Formation of a Cycotiamine Complex with Fatty Acid in Chloroform[1)]

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The formation of a complex between cycotiamine (CCT), an S-acylated type of thiamine derivative, and a fatty acid (FA) in chloroform has been studied using the solubility method, ^{1}H nuclear magnetic resonance spectroscopy (NMR), and ^{13}C-NMR for the purpose of revealing the moieties of CCT needed for an interaction with FA. The apparent stability constants for an equimolar complex between CCT and myristic acid and for CCT and stearic acid were determined (22.9 and 21.7 M^{-1}, at 298 K respectively). A similarity of the pattern of interaction in chloroform to that in 1,2-dichloroethane has been suggested. In the presence of FA, ^{13}C resonance of the carbons adjacent to N-1 in the pyrimidine of CCT were largely shifted upfield. In the presence of FA, the amino proton signal in the pyrimidine of CCT was largely shifted downfield. These results show the necessity of the N-1 nitrogen and the amino group in the pyrimidine of CCT for the interaction. In the presence of CCT, the carboxyl carbon resonance of FA shifted upfield. This can be understood to result from the destruction of a dimer of FA by the formation of the complex with CCT.

Keywords cycotiamine; fatty acid; complex; stability; association mechanism; chloroform

Thiamine disulfide (TDS), a symmetric disulfide type of thiamine derivative, forms a complex with a fatty acid (FA).[2)] The molar ratio of TDS to FA in the complex in the solid state is 1:6.[2)] The advantages of complex formation between TDS and FA (TDS–FA) to the pharmaceutical field, *i.e.* a milder taste and smell of thiamine and the possibility of controlled release, have been previously reported.[3)] Understanding the mechanism of the formation of TDS–FA is a subject of interest in the development of new pharmaceutical preparations. Recently, we have shown that the hydroxyl moiety of TDS and the carboxyl moiety of FA are required for interaction.[4)] But considering the discrepancy between the reported stoichiometry of the complex and that there are only two hydroxyl moieties in TDS, additional TDS moieties seem to be needed for interaction. On the other hand, we have tested many thiamine derivatives for their complex formation ability with FA.[5)] Cycotiamine, *N*-[1-(2-oxo-1,3-oxathin-4-ylidene)-ethyl]-*N*-[(4-amino-2-methyl-5-pyrymidinyl)methyl]-formamide (CCT) has been found to interact with FA in 1,2-dichloroethane (dichloroethane),[6)] although the complex crystal has not been isolated. On the basis of this result, it has been shown that even a thiamine derivative which lacks the hydroxyl moiety interacts with FA. In light of these results, a study of the mode of interaction between CCT and FA seemed promising for elucidating unknown aspects of the interaction between TDS and FA.

^{1}H and ^{13}C nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy are known to be powerful techniques for investigating intermolecular interactions in the liquid phase. TDS–FA has been prepared in a dichloroethane solution, but dichloroethane for NMR spectroscopy is not on the market. Deuterium chloroform is very common and available for NMR spectroscopy, and the structure is related to that of dichloroethane. Furthermore, the solubility of CCT and FA in chloroform allows the determination of these carbon signals in various molar ratios.

From these points of view, we have examined the interaction between CCT and FA in chloroform by the solubility method, the ^{1}H-NMR method, and the ^{13}C-NMR method. The association constants for the complex between CCT and myristic acid (CCT–14:0) and that for the complex between CCT and stearic acid (CCT–18:0) were determined. The CCT moieties involved in the interaction with FA were evaluated. The mode of interaction between TDS and FA is discussed in light of the mode of interaction between CCT and FA.

Experimental

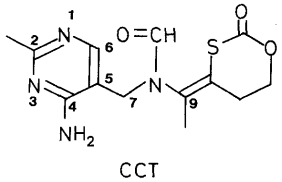
Materials Cycotiamine (CCT) was a gift of Yamanouchi Pharmaceutical Co., Ltd. Myristic acid (14:0), guaranteed reagent grade, was purchased from Wako Pure Chemical Co., Ltd, and stearic acid (18:0), guaranteed reagent grade, was purchased from Koso Chemical Co., Ltd. Chloroform, guaranteed reagent grade, was purchased from Kokusan Chemical Co., Ltd. and chloroform-*d* was purchased from Merck.

