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^{1}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}H NMR spectrum, whereas when added in concentrations above ~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}H NMR · Phthalates · Magnetic susceptibility

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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}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 is 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 ~0.5 ppm in the ^{1}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 presaturation of water for 1 s. The temperature was 310 K. A coaxial capillary with 0.75% TSP [sodium 3-(trimethylsilyl)propionate] in D_2O 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 (~20×) suction/expulsion steps with a glass Pasteur pipette.

Erythrocytes were centrifugally washed in 0.9% NaCl/D_2O 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

Chemical shift measurements using a spherical glass bulb insert with a capillary stem (Frei and Bernstein1962; 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_2O 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 *o*-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_2O 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_2O 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-L-tyrosine, which was

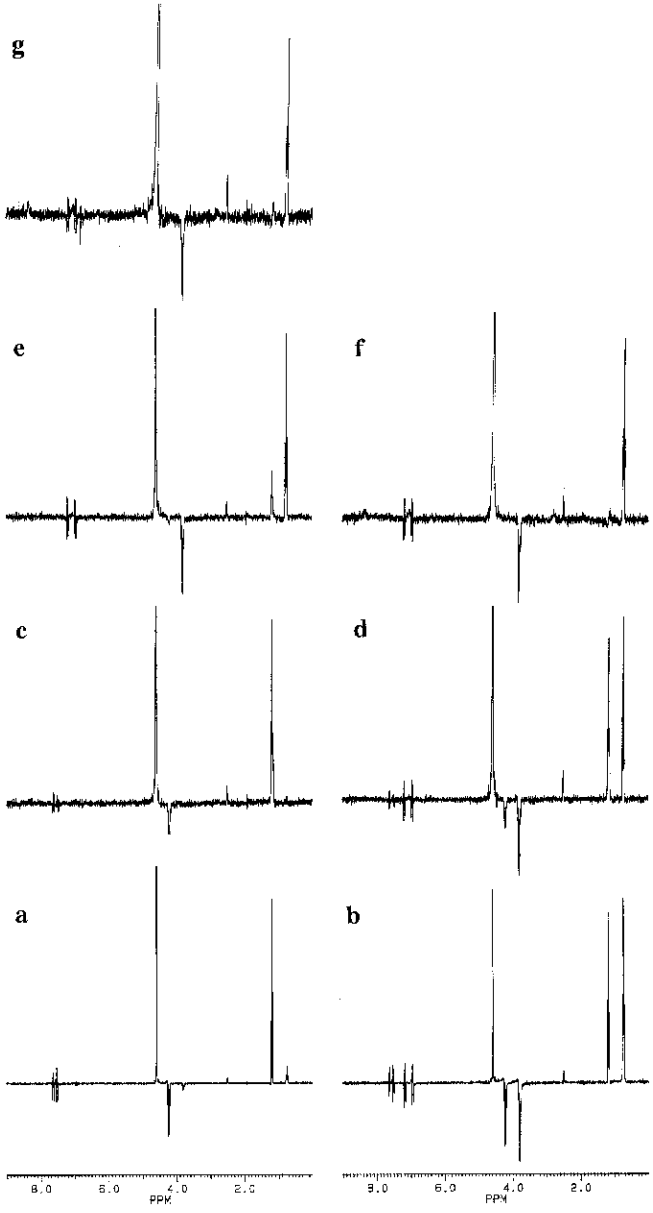

Fig. 2 ^{1}H NMR spin-echo spectra (250 MHz) of DEP: **a** 5 mM in D_2O; **b** 10 mM in D_2O; **c** 2.5 mM DEP in 0.3% (w/v) globin solution; **d** 5.0 mM DEP in 0.3% (w/v) globin solution; **e** 5 mM DEP in 0.5% (w/v) globin solution; **f** 5 mM DEP in 1% (w/v) globin solution; **g** the same solution after 5 days

slightly less effective in bringing about the low frequency resonances, and globin, which turned out to be slightly more effective, as seen in Fig. 2c–g.

