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POLARITY INDEX: A MEASURE FOR THE DESTABILIZATION EFFECT OF ORGANIC SOLUTES ON ERYTHROCYTE MEMBRANE PROTEINS

B. Paarvanova[1], B. Tacheva[1], L. Dospatliev[2], M. Karabaliev[1], I. Ivanov[1*]

[1]Department of Physics, Biophysics, Rentgenology and Radiology; Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2]Department of Pharmacology, Animal Physiology and Physiological Chemistry; Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

ABSTRACT
Cytotoxicities of thymol, diethyl maleate, pentanol, ethyl acetate, phenol, butanol, propanol, 2,6-difluoropyridine, pyridine, acetone, ethanol, methanol, N-methylformamide, dimethyl formamide, ethylene glycol, dimethyl sulfoxide, and formamide were assessed by their molar activity to decrease the structural stability of erythrocyte membrane proteins, intrinsic (anion exchanger) and pheripheral (fibrillar spectrin). The denaturation temperatures of these proteins, T_{int} and T_{sp}, respectively, were detected by thermal analysis of erythrocyte suspension admittance. At a molar concentration, C_{ex}, of the solvent these temperatures linearly decreased by ΔT_{int} and ΔT_{sp}. The molar activity of solvent to destabilize these proteins were defined as $k_{int} = \Delta T_{int}/C_{ex}$ and $k_{sp} = \Delta T_{sp}/C_{ex}$, respectively. k_{int} and k_{sp} decreased as the Snyder's polarity index, P′, of solvents increased. In semi-log plot the k_{int} and apparently k_{sp} decreased linearly within the entire scale of P′. The relative destabilization strength, k_{sp}/k_{int}, of solvents apparently changed linearly from 0 to 1, when P' changed from 0 to 9. The only exception was formamide, which had normal k_{int} value and extremely high value of $k_{sp}/k_{int} = 3.4$. This highly selective and powerful strength of formamide to destabilize spectrin could be used to design a medicine capable of specifically affecting the under-membrane network of cancer cells.

Key words: cryopreservation, vitrification solution, enzyme stability, spectrin, band3 protein, impedance

INTRODUCTION

Assessing the toxicity and neurotoxicity of environmental pollutes, among them the organic solvents, has become an increasingly important problem. Although displaying cytotoxicity, many organic solvents are frequently used for preparation of liposomes or as additives to drug preparations and blood conservation media, cryoprotectants, preservatives and even medicines in some cases (1, 2). Many widely used industrial organic solvents are potentially toxic (3). Vitreous state or ice-free cryopreservation below the glass transition temperature is becoming increasingly recognized as the most likely solution for successful preservation of tissues, organ and living organisms. Presently, it appears that cryoprotectant toxicity is the single most important barrier to achieve this solution (4).

The traditional methods for testing toxicity are both expensive and time consuming, hence a new and efficient technique for first-tier toxicity testing would be of significant importance. Organic solvents have considerable lipid solubility that affects their pharmacological action on cells. Relatedly, erythrocyte hemolysis at normal and reduced tonicities as a simple test for the membrane action of organic solvents is frequently used as a predictor of their acute toxicity (5, 6). This test does not take into account, however, the effect of organic solvents on membrane proteins which are important constituents of cells. Organic solvents exert only a weak type of interactions with the erythrocyte membrane proteins changing their conformation, stability, and consequently function. Hence, these

***Correspondence to**: *Ivan Tanev Ivanov: Dept. Physics, Biophysics, Rentgenology and Radiology, Medical Faculty, Thracian University, Armeyska Str. 11, Stara Zagora 6000, Bulgaria. E-mail: ivanov_it@gbg.bg*

solvents could have different impact on the structure of various membrane proteins that could be important for their cytotoxicity. Measuring only final hemolysis does not allow discrimination of the conformation changes these solvents produce on various portions of erythrocyte membrane proteins, peripheral and intrinsic.

Cytotoxicities of a limited number of organic solvents have been assessed by the effect these solvents produced on the denaturation temperatures T_m of erythrocyte membrane proteins, peripheral and intrinsic ones, respectively (7). The solvents with minor cytotoxicity, glycerol and erythritol, increased these denaturation temperatures, while solvents with greater cytotoxicities exhibited destabilization effect. The destabilization effect was qualitatively compared to both polarity of the solvents and their ability to produce hydrogen bonds.

The polarity index, P′, of a solvent, as determined by Snyder (8) is an integral measure expressing the ability of the solvent to interact with various test solutes. Polarity index of organic solvents is important in cryopreservation of living systems, in chromatographic separation techniques (9), in preparing cosmetic and dermatological formulations, and for predicting the stability of various enzymes in multi-phase media (10). The polarity index is also of prime interest in choosing the solvent systems for optimal extraction of biologically active compounds with beneficial health effects (11).

