An alternative to Neutral Red as a dye for environmental contaminant biomonitoring

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ABSTRACT

Many methods have been proposed for evaluating of toxic compound presence in water and then to assess the risks to both humans and animals. A common standards procedure for the measuring of contaminant toxicity strength in sea water organisms utilizes the response of a dye (Neutral Red, NR), which accumulates in the acidic vescicles of biological sensors (*i.e.* Mollusc). When marine Mollusc such as mussels are exposed to pollutants, one of the characteristic pathological alterations is a decreased integrity in the lysosomal membrane. In this work we propose a comparison with the responses obtained with another dye, Acridine Orange (AO).
The results show that the response of Acridine Orange is linear in the whole operative pH range (*i.e.*, between pH 4 and 7,4), while Neutral Red is insensitive between pH 6 and 7,4.
In addition, Neutral Red shows a protonophore behaviour. We propose therefore that the use of Acridine Orange is preferred to that of Neutral Red, as well that Acridine Orange should be alternative to Neutral Red for the monitoring of stressing environment contaminants by means of biological sensors.

INTRODUCTION

When marine molluscs such as mussels are exposed to contaminants, in order to monitor the quality of water samples [1,2], one of the characteristic pathological alterations is a decreased integrity in the lysosomal membrane [3]. This loss of integrity is assayed using the dye (accumulated or released) **Neutral Red** (***NR***).

The dye enters the lysosomes of the mussels and accumulates inside, the driving force being the internal acidic pH. The damage to the lysosomes, induced by the toxic compounds, induces a release of the dye from the lysosomes to the resuspending medium.
A spectrophotometric determination of the concentration of NR in the supernatant gives a measurement of the *status* of the cells (in the mussels), since the release of NR in the supernatant is due to damage to the ***lysosomal membrane*** (and to the structures, pumps, enzymes, *etc*., which concur to this internal acidification) caused by toxic compounds. This results in a permeability enhancement of the membrane, an increase in the internal pH, and a subsequent NR release. Therefore, the concentration of NR in the supernatant is assumed to be an index of the toxicity of the solution [4].

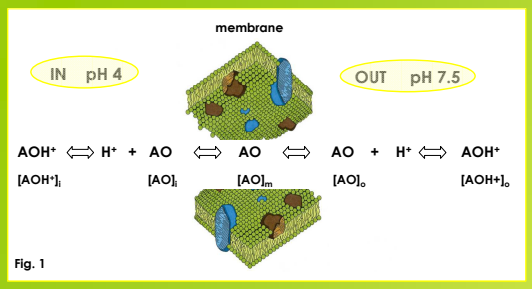

Fig. 1

Weak bases dyes as NR or **Acridine Orange** (**AO**) accumulate in the acidic compartments of the cell by means of a similar mechanism [5,6, 7], and the driving force being the internal acidic pH . **Fig.1** shows the classical mechanism which is responsible for the entry and accumulation of weak *permeant* bases in acidic vesicles. The dye (in this case AO, pKa = 10.5, although this type of behaviour is common to all weak permeant bases such as NR, pKa = 6.5 [8, 9]).) is soluble in the membrane (*phospholipidic bilayer*) in its un-dissociated form (AO). For symmetry reasons, the same situation occurs in the internal aqueous phase, where pH=4.

We believe and will demonstrate that the procedure has some weak points; the Acridine Orange dye, which is widely used for acidic pH measurements in isolated lysosomes, offers some advantages and is preferential to measurements based on the NR response.

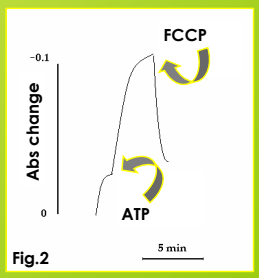

Fig.2

If the internal matrix is acidic, this induces an accumulation of the weak base in the matrix and the accumulation increases as more acidic is the internal pH (pHi). The accumulation in the matrix gives rise to an absorption change, which is probably due to a metachromatic effect [10].

