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Interaction of electrically neutral and cationic forms of imipramine with liposome and erythrocyte membranes

Hasna Ahyayauch [a,*], Félix M. Goñi [a], Mohammed Bennouna [b]

[a] *Unidad de Biofísica (CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain*
[b] *Laboratory of Pharmacology, Department of Biology, Faculty of Science, Abdelmalek Essaâdi University, BP 2121, Tetouan 93002, Morocco*

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Abstract

Imipramine (IP) is an amphiphilic amine that is clinically useful as a tricyclic antidepressant. In the present work, we have investigated the pH effects on (a) the micellization properties of IP, (b) IP-induced release of aqueous contents from artificial vesicles (liposomes), and (c) IP-induced haemolysis, under hypotonic and isotonic conditions, in the pH range 5.0–10.0. Our data show that IP-membrane interaction caused important membrane destabilisation. However, changes in pH modified strongly the effects of IP. We suggest that the parameters of IP-induced model and cell membrane lysis are sensitive to the changes in the charge of this amphiphile. IP interacts more strongly with the membrane at basic pH (smaller net charge) than at neutral or acidic pH. A simple interpretation of these data is that increasing pH causes a decrease in IP net charge, thus a decrease in the critical micellar concentration of IP, and an increased partition into the lipid bilayer. Concomitantly, pH variations both above and below 7 tend to destabilise the cell membrane, so that the protective effect of IP against hypotonic haemolysis has a maximum at neutral pH.

Keywords: Amphiphilic character; Liposomal efflux; Haemolysis

1. Introduction

Tricyclic antidepressants, such as imipramine (IP) (Scheme 1), represent a class of substances with broad therapeutic applicability. One site of action seems to be at the level of the cell membrane because of their amphiphilic character. A number of studies indicate that amphiphiles are intercalated in the lipid bilayer of membranes so that the polar group is located at the polar–apolar interface of the membrane, while the hydrophobic parts are embedded in the hydrocarbon region of the bilayer (Jain and Wu, 1977; Sallee, 1978; Frenzel et al., 1978; Brasseur et al., 1985). This intercalation is apparently the primary step that triggers alterations in membrane-associated functions. The association between amphiphilic character and ability to affect membrane functions in a wide variety of compounds can hardly be coincidental. It appears that the membrane-perturbing ability is an intrinsic consequence of the amphiphilic character.

IP is a soluble amphiphile, $pK_a = 9.5$ (Ruell et al., 2003), that is able to distribute into cell membranes and produce mixed amphiphile/lipid bilayers. At low concentrations, IP perturbs membrane structure (Ahyayauch et al., 2002, Ahyayauch and Bennouna,

* Corresponding author. Fax: +34 94 601 3360.
E-mail address: gbxahxxh@lg.ehu.es (H. Ahyayauch).

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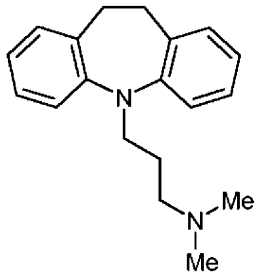

Scheme 1. Chemical structure of imipramine (IP).

