

# X-Ray Studies on Phospholipid Bilayers. X. Interactions with Chlortetracycline Hydrochloride

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Z. Naturforsch. **46c**, 133–138 (1991); received May 31, 1990

X-Ray Diffraction, Phospholipid Bilayers, Chlortetracycline Hydrochloride

This study deals with the structural perturbations that the antibiotic chlortetracycline hydrochloride (CTC) can produce on phospholipid bilayers. Two multibilayer systems, one built-up of dimyristoylphosphatidylethanolamine (DMPE) and the other of dimyristoylphosphatidylcholine (DMPC) were allowed to interact with different concentrations of CTC in the absence and presence of water. The study was carried out by X-ray diffraction methods and all experiments were done below the phospholipid main transition temperatures. The results showed that CTC produced significant perturbations on DMPC even in the absence of water, whereas they were much milder in DMPE.

## Introduction

Tetracycline antibiotics are of general use in treating a wide range of bacterial infections [1]. The site of action has been shown to be the bacterial 30S ribosomal unit, where they interfere with the peptide biosynthesis [2]. Chlortetracycline, whose structure is shown in Fig. 1, is used in medicine chiefly as its hydrochloride salt; it is a crystalline powder having a bright yellow color that suggests its brand name aureomycin [3]. Its mode of action, chemistry, toxicity and other characteristics have been the subject of many reviews [1–4].

CTC can be included amongst the drugs that present the physicochemical characteristic of being amphiphilic as it has hydrophilic groups attached to the hydrophobic four-membered ring. This type of compound is able to alter the shape of human erythrocytes [5, 6] and decrease their osmotic fragility [7]. The mechanism of these changes is still

poorly understood [8], and has been explained through the intercalation of the compounds in the lipid bilayer of the cell membrane [9] or formation of intrabilayer non-bilayer phases [6]. In fact, it has been reported that CTC induced red cell shape transformations. Interacting with calcium, it changed crenated cells (echinocytes) into cup shaped ones (stomatocytes), presumably by expanding the red-cell membrane inner leaflet relative to the outer one [10]. Additional experiments, performed in erythrocyte membranes and phospholipid monolayers, led the authors to conclude that CTC acted without the influence of pH, ionic strength or the nature of the phospholipid polar heads [11–12]. It has also been dismissed that CTC interacts with spectrin and cytoskeleton [13–15]. However, no structural studies on the interaction of CTC with the phospholipids found in the red cell membrane have been published. In the present report, X-ray diffraction techniques were used. For this purpose, CTC was allowed to interact in different concentrations with two types of multibilayers. One was made of DMPE and the other of DMPC, phospholipids that are preferentially located in the inner and outer monolayer of human erythrocyte membranes respectively [16]. Their structures under different hydrations below their main transition temperatures have been reported [17–18]. Chemically they differ in that the lipid headgroup of DMPE has an  $\text{NH}_3$  terminal group whereas it is  $\text{N}(\text{CH}_3)_3$  in DMPC. The bilayer structures are very similar in their dry crystalline phases. In fact, both have the hydrocarbon chains mostly parallel and completely extended with their

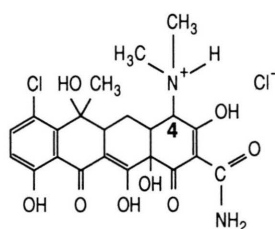


Fig. 1. Schematic structure of CTC.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen  
0939–5075/91/0100–0133 \$ 01.30/0



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polar groups lying perpendicularly to them. However, DMPE molecules pack more tightly than those of DMPC. This effect is due to its smaller polar group and higher effective charge, resulting in a very stable bilayer structure. The addition of water does not significantly perturb the packing arrangement of DMPE. However, the gradual hydration of DMPC results in water occupying the highly polar interbilayer spaces, increasing their separation [19] and the lipid undergoing a phase transition from the crystalline C to a lamellar L $\beta'$  [20]. These bilayer systems and methods have already been used in this laboratory to get an insight about the way chemicals of biological interest can perturb the structure of cell membranes [21–23].

