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NMR studies on penicillins: hydrogen bonding, self-association and micellar solutions of cloxacillin Na-salt in D_2O*

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Abstract: Self-association and formation of micellar solutions of cloxacillin sodium salt (CLO-Na) dissolved in heavy water have been investigated by NMR spectroscopy. Concentration and temperature dependence of proton and carbon chemical shifts of cloxacillin-Na in D_2O is presented and certain ^1H and ^{13}C NMR line assignments have been substantiated.

Keywords: *Penicillins; ^1H and ^{13}C concentration and VT NMR; cloxacillin; self-association; micellar solutions.*

Introduction

Practical interest in penicillins has inspired intense research activity since the Second World War [1, 2]. Although no panacea, penicillins are still widely used to control microorganisms in many diseases.

The action of these common β-lactam antibiotics is still not fully explained. One of the key points concerning their antibacterial activity seems to be related to their behaviour in solution (water-like media) and their reactivity towards some metal ions (zinc, cobalt, copper or manganese) which are present in living systems both as free ions and in many enzymes [3–9]. The stability and reactivity of penicillin in solution *in vitro* and *in vivo* is directly related to the ease of C—N bond breaking in the β-lactam ring. Therefore, studies on penicillin hydrolysis have been an important part of penicillin chemistry. Evaluation of penicillin binding sites, i.e. functionalities which are important in regard to binding of the antibiotic both to bacterial and β-lactamase receptor sites, have been an integral part of these investigations.

However, the reported results show some disagreements in regard to the mode of metal binding and origin of β-lactam hydrolysis [4, 5, 7–9].

To gain better insight into this problem we have undertaken systematic studies on penicillins in solution. NMR spectroscopy seems to be the method of choice to follow changes of individual atoms in a penicillin molecule in solution and in the presence of various metal ions. Earlier works on penicillins were carried out by use of techniques less powerful than NMR spectroscopy, or, mainly with less effective low field NMR spectrometers. Proton [2, 10], carbon [11, 12] and nitrogen [13–15] data have been reported. Reviews on ^1H and ^{13}C NMR spectra are available [10, 12]. Differences in concentration, temperature or magnetic field often make the direct comparison of spectral data problematic. Systematic reinvestigation of some penicillins with modern equipment may result in better understanding of their behaviour in solution.

Generally, penicillins are very unstable in solution [1, 2]. Therefore, a choice of a model compound, with higher stability in solution, suitable for relatively long lasting NMR experiments is important.

Cloxacillin (abbrev. CLO) is an efficient, semisynthetic penicillin resistant to acids and penicillinase [16, 17]. In contrast to benzylpenicillin G K-salt (abbrev. BPENG-K), it can be administered orally, and is widely used in treatment of neonates and during pregnancy.

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In mixture with more potent penicillins, which are destroyed by several penicillin-resistant bacteria (staphylococci and other gram-positive cocci), cloxacillin is also used as an effective β-lactamase inhibitor. Cloxacillin is used in medicine in the form of its monohydrate sodium salt ($C_{19}H_{17}ClN_3O_5SNa.H_2O$, MW 475.88, abbrev. CLO-Na) and is highly soluble in water and dimethylsulphoxide (DMSO).

A proper interpretation of cloxacillin NMR spectra in solution is essential for studying its interactions with metal ions [4, 18, 19]. However, our initial experiments with a high field spectrometer (7.05 T) revealed some interesting spectral features, not reported before. The corresponding ^1H and ^{13}C NMR line positions, and, what is more significant, the order of some lines was temperature and concentration dependent. This applied to proton resonances of the two geminal methyl groups and the lowest field carbon signals.

The purpose of this work is to indicate the influence of sample concentration and temperature on CLO Na-salt ^1H and ^{13}C NMR spectral parameters. The observed changes of chemical shifts allowed an unambiguous assignment of C-5′, C(7)=O and COO^- carbons and both α and β 2-methyl proton lines and may be interpreted in terms of hydrogen bonding with solvent and weak intermolecular interactions between hydrophobic molecular fragments, both responsible for self-association, and formation of micellar solutions in D_2O.

Results and Discussion

3′-(2″-chlorophenyl)-5′-methyl-4′-isoxazolylpenicillin (I) is known in medicine as cloxacillin. It consists of a basic penam moiety [1, 2], i.e. 7-oxo-1-thia-azabicyclo[3.2.0]heptane with an isoxazole substituent. That latter fragment is responsible for the enhanced stability of the drug [16, 17]. The whole structure reflects a state of art, where every part of the C-6 substituent was shaped to obtain the desired physico-chemical and pharmacologic properties [2, 20–22].

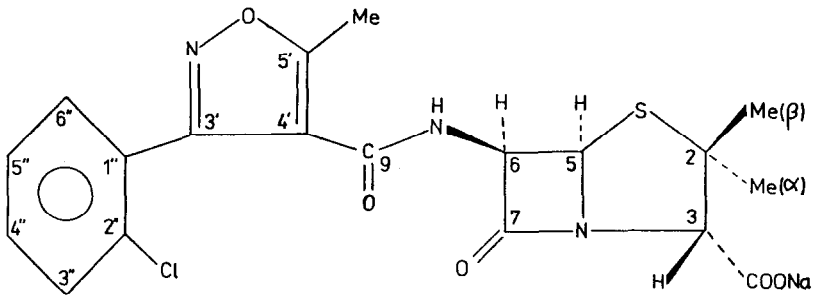
In this paper the convention of atom numbering shown in (I) is used.

CLO-Na exists in aqueous solution, i.e. in conditions similar to those in living systems, as an ionized anion (CLO-H is a fairly strong organic acid [23], $pK_a = 2.73$). The presence of three carbonyl groups (amido, β-lactam and ionized carboxylate), two amido nitrogens (one non-planar with carbonyl) and aromatic features allow many potential hydrophilic and hydrophobic sites of interaction with solvent, metal ions or proteins. There are also possible intermolecular interactions between dissolved molecules of the drug.

^1H NMR studies

Figure 1(a) shows the 300 MHz ^1H spectrum of CLO-Na in D_2O. The spectrum is simple and is both temperature and concentration dependent (see Fig. 1b and c). Both β-lactam protons (H-5 and H-6) appear as an AB system in D_2O with $J_{5,6}$ of 4 Hz, typical of *cis* vicinal protons in penicillins. However, in perdeuterated dimethylsulphoxide and acetone (DMSO-d_6 and Ac-d_6) an ABM spectral pattern is observed with $J_{NH\text{-}H(6)}$ of 9 Hz due to the lack of NH proton exchange with deuterium.