Phase Solubility Analysis The phase solubility method was carried out as described in the previous report.[6)] An excess amount of CCT was added to $0-1\times10^{-1}$ M FA chloroform solution and shaken for 24 h at 298 K until the solution attained equilibrium. This solution was filtered and the filtrate was diluted with chloroform. The amount of CCT in the filtrate was determined spectrophotometrically at 275 nm using $\varepsilon=4.97\times10^3$. FA had no effect on the absorption of CCT. All experiments were carried out at least three times and the results were highly reproducible.

^{1}H- and ^{13}C-NMR Spectroscopy The NMR spectra were measured with a JEOL GX-270 NMR spectrometer operating in the Fourier transform mode at 300 K, for ^{1}H-NMR at a frequency at 270.0 MHz and for ^{13}C-NMR with proton noise decoupling at a frequency at 68.0 MHz. Chemical shifts were measured relative to internal tetramethylsilane (TMS) and expressed according to the δ scale in parts per million downfield from TMS. These chemical shifts were estimated to be accurate within $\pm$0.01 ppm. The assignment of the spectra of CCT were based on a comparison with a previous report,[4)] and those of 14:0 were taken from Stothers.[7)]

Results and Discussion

Solubility Studies Figure 1 shows the change in solubility of CCT in chloroform depending on the concentration of FA at 298 K. The variation in measured values lies within the symbol. The solubility of CCT increased linearly with the concentration of FA, suggesting the for-



CCT

Chart 1

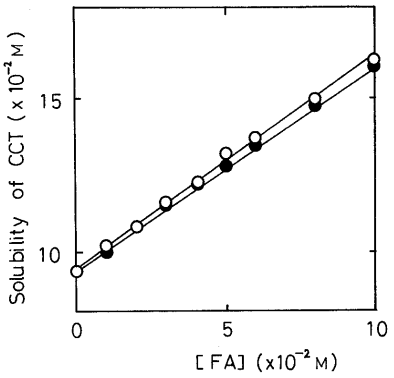


Fig. 1. Solubility of CCT as a Function of Concentration of FA in Chloroform at 298 K

FA: ○, 14:0; ●, 18:0.

TABLE I. K for CCT–FA in Chloroform at 298 K

System	K (M^{-1})
CCT–14:0	22.9
CCT–18:0	21.7

mation of a complex between CCT and FA in chloroform. The solpe of the phase solubility diagram for CCT–14:0 (0.682) was larger than that for CCT–18:0 (0.671). The apparent stability constant of an equimolar complex between CCT and FA (K) was calculated according to the equation:

$$K = \text{slope}/S_0 \cdot (1 - \text{slope}) \quad (1)$$

where slope denotes the slope of the solubility diagram and S_0 denotes the solubility of CCT in chloroform in the absence of FA. The determined K values are listed in Table I. The K value for CCT–14:0 was larger than that for CCT–18:0, although the difference was very small. These observations are in good agreement with observations made for the complex between CCT and FA in dichloroethane[6]; namely, the slope of CCT–14:0 (0.743) was larger than that of CCT–18:0 (0.719) and the K value for CCT–14:0 (116 M^{-1}) was larger than that for CCT–18:0 (102 M^{-1}). This suggests that the pattern of interaction between CCT and FA in dichloroethane is similar to the pattern in chloroform.

Each of the slopes for CCT–14:0 and CCT–18:0 in chloroform was smaller than in dichloroethane. The solubility of CCT in the absence of FA in chloroform (9.38×10^{-2} M at 298 K) was larger than that in dichloroethane (2.50×10^{-2} M at 298 K). By these two factors, *i.e.* slope and S_0 in Eq. 1, each K value in chloroform was smaller than that in dichloroethane, implying that the complexes in dichloroethane are more stable than those in chloroform. The stability of the complex may be affected by the polarity of the solvents.