### Materials and Methods

Synthetic DMPE from SIGMA (Lot 81 F-8365, A Grade, MW 678), DMPC from SIGMA (Lot 35 F-8430, A Grade, MW 636) and CTC from Lederle (MW 517) were used without further purification. Powder mixtures of DMPE:CTC and DMPC:CTC were prepared in molar ratios varying from 15:1 to 1:5. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry very slowly. The same procedure was followed with pure samples of DMPE, DMPC and CTC. The specimens thus prepared were in the form of dry and crystalline powders. Each one was placed into low absorbing 0.7 mm diameter X-ray glass capillaries and sealed. About 2 mg of each phospholipid were also put inside 1.5 mm diameter capillaries which were then filled with about 100  $\mu$ l of CTC aqueous solutions, sealed and kept for several days before being X-ray diffracted. The CTC concentration ranged from  $10^{-5}$  M to  $10^{-2}$  M. Control specimens were made of each phospholipid with pure water. The samples were X-ray diffracted in flat-plate cameras with 0.25 mm diameter glass collimators [18], rotating and cooling devices. The dry samples were also diffracted in Debye-Scherrer powder cameras of 114.6 mm diameter. The water-containing specimens were standardized by sprinkling a little calcite powder on the capillary surface. Ni-filtered  $\text{CuK}_\alpha$  radiation from a Philips PW 1140 X-ray generator was used. The relative intensities of the reflections were measured from films in a Joyce-Loebl MK III CS microdensitometer. All experiments were carried out at 60%

relative humidity and  $17^\circ\text{C} \pm 2^\circ\text{C}$ , which is below the main transition temperature of both phospholipids.

### Results

The results obtained from the interaction of the antibiotic CTC with the bilayers of the phospholipids DMPE and DMPC are presented in Fig. 2–5. Fig. 2 shows a comparison of the X-ray patterns of dry powder samples of DMPE, CTC and of their 1:1 molar mixture, all of them recrystallized from  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$  3:1. The pattern of the mixture corresponds to a superposition of those of the pure crystalline phases of DMPE and CTC indicating that CTC did not affect the structure of DMPE under the conditions these samples were prepared. Otherwise, the X-ray pattern of DMPE mixed with CTC would have differed from that of the pure lipid. Results, however, were quite different when the phospholipid was DMPC. As it can be seen in Fig. 3, the X-ray pattern of DMPC showed fewer reflections as the concentration of CTC was increased. At a molar ratio of 1:1, only four reflections in the low-angle region were observed, representing orders of a 55.8 Å period of the phospholipid bilayer, and one reflection of 4.2 Å in the high-angle region. No reflections from CTC were pres-

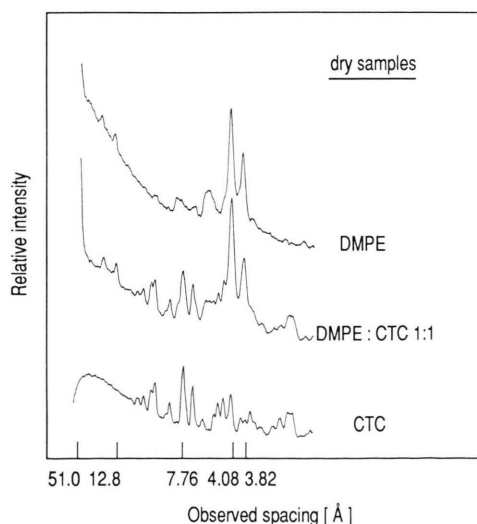


Fig. 2. Microdensitograms from X-ray diffraction diagrams of dry specimen recrystallized from  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$  3:1. Flat-plate cameras ( $D = 8$  cm).