The four aromatic protons form a complex spectral pattern near 7.55 ppm. The methyl substituent of isoxazole ring and H-3 proton give rise to single lines at 2.67 and 4.13 ppm, respectively. The geminal 2-methyl signals appear as two partly overlapped lines near 1.5 ppm. In accordance with earlier reports [2, 10] the lower field signal is assigned to β-CH_3 and the most upfield line to α-CH_3 (the validity of



(I)

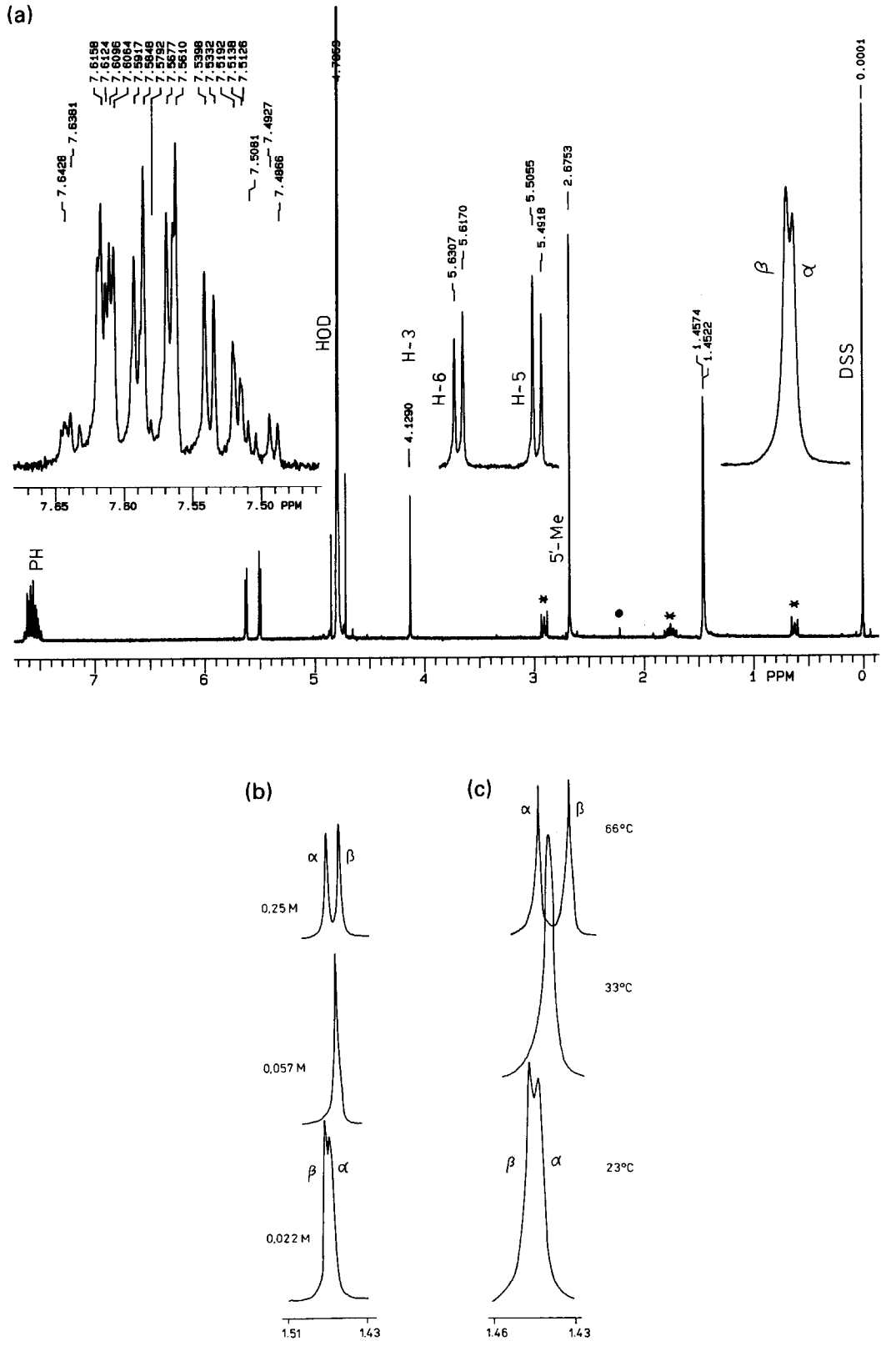


Figure 1
A 300 MHz ^1H NMR spectrum of cloxacillin-Na salt in D_2O at 23°C. (a) Whole spectrum (0.022 M solution), (b) influence of concentration on geminal 2-methyl signals, (c) influence of temperature on geminal methyl signals (conc. 0.031 M). *, DSS, ●, impurities.

Table 1
Correlation of CLO-Na NH signal (^{1}H NMR data) with some solvent features

	Solvent			
	$CDCl_3$	D_2O	Ac-d_6	DMSO-d_6
δ_{1_H} (NH) (ppm)	(6.01)*	—†	6.606	8.316
Dipole moment (D)	1.4	1.7	2.7	3.96
$\Delta\delta/\Delta T \times 10^3$ ppm °C^{-1}	—	—†	5.35	14.6
Dielectric permeability (ϵ)	4.81	78.5	20.7	46.7

*CLO-CH_3 from ref. 32.
†Not available.

this assignment will be discussed later). These geminal signals are not identical in form as evident by their different heights and line widths ($\nu_{1/2}$), a probable result of differences in their $T_{1,2}$ relaxation times [T. Kupka, unpublished results].

1. Hydrogen bonding. According to Cooper *et al.* [24], there is no evidence of intramolecular hydrogen bonding between the peptide NH proton and sulphur. This bonding is observed in penam sulphoxides (shorter distance between NH and S=O). The presence of hydrogen bonding with solvent can be studied by following the chemical shift of the NH proton in various solvents by ^{1}H NMR [25]. Its position in a proton spectrum is also temperature dependent [26].

Kessler [27], Gierash [28] and Bowen [29] used temperature coefficients of chemical shift $\Delta\delta_{NH}/\Delta T$ in studies of peptides in solution to distinguish between intramolecular NH . . . hydrogen bonds ($<3 \times 10^{-3}$ ppm deg^{-1}) and strong interactions with solvent ($>5 \times 10^{-3}$ ppm deg^{-1}). Titration of dilute solutions of compounds capable of forming a strongly solvated structure in polar solvents (e.g. DMSO-d_6) with $CDCl_3$ resulted in significant downfield shifts of the NH signal. In the present case, CLO-Na is practically insoluble in $CDCl_3$ and only very slightly soluble in acetone [30]. Nevertheless, it was possible to obtain a proton spectrum in Ac-d_6 (0.4 mg ml^{-1}) at different temperatures. Table 1 compares some ^{1}H results obtained from CLO-Na solutions in different solvents. The chemical shift of the NH proton [31] and its temperature coefficient in DMSO-d_6 is higher than in acetone-d_6, thus indicating stronger interaction with the former solvent. In both cases hydrogen bonding with solvent seems to be the dominating interaction.