1999) thereby increasing permeability. Previously, membrane leakage induced by insertion of amphiphile compounds into the erythrocyte membrane has been found to go along with acceleration of the transbilayer movement of phospholipid probes (Schneider et al., 1986; Classen et al., 1987; Rosso et al., 1988; Schwichtenhovel et al., 1992; Basse et al., 1992). Despite the considerable number of works on haemolysis by amphiphilic compounds, many aspects of this phenomenon remain unclear, in particular, the pH-dependence of the haemolysis parameters. Using hygroscopic desorption, Conrad and Singer (1979, 1981) found that amphipaths have a different interaction with biological membranes than with artificial membranes. Responsible for that would be the presence of proteins in the cell membranes, which would reduce drastically the partition coefficients. In this paper we have combined approaches using model and biomembranes. Together with haemolysis, we have studied the IP-induced lysis of liposomes (large unilamellar vesicles, LUV) and the micellization of IP in the absence of membranes. Liposomes have been widely used as models for biological membranes (Gregoriadis, 1992; Bangham, 1982) and in particular surfactant-induced release of vesicular contents has been measured using a variety of methods (Goni and Alonso, 2000). Fluorescence spectroscopy is a powerful technique to be used in conjunction with liposomes and surfactants. In our case, both IP-induced liposomal efflux and IP micelle formation have been studied using fluorescence methods. In the first section of this paper we report on the model membrane destabilisation induced by intercalation of IP in the liposome, then we describe the effects on red blood cell membranes. The pH dependence of mechano–osmotic properties is an essential piece of information for constructing any molecular explanation of IP action, thus in order to clarify the molecular mechanisms of tricyclic drug action on membrane structure we have studied the pH dependence of IP effect both on liposomal efflux and on the haemolysis of red blood cells. In both cases IP is more potent at higher pH, a condition under which its net electric charge and its critical micellar concentration are decreased.

2. Materials and methods

Imipramine (IP) was purchased from Sigma (St. Louis, MO, USA). Egg-yolk phosphatidylcholine and sphingomyelin were grade I from Lipid Products (South Nutfield, UK). Cholesterol was from Sigma. 6-Carboxyfluorescein was supplied by Eastman-Kodak (Seattle, WA, USA).

The critical micellar concentration (CMC) of IP was measured as an increase in 5 μM ANS (1-anilinonaphthalene-8-sulfonic acid) fluorescence emission (excitation at 360 nm) in 20 mM HEPES buffer at room temperature (21 ± 1 °C).

For liposome preparation, lipids were dissolved in chloroform/methanol (2:1, v/v), mixed as required, and the solvent evaporated exhaustively. Large unilamellar vesicles were prepared by the extrusion method with polycarbonate filters 0.1 μm in pore diameter (Mayer et al., 1986). Lipids were hydrated in 100 mM 6-carboxyfluorescein, 20 mM HEPES, pH 7.0 or 0.2 M sodium bicarbonate pH 10, unless otherwise stated. Non-entrapped dye was removed by gel filtration on Sephadex G-25 columns. Vesicle leakage was estimated from the increase in fluorescence exhibited by 6-carboxyflorescein upon dilution (Weinstein et al., 1977). Liposome suspensions were mixed with the same volumes of the appropriate IP solutions in the same buffer. Final lipid concentration was 100 μM. The mixtures were left to equilibrate for 1 h at room temperature. Fluorescence measurements were carried out in a 7700 Perkin-Elmer spectrofluorometer, at room temperature and with continuous stirring. Excitation light was adjusted at 490 nm. A 520 nm interference filter was used to avoid scattered excitation light.

Fresh heparinized rat erythrocytes were used in the haemolysis experiments. Blood was centrifuged at 1000 × g for 3 min, the plasma removed, and the

cells washed four times with an isotonic solution (145 mM NaCl, 5 mM sodium phosphate, pH 7.4). The hypotonic solutions at different pH were prepared as follows: pH 5, 88 mM NaCl, 1 mM sodium acetate; pH 7, 67 mM NaCl, 5 mM sodium phosphate; pH 8, 58 mM NaCl, 5 mM sodium phosphate; pH 10, 32 mM NaCl, 5 mM sodium bicarbonate. One volume of cells (3×10^9 cells/ml) was added to 3 volumes of hypotonic solution containing different concentrations of IP. After allowing equilibration at room temperature for a certain time, the samples were centrifuged ($1000 \times g$, 3 min), and the supernatants assayed for haemoglobin spectrophotometrically, using a Jenway 6050 colorimeter at 540 nm. Relative haemolysis was calculated as the ratio of A_{540} from the supernatant of cells treated with IP over that of control cells, haemolyzed in the hypotonic solution without IP.