Therefore, in the case of AO, by operating at a wavelength of 492 nm, which is the absorption maximum in water, the accumulation in an acidic matrix gives rise to a spectral change and the acidification is followed by the signal quenching which accompanies the entry of the dye. **Fig. 2** shows an example of the utilization of AO in order to follow the acidification process in isolated lysosomes, where the pH in the matrix is about 4.5 [5, 6, 11]. Analogous responses are obtained if NR, instead of AO, is used [10].

The acidic pH in lysosomes induces an accumulation of AO, with a consequent signal quenching at 492 nm (*metachromasy*). The release of the dye induced by a classical *protonophore* such as FCCP (100 nM), shows an example of release of the dye induced by membrane permeability enhancement and indicates that the transport of AO is very fast.

RESULTS & DISCUSSION

pHi	Neutral Red (NR)			Acridine Orange (AO)		
	R *	$[NR]_0$	$[NRH^+]_0$	R *	$[AO]_0$	$[AOH]_0$
4	432	$1.28\ 10^{-8}$	$1.28\ 10^{-9}$	$10^{3.5}$	$10^{-11.5}$	$10^{-8.5}$
5	44	$1.2\ 10^{-7}$	$1.2\ 10^{-8}$	$10^{2.5}$	$10^{-10.5}$	$10^{-7.5}$
6	5.18	$2.5\ 10^{-6}$	$2.5\ 10^{-7}$	$10^{1.5}$	$10^{-9.5}$	$10^{-6.5}$
7	1.29	$0.3\ 10^{-5}$	$0.3\ 10^{-6}$	$10^{0.5}$	$2.4\ 10^{-9}$	$0.4\ 10^{-5}$
7.5	1	$0.4\ 10^{-5}$	$0.4\ 10^{-6}$	1	$5\ 10^{-9}$	$0.5\ 10^{-5}$

*** R, *i.e.* ratio between the internal and external concentration of the dye**

It can be seen in the **table** on the left and from the correlated graph (**Fig. 3**) that NR is only sensitive to the pHi in the pH values ranging from 4 (4.5-5 is the pH value in the matrix of lysosomes in non contaminated cells) to 6. In the pHi values from 6 to 7.5 (which is the physiological pH value in the cytoplasm), the response and the release of NR in the supernatant is the same.

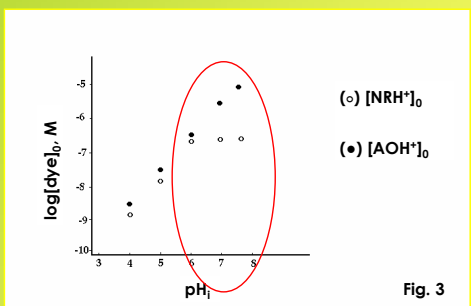

Fig. 3

A damage to the lysosomes, which induces a pHi enhancement from 6 to 7.5, gives the same response, corresponding to that given by the total destruction of the lysosomal membrane, *i.e.* a pHi of 7.5.
Therefore, NR cannot be used as probe in pHi values measurements which range between 6 and 7.5.
This problem does not occur when using AO (see the NR graph in Fig 3) and is due to the different pKa values of the two dyes.

CONCLUSIONS

- The analytical calculations indicate that the response of AO covers the whole pH range between 4 and 7.5, whereas NR is insensitive in a pH range from 6 to 7.5.
- The experimental measurements indicate that, in similar conditions, AO is not a protonophore, while NR has a protonophore effect which is similar to that induced by toxic compounds.
- Furthermore:
  - the absorption coefficient (ε) for AO is 18000, whereas it is ε = 18.500 for NR (which allows for the use of lower concentrations of AO in order to obtain the same responses as with NR);
  - the accumulation of AO is instantaneous (Fig. 2) and no incubation times are necessary (in the case of NR, the standard test implies an incubation period of three hours [12]).
- Indeed, AO is preferable to NR for toxicity measurements in aqueous samples, using biological structures (such as the mussels utilized in the standard test procedure).

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