DEP proved to be the most useful of the phthalate esters to explore the interactions of emulsions of the esters with cells and proteins. Thus, addition of DEP to freeze-thawed erythrocytes (haemolysate) revealed the same low-frequency ^{1}H NMR shift as for fresh erythrocytes and only the low-frequency (microemulsion, see Discussion) set could be observed. However, the low-frequency resonances slowly disappeared in time with no appearance of new resonances.

When DEP was added to white ghosts of erythrocytes, the ^{1}H NMR spectra appeared to be like those with the DEP in water, with both sets of resonances initially being visible.

To test the hypothesis that emulsion formation and stabilization was the basis of the two sets of resonances in the ^{1}H NMR spectra of erythrocyte suspensions containing the phthalate esters, experiments were performed with glass spherical microcells. A spherical insert of 4.5 mm outer diameter with a capillary stem was used (Frei and Bernstein 1962). For benzene the ^{1}H NMR signal from the compound in the spherical versus the stem compartment differed by 0.52 ± 0.01 ppm, with the resonances belonging to the spherical compartment being to low frequency when water was in the outer compartment (in the 5-mm NMR tube). For neat DEP the difference was 0.373 ± 0.002 ppm. For DEP saturated with water, a very similar separation of the resonances (0.394 ppm) was obtained. In an experiment with neat DEP in the spherical bulb and DEP emulsion in the outer tube, this range was again confirmed and the low-frequency set of resonances almost coincided with those of the spherical compartment.

One water-soluble compound, monomethyl phthalate, was also investigated. The resonances were observed at the same chemical shift as that of the DMSO-d_6 solution.

Discussion

The finding that all the observed low-frequency shifts were of comparable magnitude, irrespective of the specific molecular structure, and the finding that both aliphatic and aromatic resonances were subject to approximately the same shift, pointed towards a cause related to a bulk property. Furthermore, the finding that the phenomenon was observed in water solutions only as these became "milky" suggested that the low-frequency species could be related to the formation of an emulsion. An emulsion of a benzene-like molecule consists of spheres of diameter ~1–2 μm (Lawrence and Mills 1954). The spherical geometry is the basis for the low frequency (Frei and Bernstein 1962).

The concentration at which the compounds form emulsions in water is related to the length of their alkyl chain(s) and to their solubility. Thus the phthalates constitute a useful set of model compounds because their solubility and lipophilicity (expressed as log K_{OW}) vary systematically with ester chain length over a wide range.

Further evidence for the formation of an emulsion, as opposed to true micelles, came from the finding that no exchange occurred, on the NMR timescale, between the two nuclear populations giving the two sets of resonances. With increasing concentrations of solute, only an increase in the intensity of the low-frequency resonances occurred, and this did not lead to a continuous change in the chemical shifts as observed, for example, with micelles of cetyltrimethylammonium bromide (CTAB) (Eriksson and Gillberg 1966; Datema 1992;

Table 1 [1]H Chemical shifts[a] of phthalate esters in water, erythrocytes and organic solvents

Compound	δ H(ar)	δ H(ar)	δ OCH_x	δ CH_2	δ CH_3	Solvent
DMP	7.74	7.56	3.90	–	–	$CDCl_3$
	7.85	7.75	3.95	–	–	DMSO-d_6
	7.85	7.71	3.95	–	–	D_2O (solution phase)
	7.34	7.11	3.49	–	–	D_2O (droplet phase)[c]
	7.29	7.10	3.47	–	–	Erythrocytes[b]
	7.73	7.53	3.88	–	–	Neat
DEP	7.75	7.55	4.43	–	1.41	$CDCl_3$
	7.73	7.61	4.29	–	1.27	DMSO-d_6
	7.83	7.71	4.48	–	1.40	D_2O (solution phase)
	7.38	7.15	3.99	–	0.94	D_2O (droplet phase)[c]
	7.30	7.10	3.94	–	0.90	Erythrocytes[b]
	7.66	7.43	4.29	–	1.27	Neat
DPP	7.73	7.56	4.29	1.73	1.01	$CDCl_3$
	7.73	7.61	4.19	1.66	0.91	DMSO-d_6
	7.83	7.72	4.33	1.76	0.99	D_2O (solution phase)
	–[d]	–[d]	3.96	1.42	0.64	D_2O (droplet phase)[c]
DBP	7.70	7.50	4.30	1.71 1.43	0.95	$CDCl_3$
	7.71	7.63	4.22	1.62 1.35	1.35	DMSO-d_6
	7.49	7.29	4.09	1.22 ~1.5	0.70	D_2O
	7.43	7.21	4.00	1.19 1.44	0.62	Erythrocytes