3. Results

When an amphiphile, such as IP (Scheme 1), interacts with model or cell membranes, a complex equilibrium is established between, at least, surfactant monomers in solution, pure surfactant micelles, membrane-bound surfactant, and lipid-surfactant mixed micelles. The surfactant CMC is an easily measurable parameter, that provides information on the strength of surfactant–surfactant interaction. IP micellization is conveniently observed as an increase in ANTS fluorescence intensity, when this fluorophore is transferred from the aqueous to the hydrophobic environment of the micellar core. The procedure is illustrated in Fig. 1A, and the variation of CMC with pH is shown in Fig. 1B. On going from pH 5 to 10, the CMC of IP decreases by two orders of magnitude. Given the p$K_a \approx 9.5$ (Ruell et al., 2003) of the drug, the CMC of IP decrease appears to be related to the diminished net electric charge of IP, at least in the 7–10 pH interval. Changes in CMC below pH 7 must be due to factors other than IP net electric charge, because the latter is unlikely to change in the 5–7 pH interval, because of the IP p$K_a \approx 9.5$.

Large unilamellar vesicles containing phosphatidylcholine, sphingomyelin and cholesterol, all three abundant lipids in red blood cells, have been used as a simple model to explore IP-induced release of aqueous contents and to test the pH-dependence of this IP

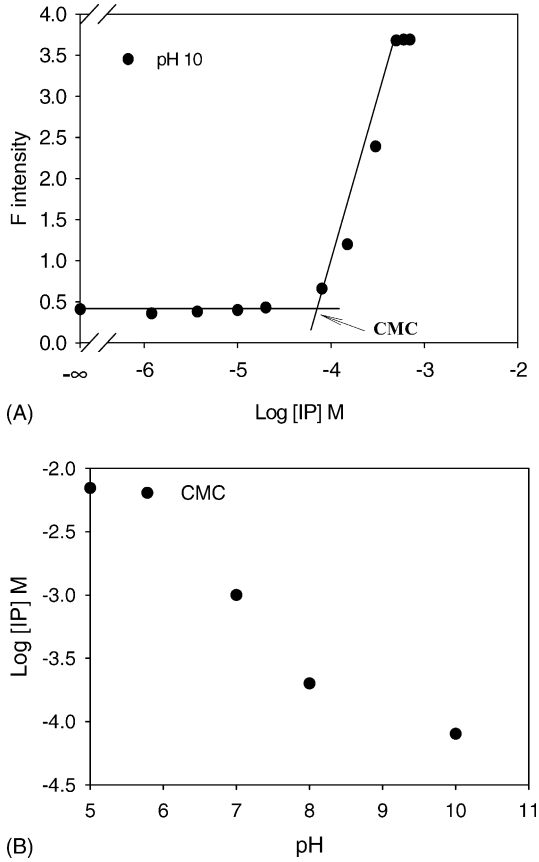

Fig. 1. (A) Fluorescence intensity of ANS as a function of increasing concentrations of imipramine at pH 10. The arrow points to the critical micellar concentration (CMC). (B) Critical micellar concentrations (CMC) of imipramine as a function of pH.

effect. 6-carboxyfluorescein was encapsulated in these liposomes and the IP-dependent release of vesicular contents could be conveniently monitored by spectrofluorometry. Preliminary experiments had shown that the fluorescent emission of 6-carboxyfluorescein did not change significantly in the 7.0–10.0 pH range. The time-course of IP-induced release of the aqueous fluorescent dye from LUV in a representative experiment at pH 7 is shown in Fig. 2. One-hundred percent release is achieved by addition of Triton X-100 at a bilayer-solubility concentration. Two useful parameters can be obtained from efflux curves as shown in Fig. 2, namely the extent of dye release at equilibrium, and the maximum slope of the fluorescence intensity versus time plot, the latter representing the maximum