A weak correlation between δ_{NH} and $\Delta\delta/\Delta T$ with solvent dielectric permeability (ϵ) and dipole moment [32] (μ) is also evident from Table 1. In water, as in DMSO, a very strong hydrogen bonding should also exist.

2. Self-association and micellar solution. ^{1}H NMR concentration studies for cloxacillin-Na in D_2O are presented in Fig. 2. All proton signals are initially shifted towards higher field with increasing concentration due to destruction of the hydration sphere, steric effects and repulsions.

These forced become more important with overcrowding in solution and increasing number of collisions. Hence, at certain concentration the opposite effects are visible.

These results may be compared with available proton data for pyrrole [25]. In the case of pyrrole in CCl_4, initially association causes a nearly exponential increase of δ_{NH} with concentration and a saturation effect is visible at higher concentrations. In contrast, its δ_{NH} dependence on concentration in DMSO-d_6 is less pronounced — initially decreasing, then rising to the certain level (solvation at low concentrations and then competition between solvation and solute–solute interactions).

Unfortunately, CLO-Na is not soluble in non-polar solvents [30], hence, observations of direct solute–solute interactions as a function of concentration or temperature are impossible. The alternative, cloxacillin methyl ester [31] soluble in CCl_4, was not available.

Hydrogen bonding with polar solvent (in a form of solute–solvent or solute–solvent–solute solvation) and direct interaction between the molecules of solute may cause

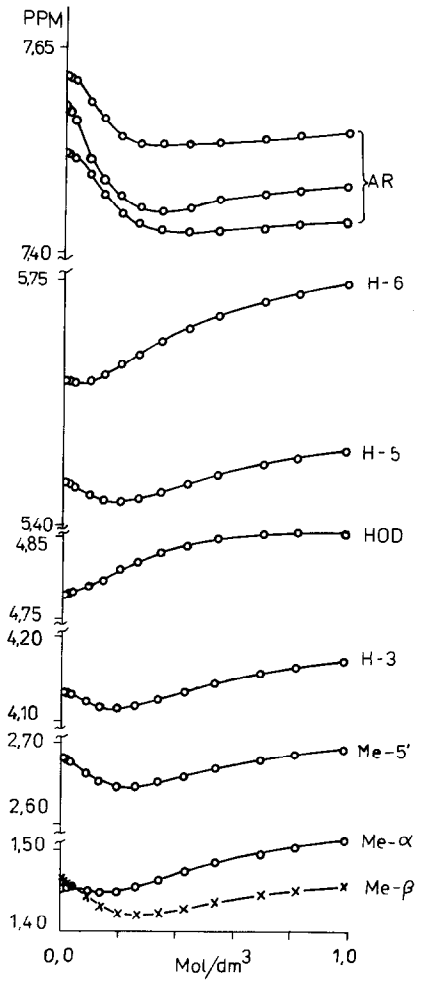


Figure 2
Concentration dependence of proton chemical shifts for cloxacillin-Na in D_2O at 23°C.

aggregation of cloxacillin. Molecules of cloxacillin, like many penicillin drugs bear an anionic carboxylate group (head) and a roughly oblong body with a hydrophobic 'tail'. Therefore, cloxacillin is shaped in a similar way to detergents or compounds capable of forming structures typical of lyotropic liquid crystals. Hence, on changing concentration, temperature or solvent, transition from isotropic solution (disorder) to some higher ordered structures (micelles or liquid crystals) should be observed.

The formation of micellar solution [33, 34] can be followed by concentration and temperature NMR studies. Aggregation of penicillin molecules cause overcrowding, stacking and repulsion of some structural fragments. Therefore, a rising concentration should be reflected in highly nonlinear behaviour of chemical shifts of the particular resonating nucleus.

From Fig. 2 it can be observed that chemical shifts of aromatic protons mainly and the other signals to lower degree, are sensitive to concentration effects.

The first reported NMR study on concentration dependence of the ^1H chemical shift of some proton signals in aqueous solution of BPEN G indeed showed nonlinear dependence [35]. These results were not interpreted. Later, Thakker *et al.* [36] published similar ^1H results. They observed that the chemical shifts of aromatic protons and $—CH_2—$ signals attached to the aromatic ring of BPEN G were the most sensitive to concentration. The observed changes in $\delta_{1_H} = f(\text{conc})$ were most pronounced at a concentration of about 0.25 M.

This value was interpreted as the critical micellar concentration (CMC), and was in good agreement with the value evaluated by non-NMR methods [37].

The chemical shift of any particular line which is assumed to be sensitive to aggregation can be described as follows [33, 34]:

$$\delta = \delta_{mic} + (CMC/C)(\delta_{mon} - \delta_{mic}),$$

where δ, δ_{mon} and δ_{mic} are the observed, the monomer and the micellar shift, respectively and C is concentration.

Plots of observed chemical shifts (signals sensitive to association) against reciprocals of the molar concentrations, $1/C$, yield two linear regions which intersect at $1/C = 1/\text{CMC}$. For $C < \text{CMC}$ the interpolated value of δ should be equal to δ_{mon} and for $C > \text{CMC}$ the value of δ_{mic} can be obtained from δ vs $1/C$ dependence for $1/C = 0$.

From the interpolated lines δ_{mon} may be assumed as the chemical shift at infinitive dilution and δ_{mic} as the interpolated signal position at $1/C \rightarrow 0$ (see both the ways of graphical presentations of data in ref. 36).

Thakkar *et al.* [36] used a concentration range of BPEN G in D_2O of 0.01–1 M and likewise found a minimum for the H-5 curve. The attracting forces were interpreted as hydrophobic interactions of aromatic 'tails' (ring stacking). The importance of aromatic ring hydrophobic interactions was suggested in a study of the mechanism of penicillin binding to serum albumin [35]. In the case of cloxacillin, hydrophobic interactions may be responsible for its very high level of binding to human serum (>90%) [2].

By applying a mathematical treatment, used

in ref. 36 to the data of Fig. 2, the formation of a micellar solution of CLO-Na in water may be assumed.

The values of the CMC for aromatics and HOD signals (evaluated according to ref. 36), collected in Table 2 are smaller than the corresponding parameters for benzylpenicillin G K-salt in D_2O [36, 37].

According to Pocsik [38–40] and Granquist [41] the processes of association, coagulation and micellation can be fitted with log-normal distribution. The dependence of the ^1H data upon concentration (Fig. 2) indeed resembles curves, described by log-normal distribution. Various graphical presentation of data from Fig. 2 are possible [$\delta = f(C)$, $\delta = f(1/C)$ or $\delta = f(\log C)$] and all of them indicate more or less clearly the changes near the CMC (see ref. 36). Near the CMC, hydrophobic and hydrophilic solute–solute or solute–solvent–solute interactions dominate over pure solvation effects.

