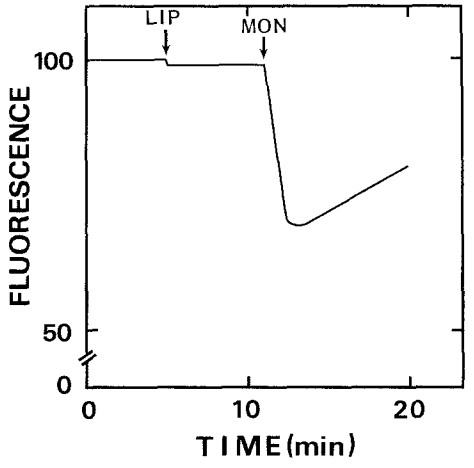

Figure 3. Fluorescence (in arbitrary units) of acridine orange in TrisCl buffer (pH = 7.4) after the addition of NaAcetate buffer (pH = 7.4) containing liposomes, and the influence of monensin.

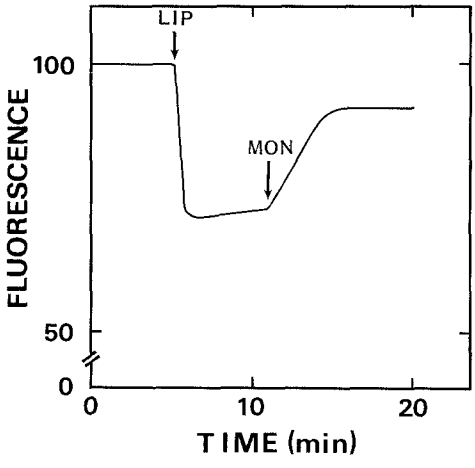

Figure 4. Fluorescence (in arbitrary units) of acridine orange in NaAcetate buffer (pH = 7.4) after the addition of TrisCl buffer (pH = 7.4) containing liposomes, and the influence of monensin.

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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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-