The aim of recent study was to better elucidate the quantitative relationship between the polarity index of a large group of organic solvents and their effect on the stability of EM proteins, both peripheral and intrinsic ones. The results of this study could be useful in assessing and predicting the cytotoxicity of various organic solvents and for finding better vitrification solutions for cryopreservation of complex living systems.

MATERIALS AND METHODS

The solvents under study (thymol, diethyl maleate, pentanol, ethyl acetate, phenol, butanol, propanol, 2,6-difluoropyridine, pyridine, acetone, ethanol, methanol, N-methylformamide, dimethyl formamide, ethylene glycol, dimethyl sulfoxide, and formamide) were commercial ultrapure grade (99.9%) from Sigma Chemical Co, St. Louis, MO, USA.

The method for determining denaturation temperatures of erythrocyte membrane proteins, peripheral and intrinsic ones, was explained earlier (12). First, erythrocytes were isolated from human blood and part of them was used to prepare resealed erythrocyte ghost membranes by hypotonic lysis. Second, we prepared suspensions of whole erythrocytes or erythrocyte ghost membranes in isotonic 60 mM NaCl/sucrose medium with or without the indicated solvent, hematocrit 0.10. Third, the suspension was heated at 3.0 °C/min heating rate and data for the temperature profile of suspension admittance, Y_s, measured at 10 kHz, were collected and stored in a personal computer. Finally, the data was processed with Microsoft Excel program to obtain the temperature profile of the first derivative of the admittance, $\partial Y_s/\partial t$, against temperature, t. The obtained temperature dependence of $\partial Y_s/\partial t$ had the same shape using whole erythrocytes **(Figure 1)** and resealed isolated erythrocyte ghost membranes (not shown).

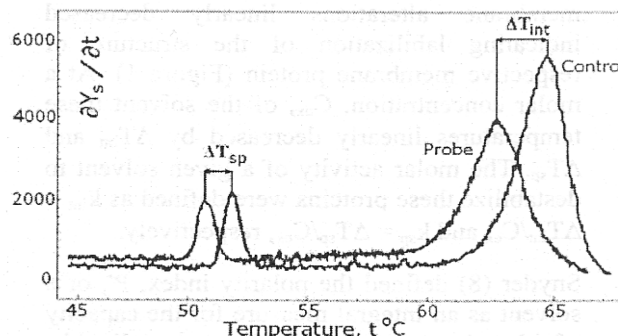

Figure 1. Changes in the temperature derivative of the admittance, $\partial Y_s/\partial t$, of erythrocyte suspension during heating. The suspension contained human erythrocytes, hematocrit 0.10, and was heated at 3.0 °C/min rate. The 52.2°C peak detects change in the beta-dispersion of cell membranes involving heat denaturation of spectrin. The 65.5 °C peak indicates egress of cytosolic ions following temperature activation of ion permeability due to a change in integral proteins. The suspension medium contained isotonic 60 mM NaCl/sucrose (the curve Control) plus organic solvent (the curve Probe).

In this way, two separate thermally-induced alterations were detected in the membrane of intact cells and their isolated ghost membranes as shown in **Figure 1.** The first one, centered at 50 °C (T_{sp}) and corresponding to the change in capacity of membranes (12), involves the heat denaturation of peripheral protein

spectrin. During the second one the ion concentration gradient dissipates in a narrow temperature interval that is detected as a sharp peak centered at 65 °C (T_{int}). This peak corresponds to the activation of passive ion permeability and involves a thermal transition in the hydrophobic domain of integral proteins, mainly the band 3 protein known as anion exchanger (13).

With cells and ghosts the reproducibility for determination of T_{int} and T_{sp} was within ±0.2 °C. Since the denaturation temperatures of both peaks were slightly displaced towards the higher temperatures all experiments with heated suspensions were carried out with the same heating rate of 3.0 °C/min. At this concentration, about 100 mM, sucrose insignificantly reduced the relative dielectric constant of the suspension medium from about 80 to 79 (14).

Before the onset of heating sufficient time (15 min) was provided for the solvent to attain equilibrium on both sides of the membranes. In the presence of organic solvents, the denaturation temperatures, T_{int} and T_{sp}, of both membrane alterations linearly decreased indicating labilization of the structure of respective membrane protein (Figure 1). At a molar concentration, C_{ex}, of the solvent these temperatures linearly decreased by ΔT_{int} and ΔT_{sp}. The molar activity of a given solvent to destabilize these proteins were defined as $k_{int} = \Delta T_{int}/C_{ex}$ and $k_{sp} = \Delta T_{sp}/C_{ex}$, respectively.

