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STUDY OF THE DERIVATIZATION OF *n*-ALKYLAMINES WITH 1-FLUORO-2,4-DINITROBENZENE IN THE PRESENCE OF AQUEOUS CETYLTRIMETHYLAMMONIUM BROMIDE MICELLES

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SUMMARY

The use of aqueous cetyltrimethylammonium bromide micelles in the derivatization of *n*-alkylamines with 1-fluoro-2,4-dinitrobenzene was investigated systematically. The rate constants of derivatization of the *n*-alkylamines (C_1–C_8) were analysed using liquid chromatography. Up to butylamine the micellar rate enhancement depends on the electrostatic interactions between the amines and cetyltrimethylammonium bromide, and beyond C_4 it depends mainly on the hydrophobic interactions. The reaction rates are also enhanced by a micelle-induced decrease of the pK_a of the amines, but to a lesser extent. The derivatization rates for the longer alkylamines are comparable with those in dipolar aprotic solvents. Pharmaceutical and biomedical science is likely to benefit from the use of micellar systems in pre-column derivatization reactions in aqueous solutions.

INTRODUCTION

In pharmaceutical and biomedical analysis, derivatization reactions are often required to improve the chromatographic properties and the detectability of drugs and their metabolites. The reaction is generally performed in aprotic solvents to increase the rate of derivatization. Because of the incompatibility of such reactions with water, the drug under investigation has to be extracted from the aqueous matrix, e.g. plasma and urine. Derivatization of the analyte in an aqueous micellar system is likely to be more advantageous than conventional techniques involving time-consuming extraction procedures.

An example of a derivatization that is enhanced by the presence of micelles is the reaction of 1-chloro-2,4-dinitrobenzene (DNClB) with amines in the presence of cetyltrimethylammonium bromide (CTAB) micelles [1].

A micellar system can affect chemical reactions in three different ways [2–13].

(A) The molecules involved in the reaction become concentrated in the small

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volume of the micellar "pseudo phase", which will result in a higher reaction probability [2]. In the case of ionic micelles it is generally assumed that polar molecules are concentrated in the so-called Stern layer of the micelle [2]. This layer consists of hydrated polar head-groups of the surfactant and ca. 60% of the counterions. Apolar molecules are distributed in the core of the micelle, which may be more or less deprived of water [3–6]. The physicochemical properties of this core approach those of hydrocarbons [7].

(B) The micelles alter the micro-environment of the molecules present in the micelle by causing changes in the dielectric constant [8] and viscosity [9] in the micelle, and the breakdown of the hydration shell of the solubilized molecules [10]. The mutual orientation of the molecules in the micelle can, owing to electrostatic and hydrophobic interactions, also enhance or inhibit the rate [2], or modify the order [11] of the reaction.

(C) The rate of reaction can be affected by the presence of a surface potential over the Stern layer of ionic surfactants. This surface potential, which is ca. ± 140 mV [8], has been reported to alter the pK_a values of amines and carboxylic acids by up to a few units [8,12,13].

Another interesting effect of micelles is their ability to solubilize molecules better than homogeneous solvents. This is because a micellar system has a wide variety of solubilization sites [14].

Although it is clear that micelles are a promising tool in the derivatization of drugs in aqueous solutions, it is remarkable that there are only a few reports on the application of micelles in pharmaceutical and biomedical analysis [15,16].

The aim of the present investigation is to gain a better insight into the possible application of aqueous micellar systems in the derivatization of drugs and metabolites under analytical conditions. With micellar systems it should be possible to circumvent tedious extraction procedures. To gain a better understanding of the underlying mechanisms of micellar catalysis, a simple test system was chosen, namely the reaction between *n*-alkylamines and a UV label, 1-fluoro-2,4-dinitrobenzene (DNFB), which was present in excess. The reaction mechanisms of DNFB with nucleophiles in aprotic solvents have been described extensively [17–19].

CTAB was used as a micellar system because it has been shown that cationic micelles can enhance the rate of the reaction of primary and secondary amines with DNFB [1]. The reaction rate is not increased by anionic and non-ionic surfactants. Under the derivatization conditions, DNFB can hydrolyse significantly to give 2,4-dinitrophenol (DNPhOH), which interferes strongly with the UV absorption of the N-alkyl-2,4-dinitroaniline (DNP) derivatives, if analysed batchwise. Therefore it was necessary to carry out a high-performance liquid chromatographic (HPLC) separation before UV detection, so that the separate kinetics of DNFB, DNPhOH and the DNP derivatives could be measured simultaneously. Factors that influence the derivatization reaction in a micellar system were investigated.

EXPERIMENTAL

Chemicals and solutions

Millipore water (Milford, MA, U.S.A.) was used throughout this study. The chemicals were of p.a. grade (Merck, Darmstadt, F.R.G.) except dioxane, which was "reinst". The methyl-, *n*-propyl-, *n*-butyl-, *n*-pentyl-, *n*-hexyl- and *n*-octyl amine (stored under nitrogen throughout) were obtained from Aldrich (Beerse, Belgium). All chemicals were used as obtained.

The DNFB reagent was dissolved in acetone at a concentration of 400 m*M* and stored at 4 °C. The *n*-alkylamines were dissolved in water at a concentration of 50 m*M*. These stock solutions were prepared afresh every week and stored at 4 °C. The standards of the N-alkyl-2,4-dinitroaniline derivatives were dissolved in acetone at a concentration of 50 m*M* and stored at 4 °C. In the case of the DNPhOH standards, known amounts of hydrolysed DNFB (checked by HPLC) were used.