[a] In ppm relative to TSP (water solutions and erythrocytes) and TMS in organic solvents
[b] High haematocrit >70%. At lower haematocrits a high-frequency set of resonances could also be seen
[c] Droplet phase refers to the emulsion droplets
[d] The aromatic resonances were not observed

Staples et al. 1997). Furthermore, saturation transfer experiments carried out by irradiating the methyl resonance of the dissolved DEP did not cause a decrease in the intensity of the low-frequency methyl resonance belonging to the species in the putative emulsion spheres. It was also characteristic of the water samples that they showed the high-frequency set of resonances alone if the sample was perfectly transparent; this could be brought about by heating the sample provided the ester concentration was not very high. Upon temperature increase the stability of the emulsion is deceased (Thomsen et al. 2000). Therefore, the high-frequency resonances seen with water solutions were assigned to the dissolved compound; the low-frequency sets were assigned to molecules in the emulsion spheres. In contrast, the spectra of the phthalate esters in erythrocyte suspensions (at high haematocrits) or in haemolysates showed only the low-frequency resonances. This phenomenon can also be explained in terms of emulsion formation, although in this case the milky colour cannot be observed. The emulsion formation is supported by the following observations: (1) identical chemical shifts of the emulsion droplets to the low-frequency set of resonances were obtained for emulsions in water; and (2) the set of resonances at low frequency did not shift with increased concentrations of esters and thus excluded micelles (see above); and (3) proteins are known to promote the formation of emulsions or, more correctly, to stabilize the phase separation as an emulsion (Dickinson 1992) (the occurrence of only one set of resonances is explained below). Alternatively, the low-frequency shift of the "droplet" resonances could have been caused by binding of the esters to macromolecules. However, the low-frequency set of resonances from D_2O mixtures and cells were both relatively narrow, thus suggesting that the compound is not strongly immobilized by binding to large macromolecules or parts of the membrane of the cells. This was supported by addition of white ghosts or membrane fragments, which did not promote emergence of the low-frequency-shifted resonances. In addition, no shift in the position of the resonances was observed with increasing concentrations of proteins. Therefore, the shift effect (occurrence of a resonance at a new position) was most likely due to formation of the emulsion "droplets" that were stabilized by the proteins.

The reason that no resonances at all are seen for DEP at concentrations below ~3.5 mM in erythrocytes is thought to be as follows. The low-frequency-shifted resonance is not present because no emulsion is formed at this low concentration and the high-frequency resonance belonging to dissolved DEP is absent because of fast exchange of the free esters with weak binding sites on large and possibly paramagnetic proteins, particularly haemoglobin. An alternative explanation for the missing high-frequency resonance at high haematocrit could be that the extracellular volume is so small that the dissolved amount of compound escapes detection. Both suggestions are supported by the finding that both sets of resonances are observed in D_2O, or in solutions with low haematocrits (Fig. 3c, d) or when only white ghosts or membrane fragments are added. In favour of