Snyder (8) defined the polarity index, P′, of a solvent as an integral measure for the capacity of this solvent to interact with various liquids. Ethanol, dioxane, and nitromethane were chosen as test probes for a solute's capacity for hydrogen-bond donor, hydrogen-bond acceptor, and dipole-type interactions, respectively. Next he proposed a formula to calculate P′,

$$P' = \log(K)_{Ethanol} + \log(K)_{Dioxane} + \log(K)_{Nitromethane}$$

based on the partition coefficients of this solvent dissolved in ethanol ($K_{Ethanol}$), dioxane ($K_{Dioxane}$), and nitromethane ($K_{Nitromethane}$). P′ is roughly proportional to the so called solvent strength parameter. For each solvent under study the Snyder's polarity index, P', was taken and averaged from several sources (15,16) and displayed in **Table 1**.

RESULTS AND DISCUSSION

For each solvent under study the molar activity to decrease the denaturation temperatures of the erythrocyte membrane proteins, k_{int} and k_{sp} is displayed in **Figure 2A** and **Figure 3**. The k_{int} and k_{sp} represent the normalized ability of respective solvent to decrease the conformation stability of intrinsic and peripheral group of erythrocyte membrane proteins. We assume the higher are the k_{int} and k_{sp} values of a given solvent the greater should be its non-specific toxicity to cells and living organisms.

Table 1. *Snyder's polarity indexes, P′, of the organic solvents under study.*

№	Solvent	Polarity index, P′
1	Diethyl maleate	1.5
2	Pentanol	2.2
3	Ethyl acetate	2.6
4	Phenol	3.0
5	Butanol	3.7
6	Propanol	4.0
7	2,6-difluoropyridine	4.0
8	Pyridine	4.3
9	Acetone	5.1
10	Ethanol	5.2
11	N-methylformamide	6.0
12	Methanol	6.3
13	Dimethyl formamide	6.7
14	Ethylene glycol	6.9
15	Dimethyl sulfoxide	7.2
16	Formamide	7.3
control	Water	9.0

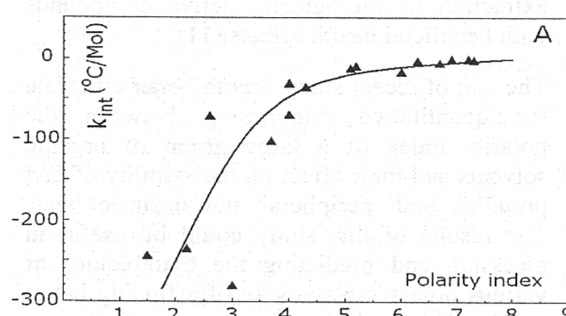

Figure 2. Effect of polarity index, P′, on the molar activity, k_{int}, of organic solvents to decrease the denaturation temperature, T_{int}, of the intrinsic proteins of erythrocyte membrane in linear (A) and semi-logarithmic (B) plot. T_{int} was determined by thermal analysis of the impedance of suspension that contained whole erythrocytes or resealed erythrocyte ghost membranes. k_{int} is defined as $\Delta T_{int}/C_{ex}$, where ΔT_{int} is the decrease of T_{int} in the presence of a solvent with concentration C_{ex} in the suspension medium. Other details are as for **Figure 1**.

Next, for each solvent the obtained k_{int} and k_{sp} values were compared to its Snyder's polarity index, P′. k_{int} and k_{sp} values showed strong species variations; nevertheless, they were clearly dependent on the polarity index of respective solvent. Generally, with increasing the polarity index the k_{int} and k_{sp} values decreased showing saturation type of dependence **(Figure 2A, Figure 3).**

In order to better refine the k_{int} /P′ dependence we further displayed the effect of the polarity index on k_{int} in a semi-logarithmic plot **(Figure 2B)**. In such a plot the k_{int} /P′ relationship is fairly good represented by a straight line indicating a first order dependence. Similar dependence was obtained and for the molar activity, k_{sp}, of solvents to destabilize the peripheral proteins (not shown). However, greater deviations of data were encountered in latter case indicating the existence of other factors, which impact k_{sp} alongside with the polarity index. We assume these factors could include the ability of solvents to form hydrogen bonds, the dielectric permeability of solvents, specific structure-dependent interactions etc.