The micelles of CLO-Na in water (by analogy with BPENG-K, a small number of molecules forming the micelle is assumed) are of anionic type, but they seem to be aggregated both by hydrophilic and hydrophobic forces.

The temperature dependence of CLO-Na proton chemical shifts in D_2O is presented in Fig. 3. The H-6 signal is the most sensitive to temperature variation possibly due to its proximity to the hydrogen bonding NH centre. In dilute solution geminal methyl signals exchange their relative position when the temperature is raised. These two lines overlap at about 33°C (see Fig. 1c).

At this point the ^1H NMR assignments of penicillins in solution should be reconsidered. In earlier reports on penicillins, the spectra were recorded at a variety of magnetic field, concentration and temperature conditions.

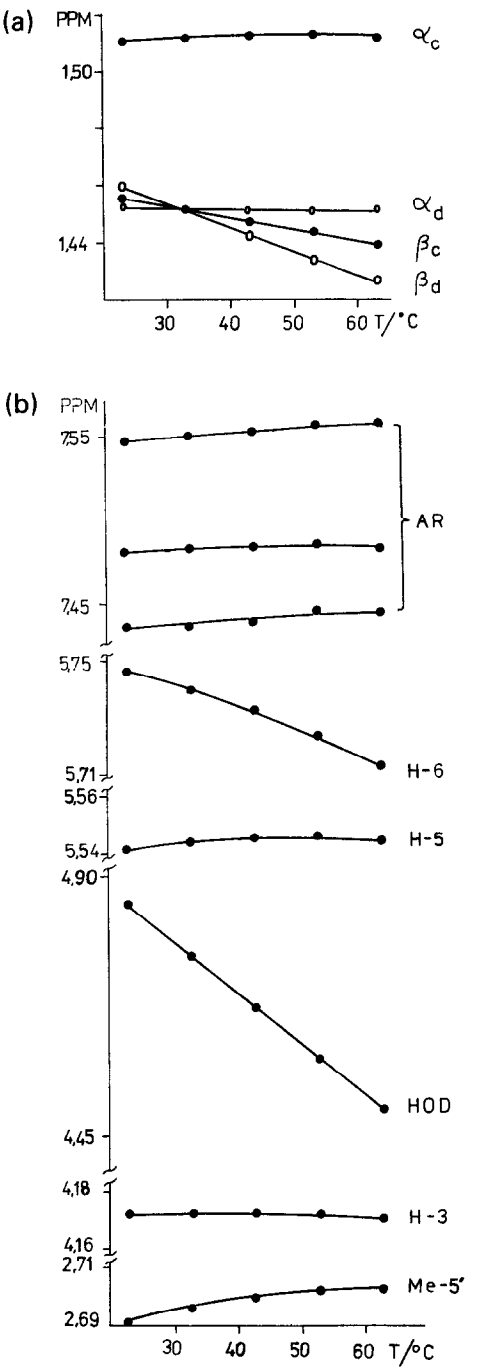


Figure 3
Temperature dependence of proton chemical shift for cloxacillin-Na salt in D_2O. (a) Geminal 2-methyl protons in concentrated (●, 0.977 M) and diluted solutions (○, 0.031 M). (b) Remaining protons in diluted solution (0.031 M).

Table 2
Critical micellar concentration for CLO-Na in D_2O at 23°C evaluated from proton and carbon spectra

Signal	Average CMC
Proton data	
Aromatics	0.0735 ± 0.0175 M
HOD	0.185 M
Carbon data	
C-2″, C-3″, C-4″, C-6″, C-5″, C-1″, C-7, C-5, C-2, 5′-C, β-Me	0.276 ± 0.025 M
CONH, COO^-, C-3′, C-4′, C-6, C-3, α-Me	0.208 ± 0.030 M

The present studies show that the ^1H chemical shifts of cloxacillin are sensitive to temperature and concentration.

Also, for future studies on complexation with dia- and paramagnetic metal ions it is

essential to check the position of α- and β-methyl groups in the proton spectrum of cloxacillin [T. Kupka, unpublished results]. It is well known [4, 6, 42] that upon addition of a paramagnetic metal ion (for example, Co(II), Mn(II) or Gd(III)) some lines of a ligand's spectrum arising from protons close to the binding site, are selectively broadened, mainly due to dipolar shortening of the transverse relaxation time, T_2. By use of this technique, α-CH_3 was assigned to the line, which was more sensitive to traces of Mn(II), i.e. to α-CH_3 which is *cis* to the 3-COO^- group.

It has been shown that the order of geminal 2-methyl signals in a proton spectrum of CLO-Na in water depends on concentration and temperature — earlier studies have not investigated this point. This may also be valid for the other penicillins. Therefore, due to various intermolecular interactions, the β-methyl protons sometimes resonate at lower magnetic field than those of α-methyl. A less probable explanation of such resonance changes is alteration of penam ring conformation with temperature or concentration.

It should be mentioned that the linear dependence of proton relaxation rates ($1/T_1$) with concentration of CLO-Na salt in water show a break near values close to the CMC [T. Kupka, unpublished results], evaluated from proton spectra. Again, this may be interpreted in terms of differences in molecular tumbling in ionic and micellar solutions.

^{13}C NMR studies

Concentration and temperature studies on cloxacillin in D_2O also indicate a strong influence of both parameters on the carbon spectrum (for line assignment see refs 11 and 12).

The 75 MHz proton-decoupled carbon spectrum of cloxacillin-Na in D_2O is shown in Fig. 4.

The enlarged parts of the spectrum, containing signals to low field of 128 ppm at various concentrations, indicate the significance of concentration for assignment of ^{13}C NMR signals in penicillin solutions (Fig. 4b).