Synthesis of the N-alkyl-2,4-dinitroaniline derivatives

The N-alkyl-2,4-dinitroanilines (DNP-alkylamines) were synthesized, but with some modifications, according to the method described by Day et al. [20]. Ca. 5 mmol of the alkylamine and 10 mmol of DNFB were dissolved in 20 ml of a mixture of a 33 m*M* borate buffer (pH 10.5) and dimethylsulphoxide (80:20, v/v). The mixture was stirred and heated to 40 °C. After the reaction was complete, residual DNFB was hydrolysed at pH 11.5. After cooling to 4 °C the precipitates (the octylamine derivative proved to be a viscous oil) were filtered and washed with 0.05 *M* sodium carbonate at 4 °C. The derivatives were dried under reduced pressure (10 mmHg), subsequently dissolved in acetone and filtered through a 0.2-μm SM 11607 cellulose filter (Sartorius, Goettingen, F.R.G.). The methyl, propyl and butyl derivatives were recrystallized twice from a diethyl ether–cyclohexane (50:50, v/v) mixture, and an ethanol–water (70:30, v/v) mixture was used in the case of the pentyl and hexyl derivatives. The derivatives were dried over phosphorus pentoxide (yield ca. 65%). The identity and purity of the products were verified by standard HPLC analysis and mass spectrometry (MS) with an MS 80 gas chromatographic–mass spectrometric (GC–MS) system (Kratos, Ramsey, NJ, U.S.A.) using a CP Sil 5 BP capillary column (25 m; Chrompack Nederland, Middelburg, The Netherlands).

Incubation procedures

The kinetics of the reaction of the *n*-alkylamines with excess DNFB in the absence and in the presence of CTAB micelles were investigated under various conditions: temperature (10–50 °C), pH (7–12.5), type of *n*-alkylamine (C_1–C_8) and concentration of reactant (0.5–8 m*M*), surfactant (0–133 m*M*) and substrate (10–500 μ*M*).

All the incubations were performed in a 33 m*M* sodium tetraborate buffer. The required amount of CTAB was dissolved in a 66 m*M* sodium tetraborate buffer by sonication for 5 min. The pH was adjusted by the addition of 1 *M* sodium hydroxide or 1 *M* hydrochloric acid. Of this CTAB stock solution, 10 ml, were transferred to a 25-ml stoppered flask. After the addition of the *n*-alkylamine,

water was added to a total volume of 20.0 ml. After the solution had been mixed, the incubation was started by the addition of the DNFB reagent (maximum 350 μl).

For the incubations in hexane, acetone and 16.5 m*M* tetramethylammonium bromide (TMeABr, in 33 m*M* borate buffer, pH 10.6), concentrations of 8 m*M* DNFB and 50 μM of propyl-, pentyl- and octylamine were used.

Unless stated otherwise the temperature was 20 ± 1 °C.

The samples for HPLC analysis were prepared according to a method described by Connors and Wong [16]. During the incubation, 500-μl aliquots were taken and transferred to a 1.5-ml stoppered polypropylene reaction vessel (Eppendorf, Hamburg, F.R.G.) containing 500 μl of a mixture of dioxane and concentrated hydrochloric acid (99:1, v/v). The acidified samples were stored at −20 °C until they were analysed by HPLC.

Chromatographic system

Of the acidified incubation samples up to 20 μl were analysed by a fully automated HPLC system, using an RP 18 column (30 cm × 4.6 mm I.D., 10 μm LiChrosorb; Merck, Darmstadt, F.R.G.). The solvent-delivery system consisted of two M 6000 A pumps and an automated gradient controller (all from Waters Assoc., Milford, MA, U.S.A.), controlled by a Model 231 automatic sampler injector (Gilson, Villiers le Bel, France). A Waters 440 absorbance detector equipped with a 365-nm filter was used. Retention times and peak areas were measured by an SP 4270 integrator (Spectra Physics, Santa Clara, CA, U.S.A.). The initial mobile phase of the gradient was methanol–10 m*M* phosphate buffer pH 7.0 (30:70, v/v). The final state of 100% methanol was reached linearly in 4 min and prolonged for 3 min.

All solvents were filtered through a 0.2-μm filter and deaerated ultrasonically prior to use.

Data analysis

The pseudo-first-order rate constant k of the derivatization of the n-alkylamines with DNFB and the hydrolysis of DNFB into DNPhOH was calculated using eqn. 1 [21]:

$$-\ln[I^{\star} - I(t)] + \ln I^{\star} = kt \quad (1)$$

where $I(t)$ is the peak area of the derivative or DNPhOH ($\lambda = 365$ nm) at incubation time t, and $I^{\star}$ is the peak area after complete conversion of the parent compound (n-alkylamine or DNFB). The rate constants depicted as k(init) represent the observed pseudo-first-order rate constant, calculated from data from the time-interval (usually two to five half-times) where the reaction obeys first-order kinetics.

Solubilization experiments

Solubilization experiments were carried out to determine the amount of DNFB and DNPhOH that can be solubilized by CTAB micelles. The solubilization data of DNFB and DNPhOH at 20 °C were obtained by adding an excess of these substrates to a 33 m*M* borate buffer (pH 7.5), which contained various amounts of CTAB (0–150 m*M*). The solutions were stirred continuously, and after 24 h triplicate samples were taken for analysis by HPLC.

Determination of pK_a values

The influence of CTAB and a non-ionic surfactant (Brij 56) on the pK_a values of the *n*-alkylamines was determined at 21°C with a 636 Titroprocessor (Metrohm, Herisau, Switzerland). A solution of 25 m*M* *n*-alkylamine hydrochloride in water, 100 m*M* CTAB or 1% Brij 56 was titrated with 1 *M* sodium hydroxide. The pK_a was determined using a standard titration program of the titroprocessor.