^{13}C-NMR Study of FA Figure 2 shows the ^{13}C-NMR chemical shift changes of 14:0 in the presence and in the absence of CCT in chloroform. In the absence of CCT, the carboxyl carbon, C-1, and the adjacent carbon, C-2, shifted downfield with an increase in concentration. This gross downfield trend in the ^{13}C resonance reflects the dimerization of 14:0 molecules. The 14:0 molecules

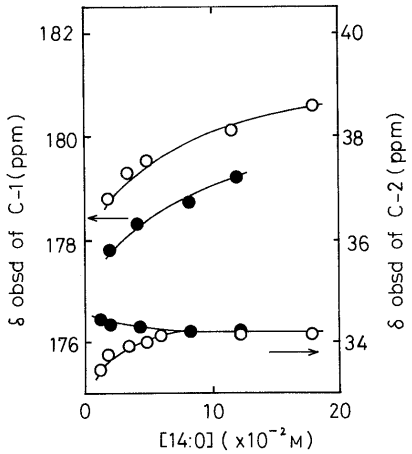


Fig. 2. ^{13}C Chemical Shift Changes of 14:0 as a Function of Concentration in the Absence and Presence of CCT in Chloroform-*d* at 310 K

○, in the absence of CCT; ●, in the presence of 3.97×10^{-2} M CCT.

undergo dimerization through hydrogen bonds in chloroform.[8] A self-association process of acetic acid in chloroform has been observed using the ^{13}C-NMR method of Maciel *et al.*[9] Their observation for carboxyl carbon is in good agreement with our observation for C-1 in Fig. 2. The formation of dimer molecules of carboxylic acid in chloroform, which is promoted by increasing the concentration, involves both the production of a hydrogen bond in which the hydroxyl group participates and the replacement of a weak hydrogen bond to the carbonyl oxygen (from a chloroform hydrogen) by a stronger hydrogen bond (from a carboxyl hydrogen). Thus, increases in hydrogen bonding to the carbonyl oxygen and by the hydroxyl hydrogen both lead to a decrease in shielding at that carboxyl carbon atom.[9] In the presence of the CCT, the C-1 and C-2 resonances shifted downfield, with no other ^{13}C resonance shifts. From this result, it is clear that the carboxyl moiety of FA is involved in the interaction with CCT in chloroform. This is exactly what was observed in our previous reports.[4,6]

The upfield shift of the C-1 in the presence of CCT seems to reflect not only the formation of a complex with CCT but also the breaking of a dimer. This can be rationalized in the following way. The similarity of the pattern of interaction in chloroform to that in dichloroethane[6] indicates that formation of CCT–FA in chloroform primarily depends on the hydrogen donating ability of FA; that is, that monomer FA can afford to form the complex. Complex formation by making a hydrogen bond should lead to a downfield shift in C-1 resonance. Therefore, the observed upfield shift upon the addition of CCT can be interpreted as resulting from the breaking of hydrogen bonds; namely, the shift in the monomer–dimer equilibrium of FA to monomer, which would be promoted by complex formation. Even if a complex was formed between dimer FA and CCT, a hydrogen bond would be made between CCT and the carboxyl oxygen of the dimer, which would lead to a decrease in shielding at the C-1 carbon. As a consequence, C-1 resonance should shift downfield, not upfield. The chemical shift change of the C-2 carbon by complexation depends on the molar ratio of FA to CCT. C-2 resonance shifted downfield in the presence of CCT

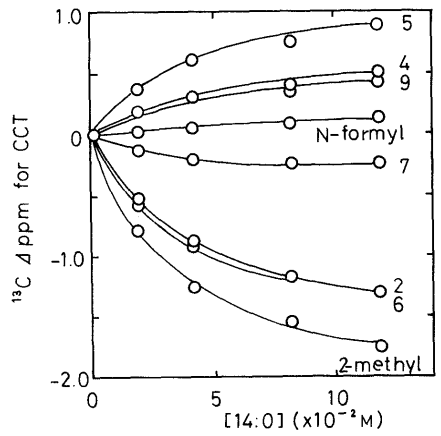


Fig. 3. ^{13}C Chemical Shift Changes of CCT as a Function of Concentration of 14:0 in Chloroform-*d* at 310 K

CCT, 3.97×10^{-2} M. The carbon positions of CCT are illustrated in Chart 1.