It is also evident from Fig. 4b that for concentrated solutions at ambient tempera-

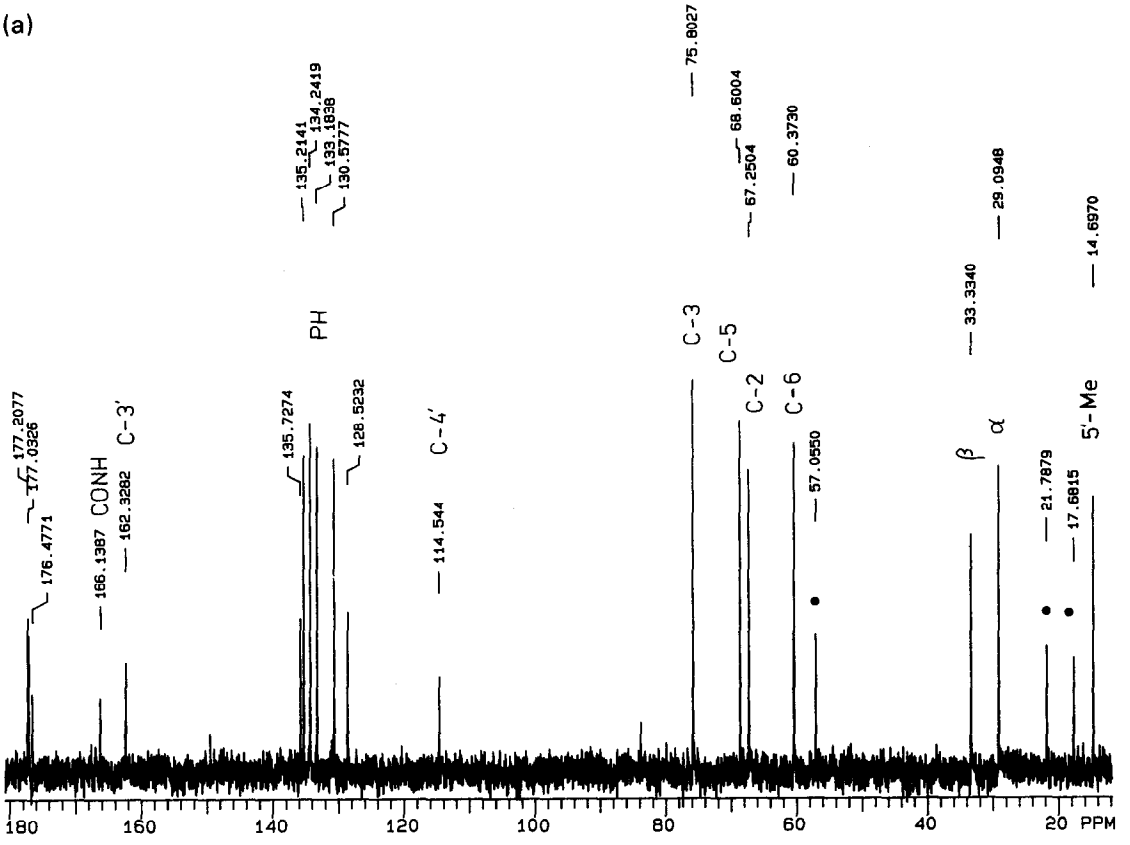


Figure 4
A 75 MHz ^{13}C NMR spectrum of CLO-Na in D_2O at 23°C. (a) Whole spectrum (0.022 M, ●, DSS). (b) The influence of concentration on the linewidths of phenyl carbons. (c) The influence of concentration on low field signals.

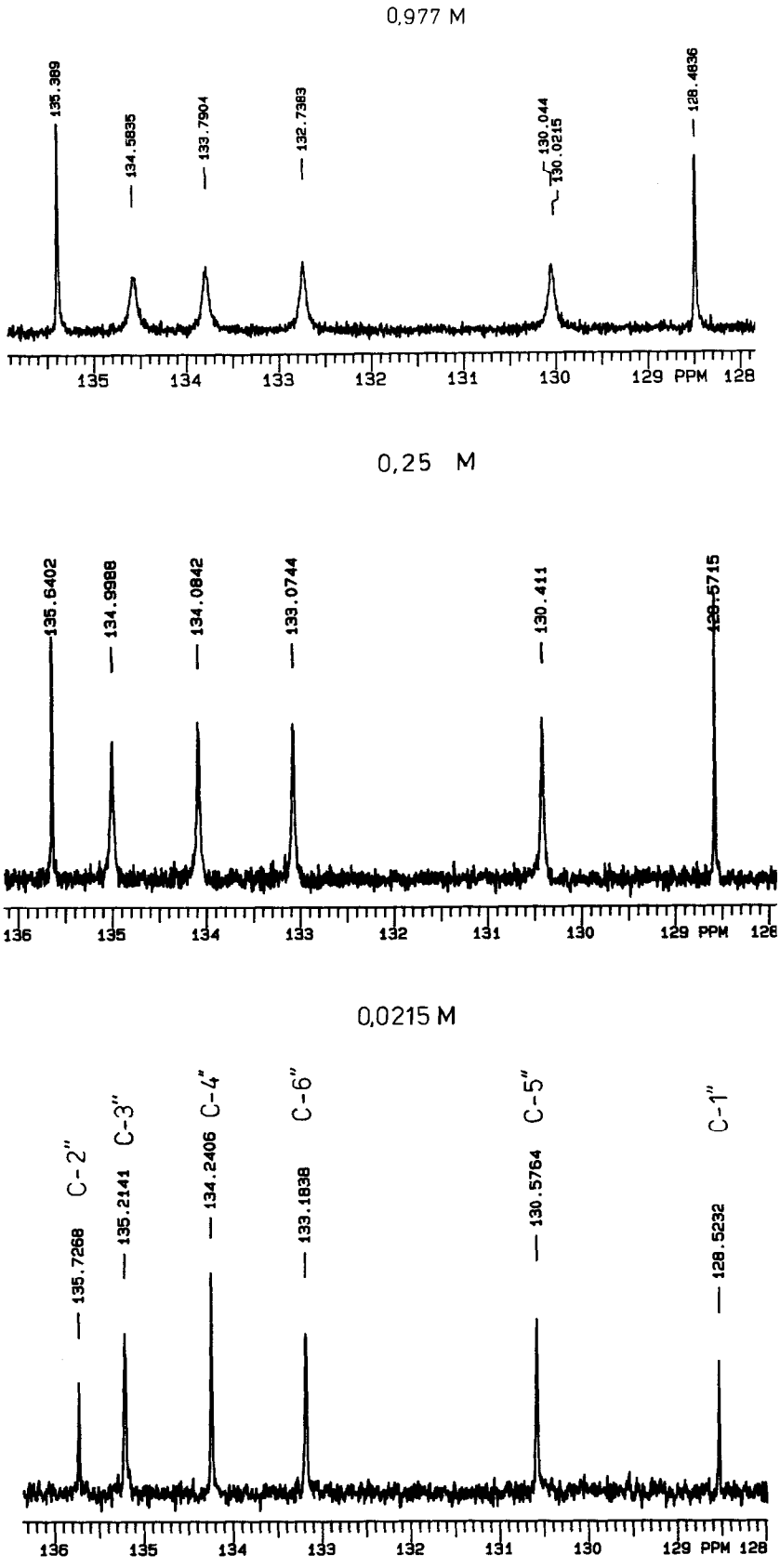

0,977 M
135.389
134.5835
133.7904
132.7383
130.044
130.0215
128.4636
135
134
133
132
131
130
129 PPM
128
0,25 M
135.6402
134.9988
134.0842
133.0744
130.411
128.5715
136
135
134
133
132
131
130
129 PPM
128
0,0215 M
C-2"
C-3"
C-4"
C-6"
C-5"
C-1"
135.7268
135.2141
134.2406
133.1838
130.5764
128.5232
136
135
134
133
132
131
130
129 PPM
128