NMR experiments

To obtain more insight into the solubilization mechanisms of DNFB and DNPhOH in CTAB micelles, 90 MHz ^{1}H NMR measurements were made at 36°C, according to ref. 22. An EM 390 ^{1}H NMR spectrometer (Varian, Palo Alto, CA, U.S.A.) was used, equipped with a 5740 frequency counter (Data Precision, Wakefield, MA, U.S.A.). DNFB or DNPhOH was titrated into a 33 m*M* borate buffer (pH 8.0) in the absence or in the presence of 150 m*M* CTAB or into hexane. All resonance line positions of CTAB and solubilisate hydrogen atoms were measured relative to tetramethylsilane (TMS) as an external standard. The reported chemical shifts are average values of three measurements.

RESULTS AND DISCUSSION

Chromatographic conditions

The acidified incubation samples are stable for at least one month if stored at −20°C. The DNP derivatives are stable at room temperature for at least 12 h in a 33 m*M* CTAB solution at pH 11.2. Within the tested concentration ranges of 0.1–8 m*M* for DNFB and DNPhOH and 5–500 μM for the DNP derivatives, linear calibration curves ($r=0.999$) were obtained that passed through the origin. A typical chromatogram is shown in Fig. 1.

Kinetic studies

Reaction mechanism. The reaction mechanism of DNFB with primary and secondary amines in aprotic solvents is an aromatic nucleophilic substitution, Sn2(ar) [16]. After the amine has attacked the carbon atom, the reaction proceeds via a so-called Meisenheimer complex (Fig. 2) [16]. Depending on the leaving group and the solvent, the breakdown of this complex may or may not be catalysed by the presence of bases [23].

The apparent rate and the yield of a derivatization are of interest from an analytical point of view. Therefore the influences of several factors on the rate and yield of the derivatization were investigated and are discussed below.

Influence of reagent. Various amounts of DNFB were added (0.5, 1.0, 2.0, 4.0 or 8.0 m*M*) to a 33 m*M* borate buffer (pH 10.6) which contained 50 μM propylamine and 33 m*M* CTAB. In all cases initial pseudo-first-order rate constants ($\pm$S.D.) were found of 3.2 ($\pm$0.1)·10^{-3} s^{-1}. During the incubation, DNFB is hydrolysed to DNPhOH. The depletion of the DNFB might negatively influence the yield and rate of derivatization. The yield of derivatization was calculated from the above experiments (Table I), and depends strongly on the excess of

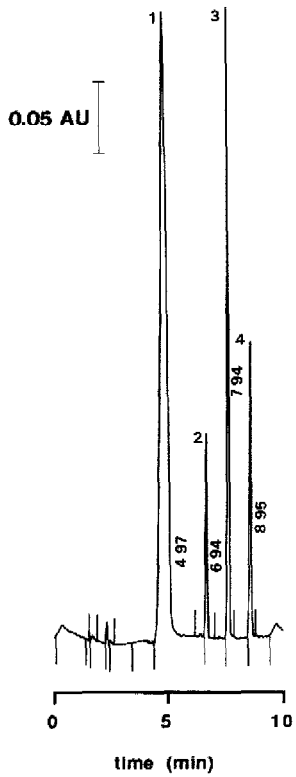

Fig. 1. Chromatogram of 20 μl of an acidified incubation sample (λ=365 nm). Peaks: 1=4 mM DNPhOH; 2=4 mM DNFB; 3=100 μM propyl-DNP derivative; 4=50 μM octyl-DNP derivative.

DNFB present. For this reason a DNFB concentration of 8 mM was used in further experiments.

Influence of CTAB. To examine the influence of the CTAB concentration on the reaction rate, 50 μM propyl- and hexylamine were incubated in the presence of various amounts of CTAB at pH 10.9. The relation between the rate constants of derivatization and the CTAB concentration is depicted in Fig. 3. Similar profiles were found by other authors [1,24,25]. Two features are of interest. First,

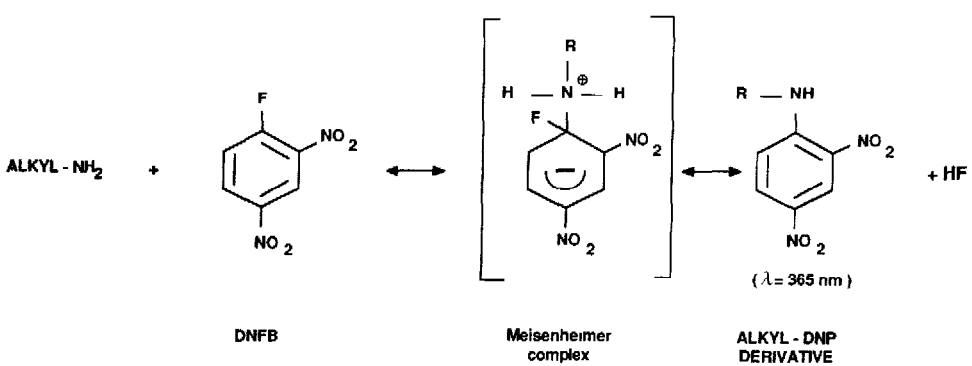

Fig. 2. Reaction mechanism of DNFB with primary and secondary amines.