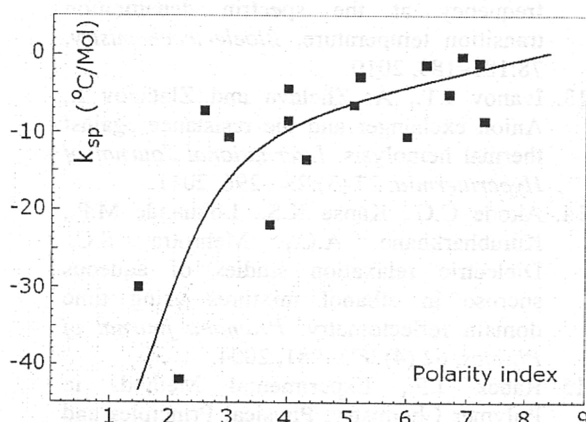

Figure 3. Effect of the polarity index, P′, of organic solvents on their molar activity, k_{sp}, to decrease the conformation stability of the peripheral proteins of erythrocyte membrane. Other details are as for **Figure 2.**

Finally, for each solvent under study we calculated the k_{sp}/k_{int} ratio, i.e., the relative strength of solvent to destabilize the peripheral proteins, k_{sp}, in respect to its strength to destabilize the intrinsic proteins, k_{int}. **Figure 4** represents the dependence of k_{sp}/k_{int} ratio on the polarity index of respective solvents. In general, the dimensionless quantity k_{sp}/k_{int} changed from 0 to 1 when polarity index changed from 0 (infinite hydrophobicity) to 9 (infinite hydrophillicity).

For the highly hydrophobic solvents with low polarity index the k_{sp}/k_{int} ratio is low and tends to zero. This tendency was clearly demonstrated with thymol, a highly lipophilic compound in which k_{int} = 2100 and k_{sp}/k_{int} = 0.06 (data not shown). The above tendency is explained by the ability of lipophilic solvents to intercalate into the lipid bilayer destabilizing the intrinsic proteins, primarily the anion exchanger, through direct interaction and indirectly by fluidizing the lipid bilayer.

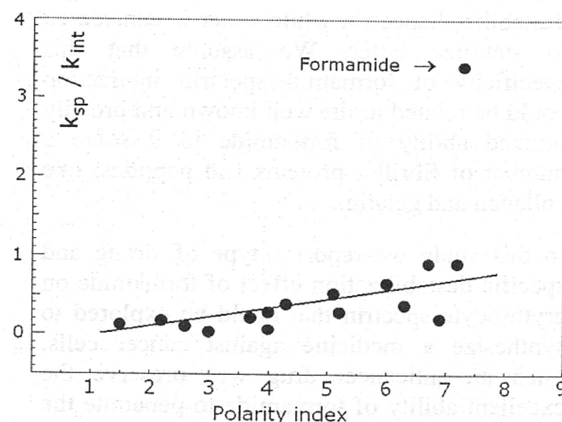

Figure 4. Effect of the polarity index, P′, of organic solvents on their k_{sp}/k_{int} ratio. Other details are as for Figure 2 and Figure 3.

The opposite happened on the other end of the polarity index scale. With increasing the polarity index to that of water (P′ = 9) the k_{sp}/k_{int} ratio linearly increased tending to appr. 1.0. This tendency reflects the increased capacity of polar solvents to interact directly with the undermembrane network of peripheral proteins, primarily spectrin, that are almost entirely exposed to the cytosole. This outcome is in line with the modern conception (4) that the same set of interactions as those between water and biomacromolecules, underlies the non-specific toxicity of highly polar organic solvents, used in cryopreservation solutions.

A strong exception from the linear k_{sp}/k_{int} dependency on P′ **(Figure 4)** is formamide as its k_{sp}/k_{int} ratio was about 3.4. On the other hand the strength of formamide to destabilize integral proteins, k_{int} ≈ 2.5 °C per mole was normal and, reportedly, coincides with the strength of this solvent to destabilize nucleic acids (17). Thus, on a molar basis formamide was about 4 times as effective in destabilizing spectrin as other solvents with similar polarity

index **(Figure 4)**. The reason for this exceptionally strong capacity of formamide to react and destabilize spectrin is not clear. This specificity towards spectrin is probably an example of a particular type of solvent/protein interaction. It could not be due to the high dielectric permeability, $\varepsilon_r = 109$, of formamide as other solvents, N-methylformamide with $\varepsilon_r = 175$ (-73 °C) and 2,6-difluoropyridine with $\varepsilon_r = 107.8$ displayed k_{sp}/k_{int} ratio in compliance with the overall dependence of k_{sp}/k_{int} ratio on P′ (Figure 4). On the other hand formamide has an extremely high hydrogen-bonding capacity, close to that of water. Nevertheless, it strongly destabilized spectrin while water is considered to stabilize latter. We assume that this specificity of formamide/spectrin interaction could be related to the well known and broadly utilized ability of formamide to dissolve a number of fibrillar proteins and peptides, like collagen and gelatin.

In this study we report a type of strong and specific destabilization effect of formamide on erythrocyte spectrin that could be explored to synthesize a medicine against cancer cells. Such an anticancer drug will preserve the excellent ability of formamide to penetrate the plasma membranes and produce specific destabilization effect on the undermembrane peripheral spectrin-like proteins of latter cells compromising their ability to survive and multiplicate.

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