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\( ^1 \text{H} \) NMR of compounds with low water solubility in the presence of erythrocytes: effects of emulsion phase separation

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Abstract When lipophilic compounds like diethyl phthalate (DEP) were added to water, two sets of resonances appeared in the \( ^1 \text{H} \) NMR spectrum, whereas when added in concentrations above \( \sim 3.5 \) mM to erythrocytes in a high haematocrit suspension, only one set of resonances was observed at the low-frequency position. The appearance of one set of resonances at lower frequency was found to be common to a series of lipophilic compounds in erythrocytes. The appearance of the NMR spectra is ascribed to the existence of an emulsion, meaning two different phases of a compound: a “droplet” (resonances to lower frequency) and aqueous dissolved phase (resonances to higher frequency). The absence of the resonances from the dissolved phase in erythrocyte solution is ascribed to exchange broadening. The absolute chemical shift of the compound in its “droplet” phase was also measured using a cylindrical/spherical microcell. This arrangement mimicked the geometry of the dissolved versus the phase-separated species and thus obviated the effect of a difference in magnetic susceptibility between the “droplet” solute and its aqueous solution. Factors influencing the formation of emulsion phases such as erythrocytes, haemoglobin and smaller proteins were investigated; they are found to be effective in the order given.

Key words Emulsion · Erythrocytes · \( ^1 \text{H} \) NMR · Phthalates · Magnetic susceptibility

Abbreviations BHT 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene) · DBP dibutyl phthalate · DEP diethyl phthalate · DMP dimethyl phthalate · DPP dipropyl phthalate · Hct haematocrit · HSA human serum albumin

Introduction

The physical form of drugs and xenobiotics in blood is of interest in many contexts (Kiehs et al. 1966; San George et al. 1984). \( ^1 \text{H} \) spin-echo NMR spectroscopy has been very useful in the study of transport, binding and metabolic transformation of compounds in erythrocytes (Brown et al. 1977; Rabenstein 1984; Guy et al. 1986; Rabenstein et al. 1988; Skibsted and Hansen 1990). In a previous study it was shown that the chemical shifts of compounds added to high haematocrit erythrocyte suspensions could be divided into three categories (Skibsted and Hansen 1990). One group, the water-insoluble compounds added to erythrocyte suspensions, showed low-frequency shifts of \( \sim 0.5 \) ppm in the \( ^1 \text{H} \) NMR spectra compared to organic solvents. The second and smaller group, the very water insoluble butylated hydroxytoluene (BHT) and dibutyl phthalate (DBP), showed the same chemical shift in erythrocytes or water, but a frequency shift compared to organic solvents. The third group, the water-soluble ones, showed no differences in shifts when data from erythrocytes, water and organic solvents were compared. The interesting feature of the chemical shift behaviour of the first group is that the shift changes from erythrocytes to organic solvent are virtually the same for all the resonances of the compounds (Skibsted and Hansen 1990). The present work was concerned mainly with the first group and grew out of the preliminary observation of a time-dependent change in resonance intensity when some of the compounds were added to human erythrocyte suspensions.

The series included the compounds dimethyl and diethyl phthalates (DMP, DEP), \( N \)-methylaniline and 3-methylstyrene (Skibsted and Hansen 1990). The
phthalates are especially interesting as di-n-butyl phthalates and the higher homologues are used as plasticizers and are known to leak from plastic used both for blood and food storage. The phthalates have been isolated from perfusion blood (Lovric et al. 1985) and from many types of food (Sharman et al. 1994) and are probably ubiquitous. The partly amphiphilic character of these compounds suggests the likely formation of micelles or emulsion droplets (Staples et al. 1997). The exchange of the compound between micelles is usually rapid and leads to an averaging of the chemical shifts of the resonances from the dissolved and the micellar species and to small changes in chemical shifts proportional to the amount of dissolved solute (Hansen and Mast 1976).

In the present work we investigated the physical state of several lipophilic phthalates (Fig. 1) using $^1$H NMR. The phthalates can be used as models for lipophilic drugs. The absorption and cellular distribution of lipophilic drugs is very important. Furthermore, the understanding of emulsion or micelle formation is central for understanding solubility data, interfacial behaviour or fate in the environment (Aveyard et al. 1994; Thomsen et al. 2000). Factors influencing emulsion droplet formation are likewise studied.