TABLE I

YIELD OF DERIVATIZATION AS A FUNCTION OF THE REAGENT CONCENTRATION AT pH 10.6 AFTER 60 MIN

DNFB concentration (m*M*)	Recovery (%)
0.5	18.2
1.0	39.9
2.0	68.8
4.0	83.9
8.0	99.3

the increase in the reaction rate occurs near the critical micelle concentration (CMC) of CTAB ($3.2 \cdot 10^{-4}$ *M* [24]). It is therefore reasonable to assume that the rate enhancement is related to the presence of micelles and not the presence of surfactant monomers. Secondly, after reaching an optimum the rate constants decrease at a concentration higher than ca. 35 m*M* CTAB. The explanation for this is that the substrates become more diluted in the micellar pseudo-phase with an increasing micelle concentration, resulting in a lower reaction probability. The optimum of the derivatization rate of hexylamine is more pronounced than that of propylamine. This could be due to the fact that hexylamine is better solubilized by micelles than is propylamine. Consequently, the effect of dilution of the micellar pseudo-phase will be more pronounced.

Influence of pH. In aqueous solutions only the deprotonated amines can react with DNFB [26]. The pK_a value of *n*-alkylamines in water (10.6) is independent of the alkyl moiety [27]. Micelles influence the pK_a of amines [8,12,13]. For this reason the influence of the pH of the medium was studied, in the absence or in the presence of 33 m*M* CTAB, on the reaction rates of 50 μM propyl- and octyl-

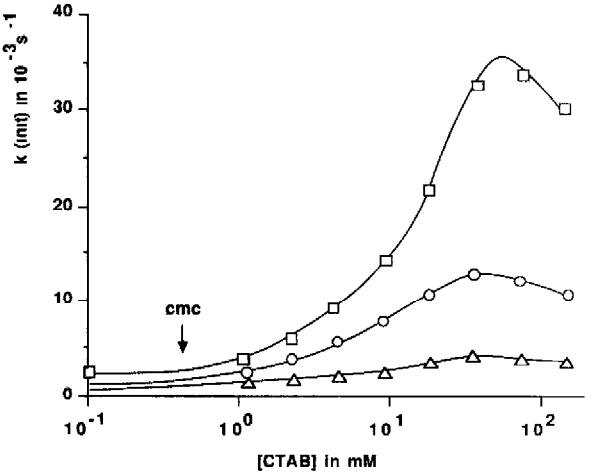

Fig. 3. Influence of the CTAB concentration on the initial rate constants, *k*(init), of the derivatization of 50 μM hexylamine (□) and 50 μM propylamine (△) and the hydrolysis of 8 m*M* DNFB (○) at pH 10.9 and 20°C.

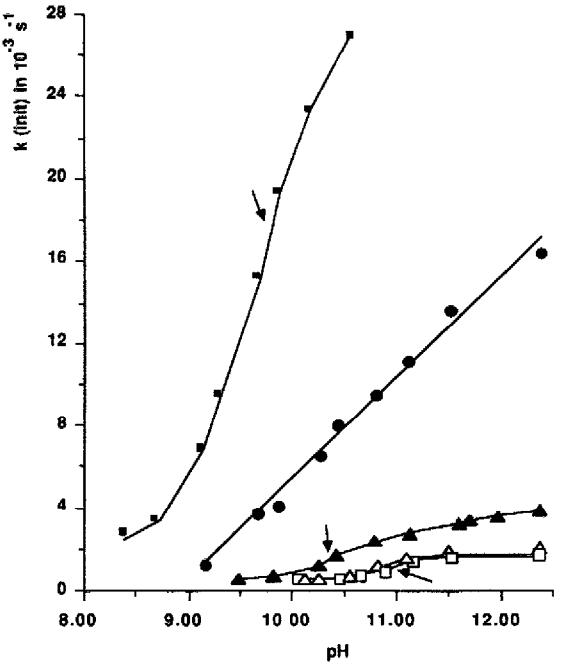

Fig. 4. Influence of the pH at 20°C on the hydrolysis of 8 m*M* DNFB (●) and the derivatization rate of 50 μM octylamine (■) and 50 μM propylamine (▲) in the absence (open symbols) and the presence (filled symbols) of 33 m*M* CTAB. The arrows indicate the apparent pK_a values of the amines under the given conditions.

amine with 8 m*M* DNFB (Fig. 4). The pH measured with a glass electrode was not significantly influenced by the presence of CTAB [28]. In the absence of micelles the rate profile of octylamine is identical with that of propylamine. The pK_a value of 10.8 derived from the point of inflection of these plots is in reasonable agreement with those found in the literature [27].

In the presence of 33 m*M* CTAB the pH profiles of the reaction rates of propyl- and octylamine are different. First, the apparent pK_a of octylamine shifts to ca. 9.7, while that of propylamine decreases to 10.3. This indicates that the micelle-induced pK_a shift of the amine depends on the length of the alkyl chain. Secondly, the rate constants do not reach a plateau at higher pH. One would expect them to do so if the reaction rate depends only on the degree of deprotonation of the amines. Therefore it is concluded that, in the presence of micelles, the hydroxide ion acts as a base catalyst.

In addition, Fig. 4 shows that hydrolysis of DNFB to DNPhOH is prominent at high pH. This, together with the fact that the derivatizations were incomplete above pH ca. 11.3, indicates that it is preferable to carry out the derivatization at lower pH.