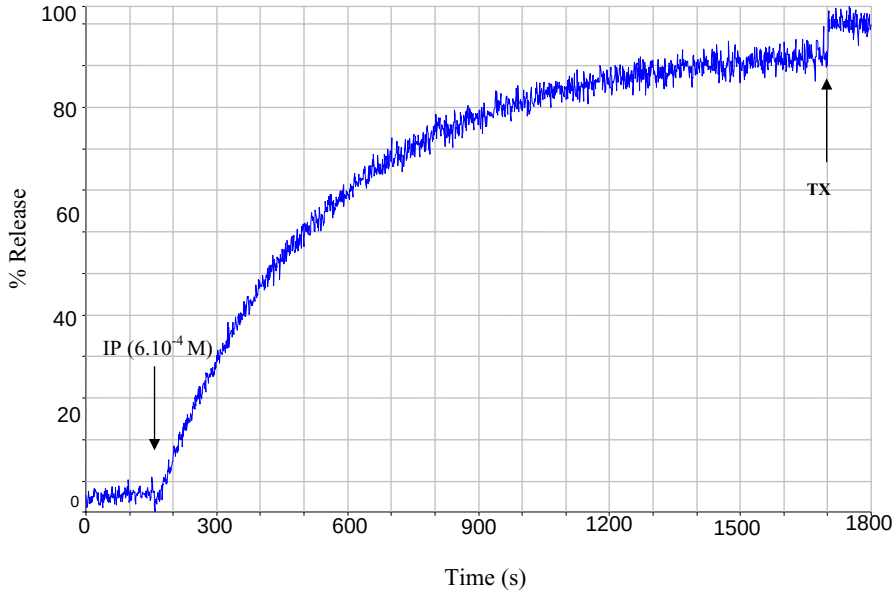

Fig. 2. Kinetic curve of percentage of 6-carboxyfluorescein released from liposomes after addition of 6×10^{-4} M of Imipramine at pH 7. TX: Triton X-100.

rate of IP-induced release of liposomal contents. Our data show that IP-induced liposomal efflux of 6-CF at pH 7.0 and 10.0 (Fig. 3). For the whole range of IP concentrations, less drug was required to obtain the same degree of dye release from the vesicles at pH 10 than at pH 7 (Fig. 3A), thus showing that the neutral form of IP is more active on membranes than the charged form. The pH effect is even more noticeable on the rates of 6-carboxyfluorescein release (Fig. 3B), the kinetics of the process being more directly related to the effective concentration of IP in the membrane. Note that changes in IP charge are likely to affect the membrane/water partition coefficient of the drug, thus effective IP concentrations in the bilayer may not be immediately correlated with the concentrations in the bulk of the solvent (total concentrations) given in the figure.

Haemolysis was studied under both isotonic and hypotonic conditions in the pH range 5–10. The results have been presented as percentage of haemolysis and relative haemolysis respectively under isotonic and hypotonic conditions, in the absence and presence of IP and at different incubation times. The results, obtained under isotonic conditions at pH 7 (Fig. 4), show clearly an important increase in percentage of haemolysis above certain drug concentrations. This phenomenon appears to be a function of both IP concentration and incubation time. Indeed, for the same concentration of amphiphile agent, percentage of haemolysis increases with incubation time. (Fig. 4A). In the same way, for the same incubation time, percentage of haemolysis increases significantly with IP concentration (Fig. 4B).

Contrary to the isotonic conditions, a biphasic behavior of IP-induced haemolysis has been observed under hypotonic conditions at pH 7 (Fig. 5). Relative haemolysis decreases above 10^{-6} M, the drug protecting the cells from hypotonic haemolysis, and reaches a minimum at 8×10^{-4} M. Above this concentration, relative haemolysis increases because the haemolytic effect predominates, i.e. relative haemolysis becomes >1. This behaviour remains almost invariant with time after 1 h (data not shown).