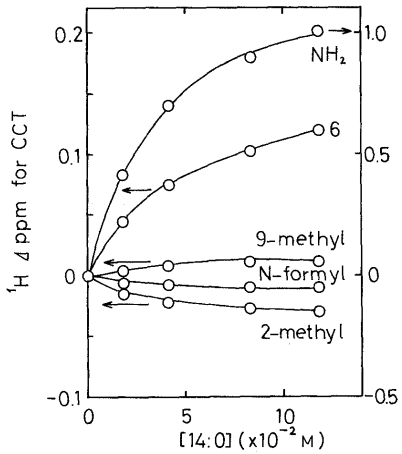


Fig. 4. ^{1}H Chemical Shift Changes of CCT as a Function of Concentration of 14:0 in Chloroform-*d* at 300 K

CCT; 3.97×10^{-2} M. The proton positions of CCT are illustrated in Chart 1.

over a range of 14:0 under 8.23×10^{-2} M, which corresponds to a molar ratio of CCT to FA of about 1:2, although monomerization of FA leaded an upfield shift of the C-2 resonance. From these results, it appears that the effect of complex formation compared to the effect of monomerization on C-2 resonance is larger than that on C-1 resonance.

^{13}C- and ^{1}H-NMR Study of CCT Figure 3 shows the ^{13}C-NMR chemical shift changes of CCT depending on the 14:0 concentration. By increasing the 14:0 concentration, large upfield shifts were observed for 2-CH_3, C-6, and C-2, and a smaller downfield shift was observed for C-5. The chemical shift changes for C-4, C-9, C-7, and N-formyl were smaller than the above changes. Only a slight shift was observed for the ^{13}C resonances assigned to carbons in the oxathine ring. In summary, chemical shift changes were observed primarily in the pyrimidine ring, but not in the oxathine ring of CCT. This observation implies that the pyrimidine ring, but not the oxathine ring, of CCT is involved in the interaction with FA.

The carbon atoms whose resonances underwent the largest shifts are adjacent to the N-1 nitrogen atom of the pyrimidine. These effects are similar to those found for ^{13}C chemical shift changes in the pyrimidine ring of thiamine upon protonation in D_2O,[10] although they are much larger in magnitude. The resonances of the carbon atoms adjacent to the N-1 nitrogen atom in the pyrimidine, which is known to be a basic site of thiamine, shift upfield upon N-1 protonation (C-2, C-6, and 2-CH_3 in the pyrimidine from pH 7.5 to pH 0.9, 3.3, 7.9, and 12.0 ppm, respectively).[10] The inflection in the chemical shift curve of CCT in Fig. 3 can be explained in a similar fashion to the inflection in chemical shift curve due to changed pHs by the protonation of the N-1 nitrogen. The fact that the effect of CCT complex formation on the ^{13}C chemical shift of the pyrimidine ring carbons is small compared to the effect of thiamine protonation on the ^{13}C chemical shift suggests that the complex, CCT–FA, is formed through hydrogen bonding.

Figure 4 shows the ^{1}H-NMR chemical shift changes of CCT depending on the concentration of 14:0. With increasing 14:0 concentration, the signal of the NH_2 protons shifted largely downfield; the C-6 and 9-CH_3 proton signals also shifted downfield, but to a much lesser extent, and the 2-CH_3 and N-formyl proton signals shifted upfield. The signals of all other protons in CCT were hardly affected by an increasing 14:0 concentration. Similar behavior has been observed in the ^{1}H chemical shift changes of thiamine caused by protonation of the pyrimidine ring, although the C-6 and 2-CH_3 proton signals shift in opposite directions. The results shown in Fig. 4 support the results obtained by the ^{13}C-NMR method. The difference in the direction of the shift of the C-6 and 2-CH_3 protons may be explained by the differences in the properties of the solvents. The effect of the hydrogen bond to N-1, which changes the electronic environment of all nuclei in the pyrimidine ring, may be transmitted through space *via* a short circuit provided by solvent molecules, as in the case of thiamine protonation.[11]