Influence of the substrate concentration. The influence of the substrate concentration on the derivatization rate was investigated at pH 10.9, in the presence of 16.5 m*M* CTAB and 8 m*M* DNFB. At concentrations of 10, 50, 100 and 500 μM propylamine, identical pseudo-first-order rate constants of 2.19 (± 0.12)·10^{-3}

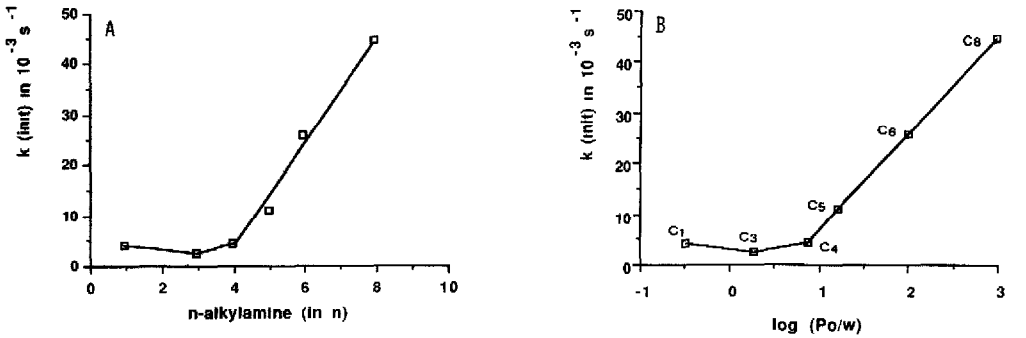

Fig. 5. (A) Dependence of the derivatization rate on the chain length (n) of 50 μM n-alkylamines in the presence of 8 mM DNFB and 33 mM CTAB at pH 10.9 and 20°C. (B) Rate constants of n-alkylamines as a function of the octanol-water partition coefficient log ($P_{o/w}$).

s^{-1} were found in the presence of CTAB. In a 33 mM borate buffer, which was saturated with DNFB, the rate constants were 0.30 (± 0.07) $\cdot 10^{-3}\,s^{-1}$. Therefore in both cases the rate constants appeared to be independent of the substrate concentration.

Influence of chain length. From Figs. 3 and 4 it can be concluded that the alkyl chain of the amine has an important modulating effect on the reaction rate in the presence of micelles. This effect was therefore studied in more detail. First the effect of the hydrophobicity of the n-alkylamine on the reaction rate was examined in the presence of 33 mM CTAB, 8 mM DNFB and 50 μM n-alkylamine. To exclude possible interference due to a different degree of deprotonation of the amines, these experiments were carried out at pH 10.9. As is depicted in Fig. 5A, the rate constants increase linearly with the chain length beyond butylamine, whereas for shorter chain lengths no influence of the chain length on the reaction rate can be seen. This behaviour could be related to the partitioning of the amine between buffer and CTAB micelles.

It would be difficult to determine the partition coefficients for these substrates in a micellar solution, so instead the partition coefficients between n-octanol and water ($P_{o/w}$) were used (Fig. 5B) [29]. These partition coefficients have been tabulated for many drugs [30].

From Fig. 5B it can be seen that beyond n-butylamine the derivatization rates are linearly related to log($P_{o/w}$), and therefore to the hydrophobicity of the substrates. With the short-chain n-alkylamines there is apparently no hydrophobic contribution to the rate-enhancing effect of the micelle. This conclusion is supported by the fact that it has been reported that alkanols as short as propanol do not penetrate the micelle core. This in contrast to the longer alkanols [31]. The fact that amines are more hydrophilic than the alkanols [32] could explain why a hydrophobic interaction can only be seen beyond n-butylamine.

The positive deviation from this relation with the hydrophobicity that can be seen with the short alkylamines could be explained by the presence of an electrostatic interaction between the amines and the Stern layer of the micelle.

Not only was the direct influence of hydrophobicity of the amines on the derivatization rate studied, but also the influence of the chain length on the micelle-

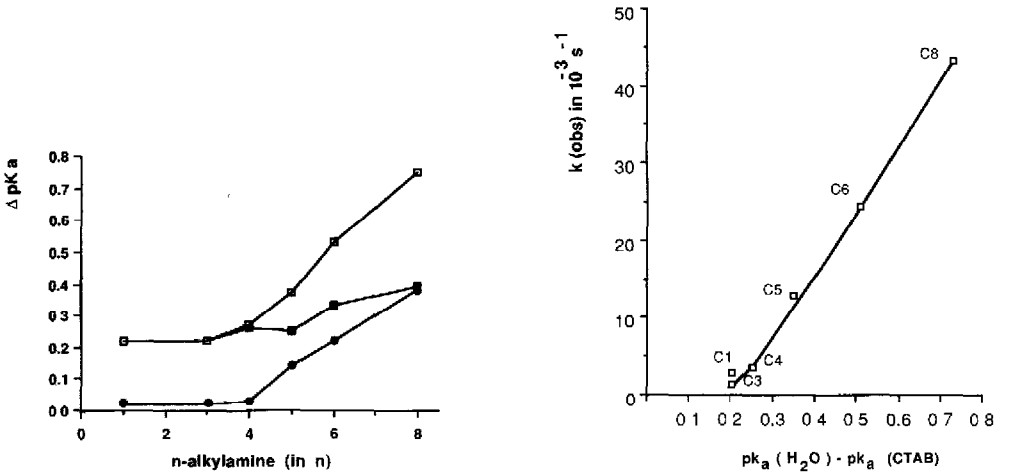

Fig. 6. The pK_a shift of *n*-alkylamines (25 m*M*) induced by the presence of 100 m*M* CTAB (□) and 1% Brij 56 (●), compared with those in aqueous solutions, and the difference in the pK_a shifts induced by CTAB and Brij 56 (■).