A summary of the pH effects on IP-dependent haemolysis is presented in Figs. 6 and 7. The data correspond respectively to the variation of haemolysis and relative haemolysis at a given pH after 4 h incubation under isotonic or hypotonic conditions. It appears clearly from these results (Fig. 6) that percentage of haemolysis varies significantly with pH. For the same IP concentration, pH increases are

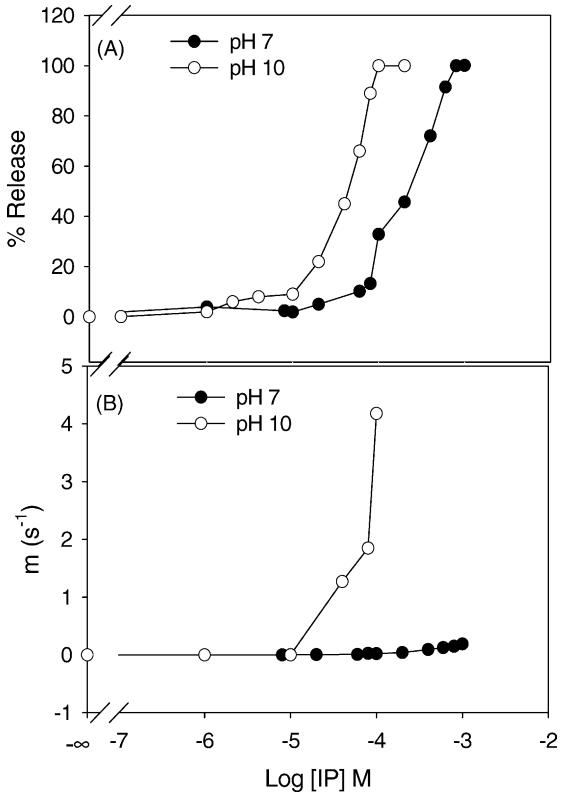

Fig. 3. (A) Percentage of released 6-carboxyfluorescein from large unilamellar vesicles at pH 7 (●) and at pH 10 (○). Vesicle composition was egg phophatidylcholine:egg sphingomyelin:cholesterol (6:1:1 molar ratio). Lipid concentration was 100 μM. (B) Rates of leakage induced by imipramine at pH 7 (●) and at pH 10 (○). Rates are measured as slope (*m*) of the fluorescence vs. time curve.

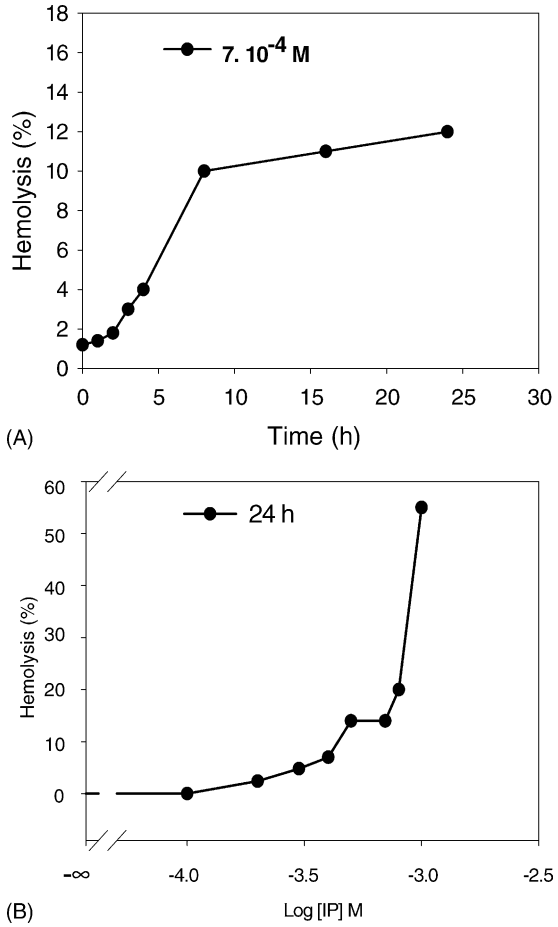

Fig. 4. Imipramine induced haemolysis at pH 7 under isotonic conditions. (A) Time course of haemolysis during the incubation of erythrocyte suspension in the presence of 7×10^{-4} M imipramine. (B) Percentage of haemolysis at pH 7 after 24 h of incubation with increasing concentrations of imipramine.