Figure 4(b)

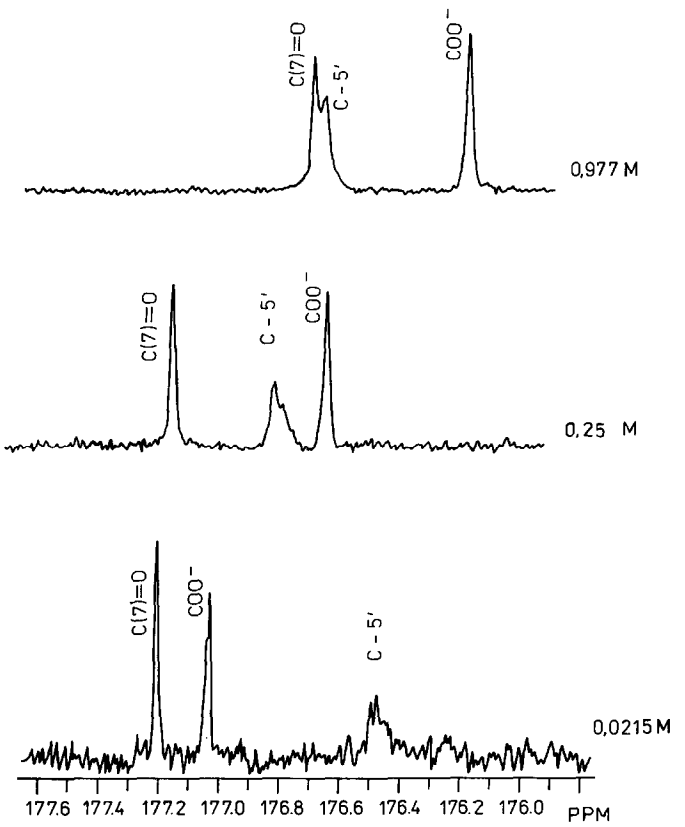


Figure 4(c)

tures the signals of protonated phenyl carbons are broader than those of quaternary carbons. On increasing dilution a decrease in line widths is observed indicating the diminishing contribution of intermolecular interactions.

The C-5′, C(7) = 0 and COO^- signals change their relative positions upon variation of concentration [Figs 4(c) and 5] or temperature (Fig. 6). The line order in Fig. 4(c) was additionally confirmed by selective broadening and eventually disappearance of the COO^- signal upon addition of Co(II).

Proton and carbon signals of geminal methyl groups were also correlated with the aid of a two dimensional HETCOR experiment [T. Kupka, unpublished results]. The low and high field carbon signals (assigned to β-CH_3 and α-CH_3, respectively according to ref. 11 and 12) in a concentrated solution of CLO in D_2O at 23°C corresponded to high and low field proton signals, i.e. β- and α-CH_3 protons. This experiment was also repeated in the presence of Co(II) ions and the corresponding low field proton signal was selectively broadened. Therefore, the assignment of 2-methyl signals in proton spectra of CLO-Na in earlier reports [10] should be substantiated.

The changes in positions of individual cloxacillin carbon signals upon rising concentration are shown in Fig. 5: highest sensitivities to concentration changes are apparent for CONH, COO^-, C-3′, C-4′, C(7)=O and C-5′ signals.

Temperature dependence of carbon chemical shifts evaluated from both diluted and concentrated solutions also indicate the probable sites of interaction of hydrated CLO anions in dilute solutions with water molecules [mainly CONH and COO^-, see Fig. 6(a)] and solute–solute interactions, dominating in a concentrated, i.e. micellar solutions of CLO-Na in water [see Fig. 6(b)].

Carbon data from Fig. 5 enabled the evaluation of the CMC (from $\delta = f(1/C)$ dependence); two different values were obtained (see Table 2) indicating the varying sensitivity of particular sites of cloxacillin molecule to self-association. COO^-, CONH, C-4′, C-3′, C-6, C-3 and α-Me signals, which to some extent take part in formation of hydrogen bonds and