Fig. 7. Comparison between the pK_a shift of *n*-alkylamines and the observed rate constants (k) in the presence of CTAB micelles.

induced pK_a shift. The pK_a values of the hydrochloride salts of the *n*-alkylamines at a concentration of 25 m*M* were determined in the presence of 100 m*M* CTAB or 1% Brij 56 and compared with those determined in water. In the presence of CTAB, a constant shift of 0.2–0.3 units in the pK_a values were observed with the shorter *n*-alkylamines, whereas beyond butylamine the induced pK_a shift increased linearly with the chain length of the amine (Fig. 6). When a non-ionic surfactant (Brij 56) was used, an identical relation was found, only without the constant bias of ca. 0.25 pH units that was found with CTAB micelles (Fig. 6). This bias is therefore apparently induced by the surface potential of the CTAB micelle [8,12,13].

These results also support the previous conclusion that the hydrophobic interactions between the *n*-alkylamines and CTAB become significant only beyond *n*-butylamine.

The titrimetrically determined pK_a values of propylamine and octylamine are 10.4 and 9.8, respectively, in the presence of CTAB (Fig. 6). The pK_a values calculated from the pH dependence of the derivatization of propylamine and octylamine are 10.3 and 9.7, respectively, in the presence of the same micelles (Fig. 4). This similarity indicates that in the presence of micelles also only the deprotonated form of the amine can be derivatized. Furthermore, a linear relation can be seen between the pK_a shift and the derivatization rate of an *n*-alkylamine (Fig. 7).

From the above results it can be concluded that the hydrophobicity of the *n*-alkylamine is (directly or via the micelle-induced pK_a shift) a major factor that influences the derivatization rate of *n*-alkylamines in micellar solutions, via a

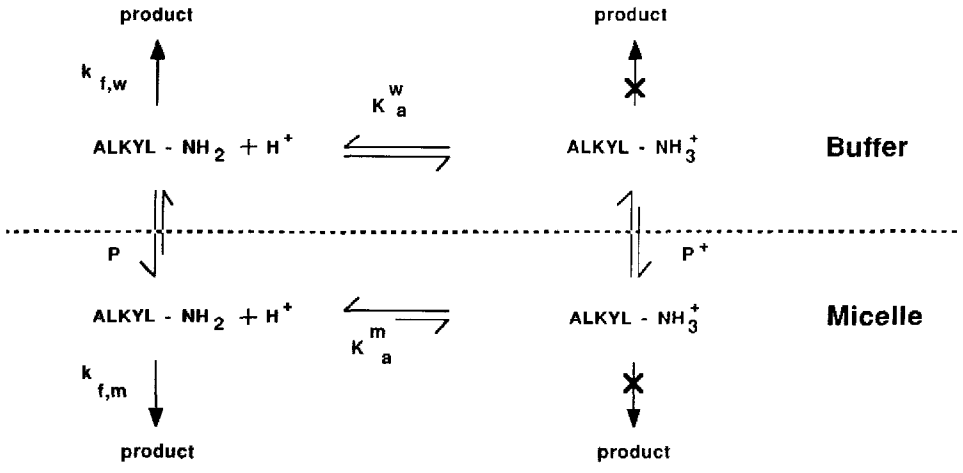

Fig. 8. Equilibrium system in micelle and buffer.

complex system of equilibria (Fig. 8). The degree of protonation of the amine in the micelle (K_a^m) and buffer (K_a^w), and the partition coefficients (P^+ and P) of the protonated (BH^+) and deprotonated (B) amine species between the micellar pseudo-phase and buffer, will affect the derivatization rate. The rate of hydrolysis has been found to be much greater than the partition rates of the amines [33,34].

Finally, the ratio of the micellar ($k_{f,m}$) and the aqueous ($k_{f,w}$) reaction rates will have a strong influence on the observed reaction rate. The latter two constants will also depend on the partition behaviour of the reagent.

From Fig. 8 it can be concluded that the equilibria within the micellar system favour the formation of deprotonated alkylamine species in the micellar pseudo-phase. This factor, together with changes in the micro-environment of the amine, will accelerate the derivatization within a micellar system.

Comparison with other systems. Some properties of the structural elements of a micelle, such as the alkane-like core and the cationic Stern layer, could be more or less mimicked by hexane and an aqueous buffer containing TMeABr, respectively. The pseudo-first-order rate constants of the derivatization of 50 μM propyl-, pentyl- and octylamine with 8 mM DNFB in hexane or a 16.5 mM TMeABr solution at pH 10.6 are given in Table II. From this table it can be concluded that

TABLE II

INFLUENCE OF THE SOLVENT ON THE DERIVATIZATION RATE

Values are k(init) in 10^{-3} s^{-1}.

Amine	Acetone	Hexane	Buffer	16.5 mM TMeABr	33 mM CTAB	Ratio CTAB/acetone
C_3	37.1	0.84	0.26	0.28	2.7	0.07
C_5	36.4	0.83	0.27	0.25	10.6	0.29
C_8	40.2	0.81	0.26	0.26	42.0	1.04

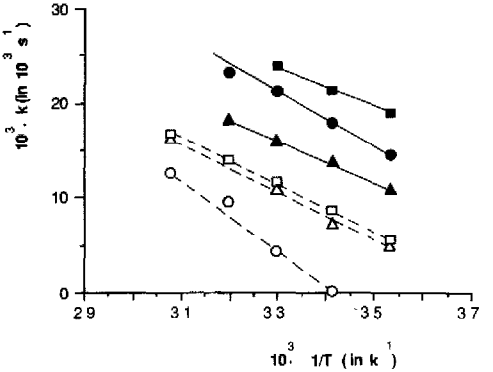

Fig. 9. Arrhenius plots of the hydrolysis of 8 m*M* DNFB (○, ●) and the derivatization of 50 μM propylamine (△, ▲) and 50 μM hexylamine (□, ■) in the absence (open symbols) and the presence (filled symbols) of 33 m*M* CTAB.

the presence of the Stern layer is essential for the catalytic effect of cationic micelles. This could be explained on the assumption that the charged Stern layer can stabilize the transition state, whereas the alkane-like core cannot.