accompanied by a concomitant increase of percentage of haemolysis. Note, however, that no differences are observed between pH 5 and 7. pH effects are more marked at higher IP concentrations, indicating that they are due to changes in the IP molecule, rather than in the red blood cell membranes. The results in Fig. 7 show that the biphasic behavior of IP is also pH dependent. The minimum of relative haemolysis occurs at a lower IP concentration the higher the pH. This suggests that the lytic phase is facilitated by the high pH, in agreement with the isotonic haemolysis and liposome leakage data. It is interesting that the larger protection effect against hypotonic haemolysis (at 10^{-3} M IP), occurs at pH 7, presumably when the cell membranes are in optimum condition. Thus resistance to hypotonic haemolysis depends probably on the combined properties of the membranes and the drug.

4. Discussion

The interaction of IP with liposomal membranes causes an important leakage of 6-CF at pH 7 (Fig. 3). This efflux of 6-CF is explained by the membrane destabilisation caused by the intercalation of this drug in the liposomal membrane (Ahyayauch and Bennouna, 1999; Ahyayauch et al., 2002).

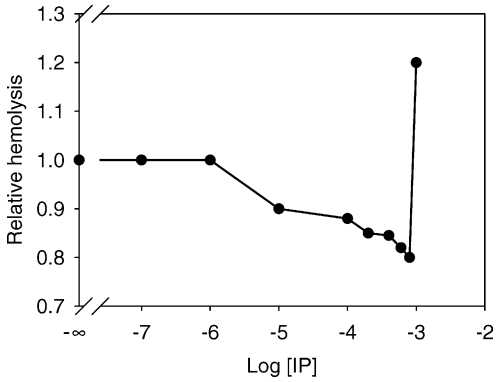

Fig. 5. The antihemolytic and hemolytic activity of imipramine after 24 h incubation of erythrocytes under hypotonic conditions.

However, the fluorescence increase observed at pH 10 is more important than at pH 7. This demonstrates the higher destabilising property of the amphiphile in neutral form. This result suggests that the hydrophobic interaction induces defects of membrane structure resulting in a rapid release of 6-CF.

Our results of percentage haemolysis variation under isotonic conditions according to IP concentration and times of incubation (Fig. 4) reveal that the interaction of this cationic amphiphile with red cell membranes generates a destabilisation of the membrane structure. So, the interaction of this amphiphile with the membrane decreases the order and structural organization of lipid “packing” and increases the permeability to the small ions (Isomaa et al., 1986).

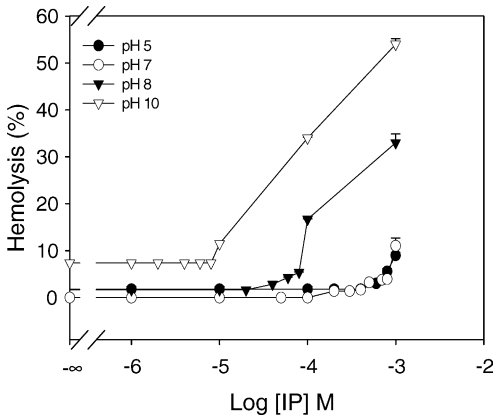

Fig. 6. pH-dependent effect of imipramine on hemolytic percentage after 4-h incubation under isotonic conditions.

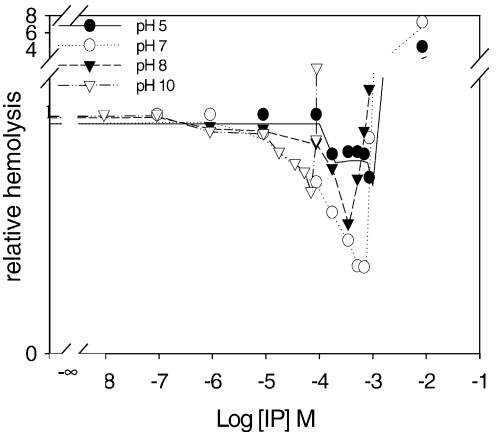

Fig. 7. pH-dependence of imipramine-induced biphasic behaviour after 4 h incubation with increasing concentrations of imipramine under hypotonic conditions.