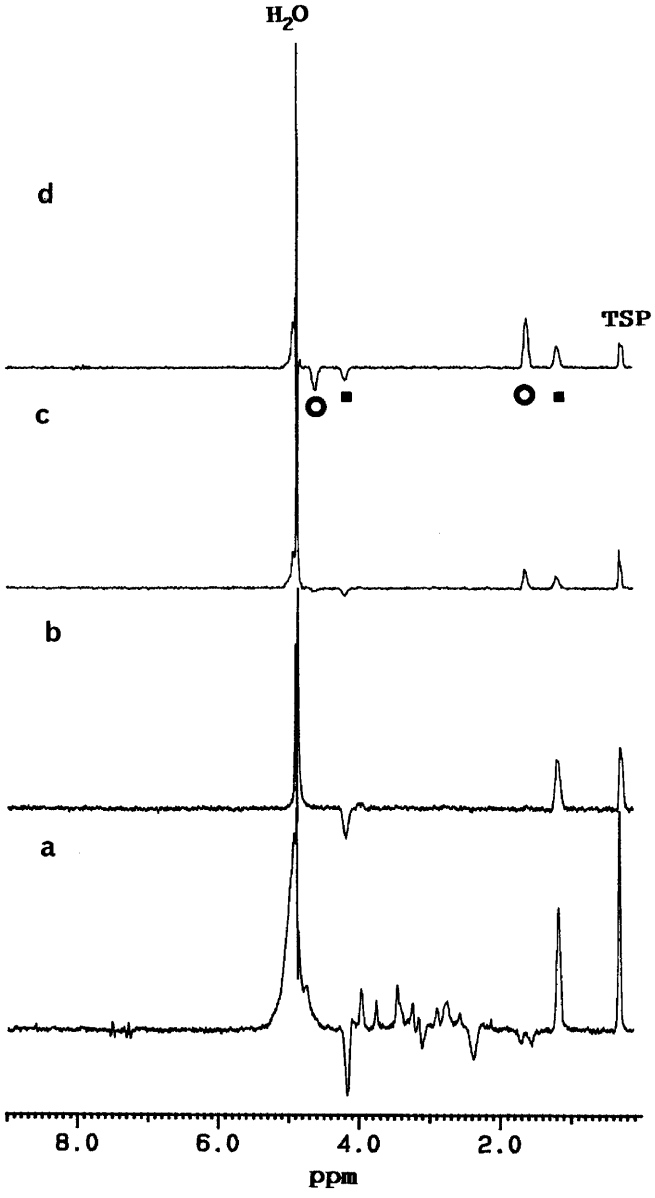

Fig. 3 ^{1}H NMR spin-echo spectra (250 MHz) of DEP in a suspension of erythrocytes: **a** 10 mM DEP in a suspension of erythrocytes (Hct = 0.9); resonances from DEP as well as erythrocytes are seen; those of DEP [CH_3, CH_2 and CH (arom)] are marked with *squares* for the droplet form and with *circles* for the dissolved form; **b** Hct = 0.4; **c** Hct = 0.11; **d** Hct = 0.04; H_2O marks the residual water and TSP is the reference resonance

the first suggestion is the finding that, at concentrations above ~3.5 mM DEP, only the low-frequency resonance is seen in solutions with haemolysed erythrocytes. In this case the amount of solvent is much larger than for erythrocyte suspensions.

Summary

Various types of chemical shift behaviour of phthalate esters and two benzene derivatives, with low water solubility, could to be accounted for as follows:

1. For DEP and similar compounds, the low-frequency set of resonances seen with erythrocyte suspensions of high haematocrit was due to the presence of a "droplet" phase.
2. BHT and DBP are so water insoluble that they only form the "droplet" phase with little free solute in the aqueous phase. This is also true for benzene in a microemulsion in water (Datema 1992). Even in erythrocyte suspensions of low haematocrit, this was the case. Thus, the resonances in water were of the low-frequency type and no chemical shift was seen on addition to erythrocytes, whereas a shift was evident when organic solvents were used.
3. For the compound with the highest water solubility (DMP), only one unshifted resonance relative to organic solvents was seen on addition (< 50 mM concentrations) to erythrocytes.

In view of these findings we are able to predict the behaviour of the emulsion-dependent shift effect for all lipophilic compounds added to water or to erythrocytes. In addition, similar shifts are observed for ^{19}F chemical shifts of fluorocarbons in water or in erythrocytes (Hansen PE, Blom K, Grauert C, unpublished results). Understanding magnetic susceptibility effects is of great importance in the interpretation of magnetic imaging pictures (Mahallati et al. 1999). It is also relevant to note that a similar shift has been observed in ^{1}H NMR spectra of methyl resonances of globular proteins, after correction for ring current effects (Feeney J, personal communication); so this can possibly be interpreted as an emulsion-phase-like effect similar to that which has been reported above.

Erythrocytes, haemoglobin and globin turned out to be very good emulsion promoters, human serum albumin is good whereas polylysine and BPTI were less effective.

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