The lack of a catalytic effect of TMeABr could be attributed to the lack of a pseudo-phase in which the substrates can be concentrated. Furthermore, the enhancement of the derivatization rate is not due to the presence of monomeric tetraalkylammonium compounds. This was also concluded from Fig. 3, where CTAB was used.

From an analytical point of view it is of interest to compare (Table II) the micelle-enhanced reactions with those in the frequently used aprotic solvents. From Table II it can be concluded that the derivatization rates of the longer *n*-alkylamines are comparable with those in aprotic solvents. This means that the application of micellar systems in the derivatization of the more hydrophobic amines is competitive with aprotic systems.

Influence of the temperature. The influence of the temperature on the reaction rate of 8 m*M* DNFB with 50 μM of propyl- or hexylamine in the absence or in the presence of 33 m*M* CTAB at pH 10.6 was investigated. The results are shown as Arrhenius plots (Fig. 9). The derivatization yield of the *n*-alkylamines after 45 min was ca. 100% at all elevated temperatures. Therefore it is possible to perform derivatizations in the micellar system at elevated temperatures. Not only are higher derivatization rates obtained, but also the viscosity of the micellar solutions is decreased at higher temperatures. From the Arrhenius plots one can calculate, using eqn. 2, the enthalpy of activation ($dH^\star$) of the reaction, as shown in Table III.

$$\ln k = -\frac{dH^\star}{R} \times \frac{1}{T} + \ln A \qquad (2)$$

where k is the rate constant, T is the absolute temperature and R is the gas constant.

It is clear that the $dH^\star$ values of the derivatizations are decreased by ca. 15 kJ mol^{-1} in the presence of micelles. This indicates that the transition state of the reaction is destabilized because of interactions with CTAB micelles.

TABLE III

ENTHALPIES OF ACTIVATION OF THE PSEUDO-FIRST-ORDER RATE CONSTANTS OF DINITROPHENYLATION AND HYDROLYSIS

		dH^* (kJ mol^{-1})
Hydrolysis	Buffer	123
	Micelle	80
Propylamine	Buffer	80
	Micelle	66
Hexylamine	Buffer	78
	Micelle	64

The Arrhenius coefficient (A) of the reaction was not calculated from these data, owing to the inherent inaccuracy of this method [21].

Distribution of DNFB and DNPhOH in the CTAB micelles

From the solubility plots determined for DNFB and DNPhOH (Fig. 10A), it can be concluded that in a solution containing 33 mM CTAB the solubility of DNFB at pH 7.5 is ca. 8 mM.

Under these saturated substrate conditions the partition coefficient (K) of DNFB and DNPhOH between the buffer and the micellar phase can be calculated by using eqn. 3 as described by Bunton and Robinson [24]:

$$\frac{K}{N} = \frac{Q}{(1-Q)} \cdot \frac{1}{(C_d - CMC)} \qquad (3)$$

where Q is the fraction of substrate solubilized in the micellar phase (Fig. 10B), C_d is the surfactant concentration, CMC is the critical micelle concentration and N is the aggregation number of the micelles. Taking the linear part of the plots (Fig. 10B), and assuming $N=100$ [35] and $CMC = 3.2 \cdot 10^{-4}\ M$ [24], one finds the following values: $K(DNFB) = 4500$ and $K(DNPhOH) = 2700$. The calculated value of $K(DNFB)$ is comparable with that of $K(DNClB) = 4600$ (using $N=61$) [24]. During the solubilization experiments using particularly DNPhOH,

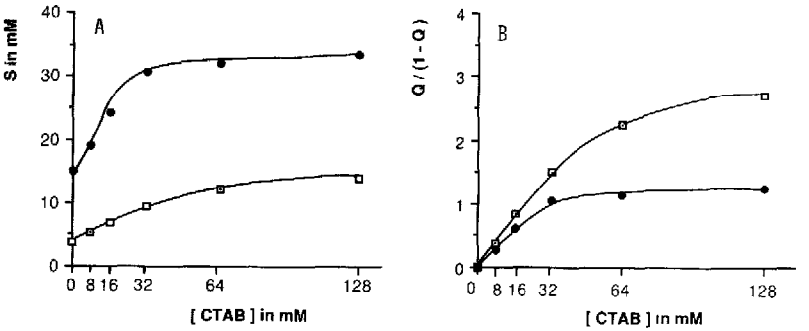

Fig. 10. (A) Dependence of the solubility of DNFB (□) and DNPhOH (●) on the CTAB concentration at pH 7.5 and 20°C. (B) Relation of $[Q/(1-Q)]$ and concentration of CTAB.