The increase in membrane permeability due to insertion of the IP in the red cell membrane is due to the phospholipid redistribution between the internal and external face of the membrane (Schneider et al., 1986; Classen et al., 1987; Rosso et al., 1988; Basse et al., 1992; Schwichtenhovel et al., 1992). The lysis caused by IP is explained by the colloid–osmotic mechanism. In other terms, the interaction of the IP with red cell membrane induces the ions to be in equilibrium between the interior and outside of the membrane. The movement of Na^+ and K^+ through the membrane has an important significance in the regulation of the cellular volume (Sachs et al., 1975; Canessa, 1988; Lux and Palek, 1995). However, the presence of hemoglobin inside the cells is going to create an osmotic pressure difference leading to water influx and therefore to haemolysis. This haemolysis could be explained by the large and steady hole opening in membrane that is going to permit the efflux of hemoglobin (Hoffman, 1962; Bodemann and Passow, 1972; Jung et al., 1973; Schwoch and Passow, 1973; Steck, 1974; Johnson, 1975; Billah et al., 1977; Johnson and Kirkwood, 1978; Bejerrum, 1979; Kanda et al., 1979). The size of this pore seems to be concentration dependent. Variation in haemolysis with incubation time could be explained by certain studies that showed that the haemolysis hole reaches a maximal size after a prolonged incubation. This size is extremely dynamic and steady during several days. Under hypotonic conditions, our data show a

biphasic behavior (Fig. 5) characterized at a first stage by a significant reduction in relative haemolysis. This decrease is concentration dependent, when arrived to its minimum the second phase begins. The latter is characterized by an important increase of the relative haemolysis. The decrease in relative haemolysis could be explained by a phospholipid rearrangement that provokes an increase of permeability and therefore a fast ion efflux. This efflux decreases the osmotic difference existing between the inside and the outside of the cell (Hagerstrand and Isomaa, 1991). This osmotic resistance could also be explained by the intercalation of amphiphiles in the membrane, thus increasing the cellular surface/volume and therefore the haemolytic critical volume (Machleidt et al., 1972; Seeman, 1972). However, this increased critical volume, due to the incorporation of amphiphiles in the bilayer, is not a general mechanism that explains the anti-haemolytic effect and, in addition, it is not valid for all amphiphiles. The increase in relative haemolysis is explained by the colloid–osmotic mechanism (as discussed earlier in the text). Membrane stabilization is apparently limited and when the number of amphiphile molecules exceeds a certain level, the degree of bilayer destabilisation increases and haemolysis takes place.

Otherwise, our results show that the haemolytic effect obtained under isotonic conditions (Fig. 6) and the biphasic behavior of the IP recorded under hypotonic conditions (Fig. 7), vary according with pH, when pH increases the haemolytic effect and the biphasic behavior appear at lower concentrations. In the same way, for the same drug concentration, the IP effect obtained at pH 10 is more important than the one recorded at pH 7 and 5; this shows that the interaction of the neutral form with the membrane is more important than that of the charged form (see Scheme 1). At higher pH, IP molecules are deprotonated, thus becoming electrically neutral, and the hydrophobic attraction forces of the IP-non-polar moieties to be more important than the electrostatic interactions of the charged IP amino groups, leading to easier intermolecular interactions, i.e. lower critical micellar concentrations, and lower haemolytic concentrations (Figs. 6 and 7). The critical micellar concentration of pure IP decreases with pH, in parallel with the minimal drug concentrations causing haemolysis (compare Figs. 1B and 7). The situation is qualitatively similar to what occurs when the amphipathic drug chlorpromazine interacts with lipid bilayers (Ahyayauch et al., 2003) except that the pH effect on the micellization of chlorpromazine is larger than that of IP (four orders of magnitude versus two orders of magnitude). In summary, high pH facilitates IP-membrane interaction, thus membrane lysis, through a decrease in net charge of IP; neutral pH leads to a more stable cell membrane, thus to a larger extent of protection against hypotonic haemolysis.

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