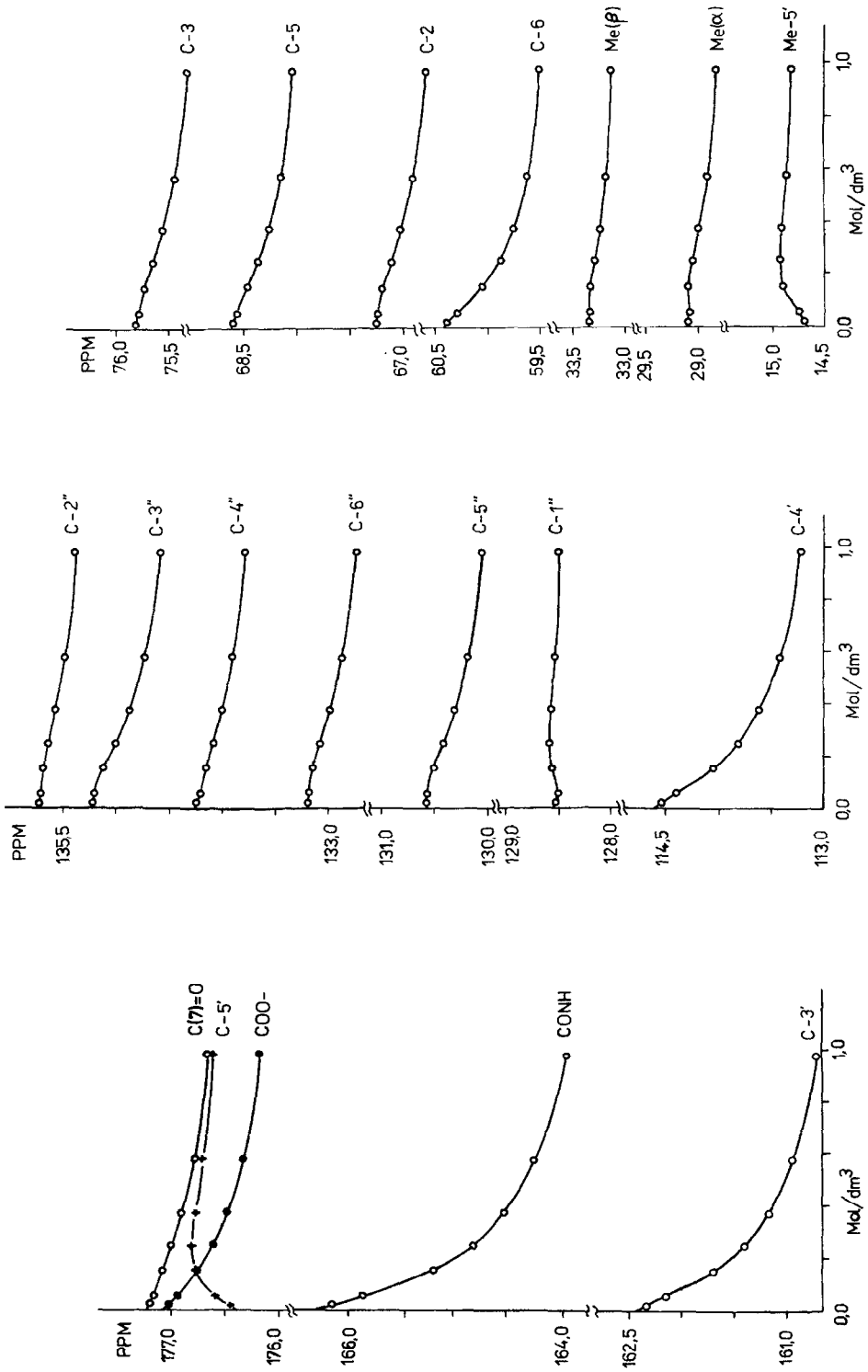


Figure 5
Concentration dependence of CLO-Na carbon signals in D_2O at 23°C.

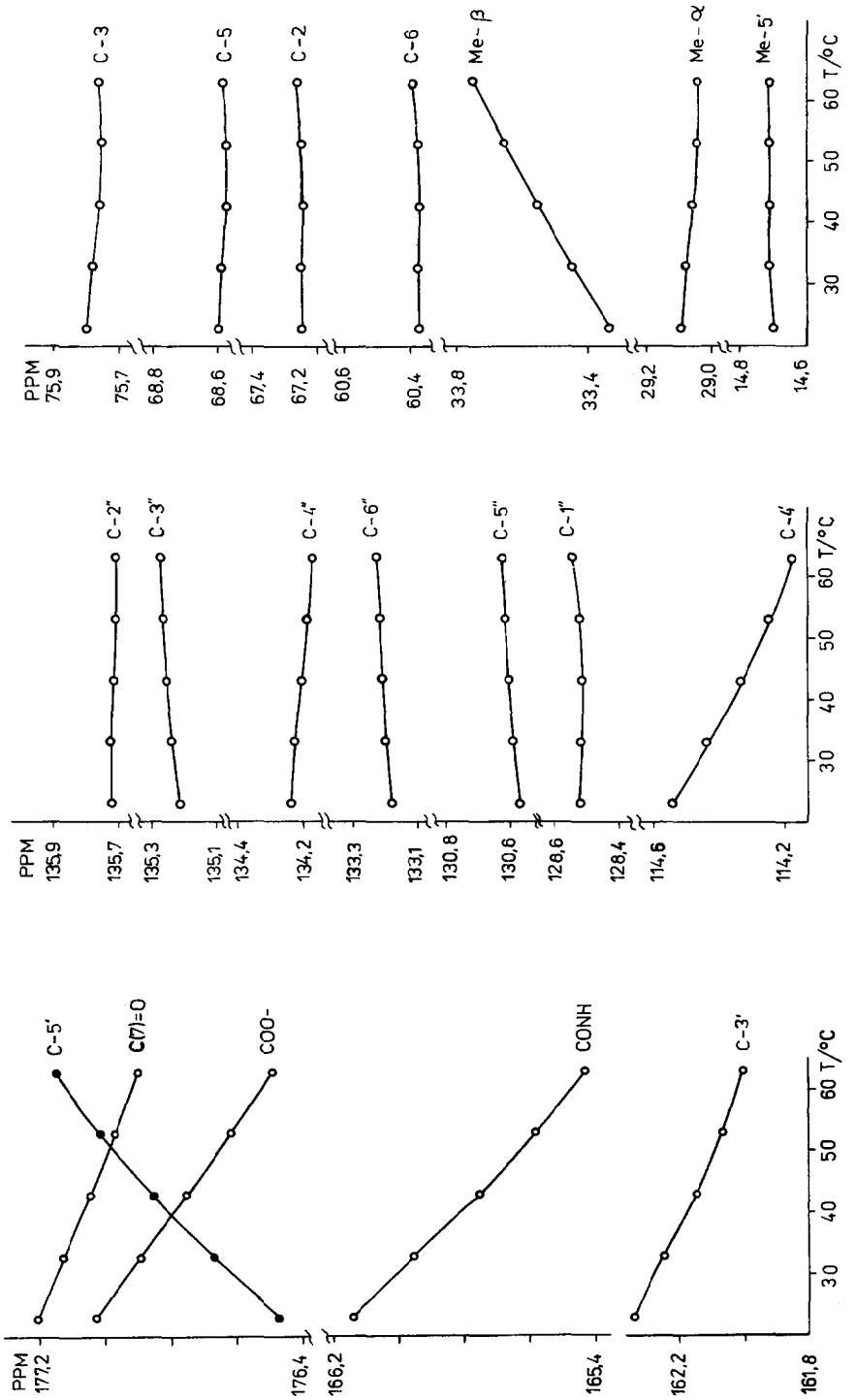


Figure 6
Temperature dependence of CLO-Na carbon signals in D_2O at concentration: (a) 0.03 M; (b) 0.98 M.