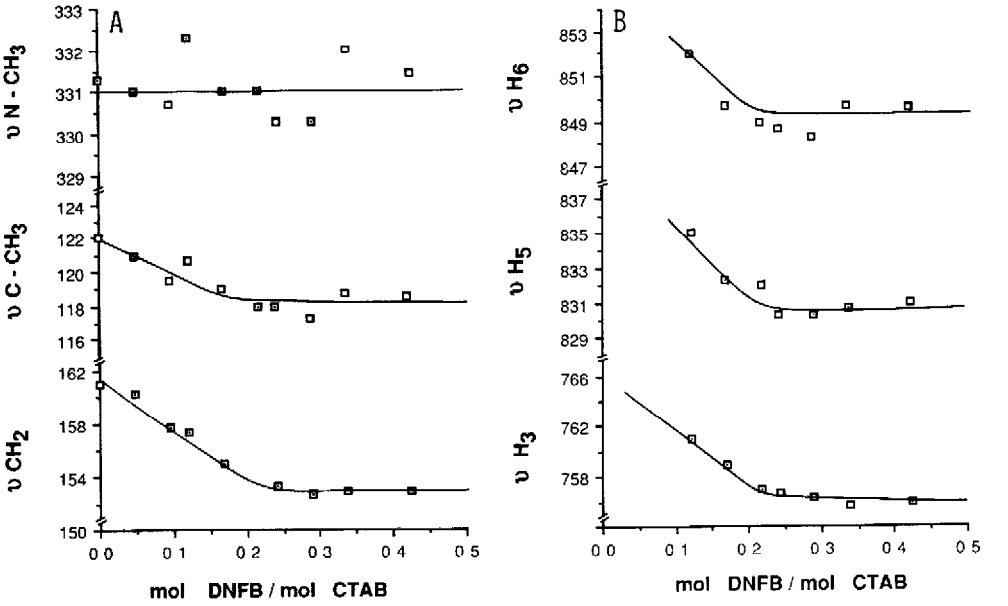

Fig. 11. 90 MHz ^{1}H NMR resonance line-shifts (in Hz) due to the solubilization of DNFB in 150 m*M* CTAB. (A) Line-shifts of CTAB hydrogens; (B) line-shifts of DNFB hydrogens. The shifts are relative to TMS (0 Hz).

highly viscous solutions were observed at higher concentrations of DNPhOH and CTAB. Increasing viscosity was also found when DNPhOH was added to a solution of 33 m*M* CTAB at pH 10.6. When the concentration of DNPhOH exceeded 12 m*M* the viscosity of the medium increased drastically. Because of this increasing viscosity it is not advisable to add, at room temperature, large amounts of DNFB to compensate for the hydrolysis to DNPhOH.

The nature of this viscoelastic behaviour of CTAB micelles in the presence of anionic substituted aromatics has been described in the literature [36–38].

NMR data

The aromatic anisotropic shifts of the protons of CTAB and DNFB of the titration of CTAB with DNFB are given in Fig. 11A and B, respectively. Owing to severe linewidth broadening it was not possible to measure the resonance line-shift of the α- and β-methylene groups of CTAB, nor was it possible to determine shifts of DNFB signals at low DNFB concentrations in a micellar solution.

Owing to the poor solubility of DNFB in hexane and borate buffer, the resonance line-shifts of DNFB in these solutions were not measureable. All these limitations made it impossible to perform a good quantification of the solubilization behaviour of DNFB, but it appears that the titration plots of CTAB with DNFB are similar to those for isopropylbenzene and nitrobenzene [22], because the addition of the solubilizate causes the $-CH_2-$ and the $-CCH_3$ hydrogen signals to shift rapidly towards higher fields, leaving the $N-CH_3$ hydrogen resonances almost unaffected. For isopropylbenzene and nitrobenzene, Eriksson and Gill-

berg [22] concluded that these substrates are preferentially solubilized in the core of CTAB micelles. It is therefore plausible to conclude that DNFB is also solubilized in this region.

The apparent maximum solubility of DNFB in a 150 m*M* CTAB solution at 36°C can be deduced from Fig. 11. A plateau value of the anisotropic shift occurs at a mole ratio of 0.25, which is ca. 30 m*M*. The solubility, which can be derived from the solubility experiments, is 15 m*M* at 20°C (Fig. 10). If these values are compared reasonable agreement is found, provided that the difference in temperature is taken into account.

GENERAL CONCLUSIONS

From the results reported here some general conclusions can be drawn.

The results clearly indicate that the hydrophobicity of the alkylamines in the presence of CTAB micelles is a major factor that controls the reaction rate between the amine and DNFB, via two mechanisms. (1) The hydrophobicity of the amine determines the partition coefficient between the buffer and the micelle, and therefore the degree of penetration into the micelle. (2) The penetration of the amine into the micelle leads to a hydrophobicity-dependent decrease of the pK_a of the amine, which results in an increasing amount of the derivatizable form of the amine.

The reaction site of the derivatization of the *n*-alkylamines with DNFB is very probably located on the inside of the Stern layer. This may be rationalized by the fact that the Stern layer is essential for the rate enhancement of the derivatization in the CTAB micelles. Furthermore, the dependence of the reaction rate on the hydrophobicity of the *n*-alkylamine and the distribution of the reagent into the micelle also supports this assumption. However, more detailed studies are needed to verify this hypothesis.

The reaction rates at room temperature are comparable with those in a dipolar aprotic solvent if hydrophobic substrates are used. At room temperature the derivatization time (ten half-lives) for octylamine in the micellar system is ca. 4 min. The apparent relations between the derivatization rate constant in micelles, the micelle-induced pK_a shift of the amine and the partition coefficient of the amine between *n*-octanol and water could be used to investigate in advance the possible application of a micelle-enhanced derivatization of a particular drug.

Some comments can be made on the practical use of micelle-enhanced derivatization reactions. If a conventional derivatization procedure, e.g. with carboxylic acids, involves a tedious sample pretreatment such as extraction of the substrate into an appropriate derivatization solvent, it is evident that direct derivatization in an aqueous micellar solution is likely to be a very useful tool in pharmaceutical analysis. Furthermore, the aqueous micellar derivatization reactions give the interesting opportunity of an on-line pre-column derivatization of aqueous samples prior to HPLC analysis.

The micelle-enhanced derivatizations of drugs (containing amine or carboxylic functional groups) present in biological matrices are under current development.

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