The C-7 methylene signal was a broad singlet. This may be explained as resulting from an intramolecular hydrogen bond between the N-formyl oxygen and a hydrogen of the 2-amino group of the pyrimidine ring. The temperature-dependent signal broadening of the methylene protons attached to the pyrimidine ring of disulfide type thiamines has been explained as resulting from hydrogen bonding between the N-formyl and 2-amino groups of the pyrimidine ring, and from the increase in molecular size caused by the disulfide bond.[12] The change in the chemical shifts of the C-7 protons and carbon by the addition of 1.2×10^{-1} M 14:0 were very small (0 and 0.25 ppm upfield, respectively) compared to the other chemical shift changes in CCT shown in Figs. 3 and 4. The changes in chemical shift of the N-formyl proton and carbon were very small, and leveled off at the concentration of 14:0 over 8.23×10^{-2} M. These small changes can be interpreted as reflecting the hydrogen bonding to the N-1 of the pyrimidine ring transmitted by solvent molecules. These results support the existence of intramolecular hydrogen bonding between the N-formyl and NH_2 group of the pyrimidine. Due to this intramolecular hydrogen bonding, there may be no interaction between the N-formyl and FA.

The shift of signal of the NH_2 protons depending on the concentration of 14:0 was more than 10-fold relative to the other signals, as shown in Fig. 4. The same behavior has been observed for the signal of the amino protons in thiamine upon protonation of the N-1 of the pyrimidine,

even though the amino group is not protonated.[11] This has been explained by the fact that the amino protons are closer to a center whose charge changes when protonation occurs and results from the contribution of an immonium-type resonance form where the nitrogen atom is positively charged.[11] Considering the similarity of the effect of complexation on changes in the electronic environment of the nuclei of CCT to the effect of thiamine protonation, there may be a small charge change in the NH_2 group when complexation occurs. This indicates the possibility of an interaction between the NH_2 group and FA.

Interaction between CCT and FA, and TDS and FA From this series of experiments, the complex between CCT and FA in dichloroethane and in chloroform solution can be described as follows: CCT–FA is a hydrogen-bonded complex, in which FA primarily acts as a hydrogen donor and CCT primarily acts as a hydrogen acceptor. The N-1 nitrogen atom and possibly the NH_2 in the pyrimidine ring of CCT are required for the interaction.

The aminopyrimidine moiety and the N-formyl moiety, which are related to the interaction with FA, are also present in TDS. Therefore, it is reasonable to assume that the pyrimidine N-1 and amino group, in addition to the hydroxyl group in TDS, are involved in the interaction with FA in solution. From this result, the number of TDS moieties needed for the interaction with FA amounts to 6. This perfectly agrees with the reported stoichiometry of TDS–FA, 1 : 6. Therefore, this can perhaps be applied to interaction in the solid state. According to recent studies on the crystal structure of thiamine thiazolone[13] and thiamine tetrahydrofurfuryl disulfide,[14] an intramolecular hydrogen bond between the 4-amino group in the pyrimidine and the N-formyl oxygen, and hydrogen bonds which connect the molecules *via* the 4-amino hydrogen and the N-3 nitrogen in the pyrimidine and the hydroxyl hydrogen and the N-1 nitrogen in the pyrimidine are present. From these observations, our findings seem to be reasonable.

The formation of a complex in solution is a different subject from the isolation of the crystal from solution. There are many problems we have to solve before isolating crystals of this complex, such as the problem of the interaction between the solutes and the solvent and the interaction between the complex and the solvent. But the interaction of the solutes, even in solution, is a prime requisite for isolating the complex. Therefore, our findings concerning the mode of interaction between CCT and FA is very helpful for developing new complexes not only between FA and thiamine derivatives, but also between FA and compounds whose structures are similar to thiamine.

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