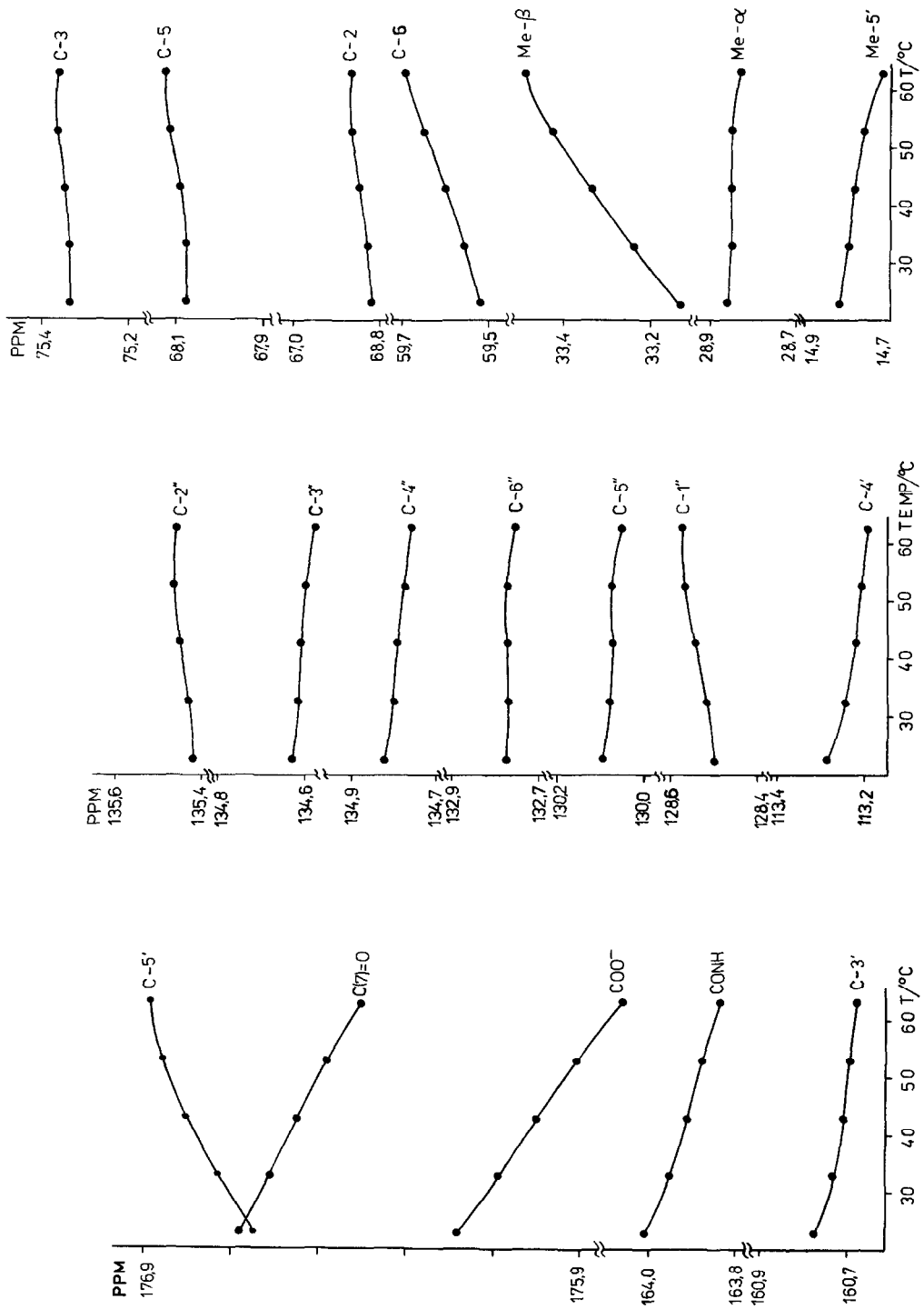

PPM
C-3
C-5
C-2
C-6
Me-β
Me-α
Me-5'
T/°C
C-2''
C-3''
C-4''
C-6''
C-5''
C-1''
C-4'
TEMP/°C
C-5'
C(7)=O
COO⁻
CONH
C-3'
T/°C

Figure 6(b)

decrease the freedom of a local molecular movement, show lower CMC values.

These data indicate the significance of interaction between the isoxazole, and to a lesser extent, the phenyl rings, as well as hydrogen bonding during self-association.

The results of our further studies on cloxacillin in solution, including works on molecular motions and complexation with dia- and paramagnetic metal ions will be discussed in a separate paper.

Experimental

Materials

Cloxacillin-Na was supplied by Pharmaceutical Works, Polfa, Tarchomin (Poland) and was used without further purification. D_2O (99.75%D) and DMSO-d_6 were obtained from OPIDI IBJ Świerk k/Otwocka (Poland) and Ac-d_6 from IChO PAN Warszawa.

Upon dissolving CLO-Na in D_2O a foam was formed (as seen for detergents) and concentrated solutions were very viscous. It was difficult to adjust homogeneity of magnetic field when running NMR spectra from concentrated solutions (therefore the changes in linewidths are not discussed in this work).

NMR measurements

300 MHz proton and 75 MHz carbon spectra (decoupled from protons) were recorded with a Varian VXR-300 NMR spectrometer. Freshly prepared solutions at different concentrations (0.001–1 M) were placed in 5 mm o.d. tubes and some measurements were repeated several times to check the reproducibility of chemical shifts. In DMSO-d_6 and Ac-d_6 TMS was used as internal standard and similarly DSS was used in D_2O solutions. Spectra were carefully phased to obtain the claimed accuracy of chemical shifts of +0.002 ppm for protons and +0.003 ppm for carbon spectra. Temperature changes within ±1°C accuracy were controlled with a Varian VT unit. The most important ^1H measuring parameters were as follows: spectral width, 3600 Hz; pulse width 7 μs (*ca* 33° flip angle); acquisition time, 3.6 s; number of scans, 16 (500 in diluted acetone solution). Carbon spectra decoupled from protons with WALTZ pulse sequence were recorded with spectral width, SW corresponding to 190 ppm. Five hundred to 10000 scans, each of 1.6 s, with pulse width, PW of 5 μs (30° flip angle) and without additional delay between the pulses were acquired. Proton and carbon spectra were recorded with digital resolution of 0.084 Hz/point for ^1H and 0.22–0.45 Hz/point for ^{13}C. To improve the signal-to-noise ratio in diluted solutions the exponential weighting function was used before FT (corresponding to the line broadening, LB of 0.3 and 1.0 Hz for proton and carbon, respectively). In the case of partly overlapped geminal methyl protons additional spectral resolution enhancement was applied to obtain accurate line positions [43–45].

Conclusions

The dependence of line position and their order in proton and carbon spectrum of cloxacillin Na-salt on concentration and temperature has been demonstrated and the assignment of certain resonances (C-5′, C(7)=O, COO^- carbons as well as α- and β-2-CH_3 protons) substantiated.

The results of systematic concentration and variable temperature studies of aqueous solutions of CLO-Na by ^1H and ^{13}C NMR indicate the formation of hydrogen bonding at CONH, COO^- and C(7)=O sites. Hence, the most pronounced changes in chemical shifts were observed for these proton and carbon signals and for the atoms, close to the hydrogen bonding centres.

Additionally, some proton data from DMSO-d_6 and Ac-d_6 solutions support the existence of strong solvation.

The formation of a micellar solution of CLO-Na in D_2O, as earlier reported for BPEN G solution, originating both from hydrogen bonding and weak interaction between hydrophobic molecular fragments is proposed. The evaluated values of CMC are different for various parts of the molecule and higher for the carbons (about 0.2–0.28 M) than for aromatic protons (0.07 M). The greater sensitivity of phenyl protons, which are inside the micelle, to concentration effects indicate the formation of micellar order at lower concentration than the CMC. The value of CMC for HOD (about 0.2 M) is close to the value obtained from carbon data and is similar to the literature data.

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