**Materials and methods**

**Compounds**

DMP was purchased from Fluka (Buchs, Switzerland), DEP from Aldrich (Weinheim, Germany), DBP from Merck (Darmstadt, Germany) and dipropyl phthalate (DPP) from TCI (Japan). The monomethyl ester was bought from Aldrich. All compounds were used without further purification.

**NMR**

$^1$H NMR spin-echo spectra were measured at 250 MHz using a Bruker AC 250 NMR spectrometer. The Hahn spin-echo pulse sequence (Hahn 1950) with $\tau = 68$ ms was used in combination with continuous pre-saturation of water for 1 s. The temperature was 310 K. A coaxial capillary with 0.75% TSP [sodium 3-(trimethylsilyl)propionate] in D$_2$O was normally used for field/frequency locking and as a chemical shift reference.

The organic compounds were dissolved in DMSO-$d_6$ (∼1 M) and 3–5 μL of this stock solution was added to the aqueous samples; vigorous mixing was achieved by repeated (∼20x) suction/expulsion steps with a glass Pasteur pipette.

Erythrocytes were centrifugally washed in 0.9% NaCl/D$_2$O and pelleted as described previously (Skibsted and Hansen 1990). Haemolysates were prepared in the NMR tubes by three freeze-thaw cycles. White erythrocyte ghosts were prepared as described previously using a hollow-fibre filtration procedure (Price et al. 1989).

![Fig. 1 Phthalates](image)

Chemical shift measurements using a spherical glass bulb insert with a capillary stem (Frei and Bernstein 1962; Chu et al. 1990) were carried out at 301 K and 400 MHz in a Bruker AMX 400 WB spectrometer.

**Results**

Several phthalate esters, that are only sparingly soluble in water, were added to erythrocyte suspensions of high haematocrit (> 75% w/v). The subsequent $^1$H NMR spectra showed one set of sharp resonances for each compound, but all resonances were shifted to high frequency compared with the solutions of the esters in organic solvents (Skibsted and Hansen 1990). However, at low haematocrit (11% w/v), both sets of resonances were seen (see Fig. 3c). Note also that for a high haematocrit (> 85% w/v) suspension of erythrocytes, no resonances from DEP were observed at all when the DEP concentration was below ∼3.5 mM.

Phthalates in D$_2$O gave rise to two sets of resonances separated by approximately 0.35 ppm. This was true not only for the four phthalate esters but also for $\alpha$-bromotoluene and 3-nitro-4-fluorotoluene (data not shown), when sufficient compound was added to make the mixtures appear milky. In this case, two different sets of resonances were observed, one at the low frequency described above, and one at the chemical shift found with organic solvents (the high-frequency resonance). For DEP, both sets of resonances were seen at concentrations above ∼5 mM, which is at the limit of its solubility; in other words, this is its “critical phase-separation concentration” (Fig. 2a and b). For DPP, this limit was shown to be significantly lower. The group of high-frequency resonances was ∼25% of the intensity of the normal (organic solvent or aqueous solution phase) set at a concentration of 5 mM. However, for DMP solutions in water the concentration had to be greater than 50 mM before the low-frequency resonances appeared. Most of these milky emulsions made with water or D$_2$O were not stable and phase separated over several minutes. This led to the disappearance of the low-frequency set of resonances, but not of the “normal” (high-frequency) set. The basis for this was shown to be the sedimentation (macroscopic/bulk phase separation) of the neat compound liquid out of the sensitive volume of the NMR spectrometer probe.

The chemical shifts of DMP, DEP, DPP and DBP in different solvents are given in Table 1. It can be seen that the differences between the chemical shifts of the neat compound and that in the presence of erythrocytes were close to 0.35 ppm for all the resonances. The same extent of shift was seen if a protein, for example human serum albumin (HSA), in a concentration larger than ∼0.3% (w/v) was added to a D$_2$O solution of DEP. With addition of HSA, less DEP was needed to bring about the low-frequency set of resonances. With a concentration of 10 mM DEP and 0.5% (w/v) of HSA, only the low-frequency resonances were seen. Other proteins showed a similar effect: poly-$\alpha$-tyrosine, which was