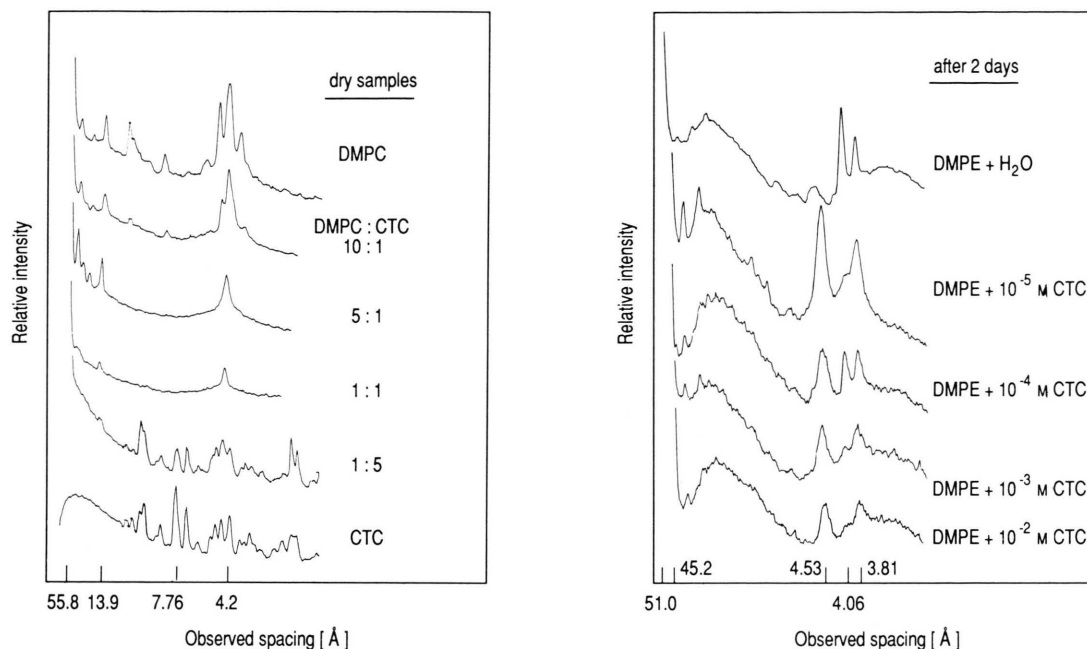


Fig. 3. Microdensitograms from X-ray diffraction diagrams of dry specimens recrystallized from  $\text{CHCl}_3$ :  $\text{CH}_3\text{OH}$  3:1. Flat-plate cameras ( $D = 8$  cm).

ent up to this concentration. However, when it reached a lipid:CTC 1:5 ratio the X-ray pattern was essentially that of CTC as the only reflections from DMPC that remained were the considerable weakened of 55.8 Å and 4.2 Å. These results imply that CTC was incorporated into the phospholipid bilayer producing an important perturbation on its structure. This is confirmed by the 4.2 Å reflection present in the 5:1 and 1:1 mixtures. This reflection has been observed in lecithin:water mixtures below their main transition temperatures ( $\beta$  and  $\beta'$  phases) and it arises from the stiff and fully extended hydrocarbon chains organized with rotational disorder in an hexagonal lattice [20].

When DMPE samples were allowed to interact with CTC aqueous solutions two types of results were observed. One, shown in Fig. 4(a), corresponds to specimens kept for two days after preparation before being X-ray diffracted. As it can be seen, the pattern of pure hydrated DMPE is nearly the same as that of the dry specimen. However CTC, in a concentration as low as  $10^{-5}$  M, produced considerable changes in the pattern. Not only the reflections from DMPE have different

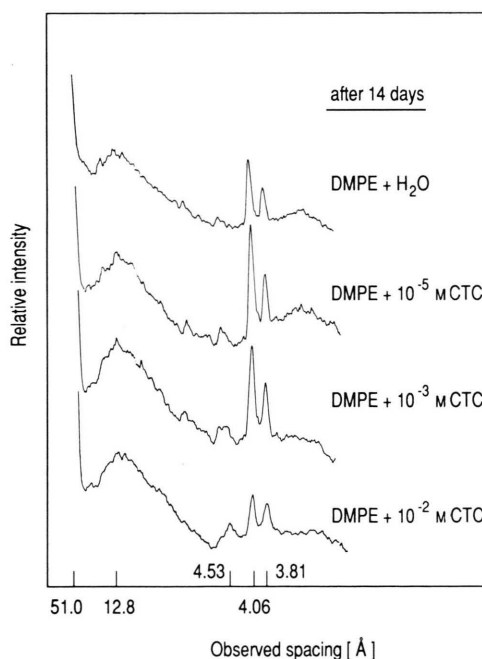


Fig. 4. Microdensitograms from X-ray diffraction diagrams of aqueous mixtures of DMPE. (a) Two days after preparation; (b) two weeks after preparation. Flat-plate cameras ( $D = 8$  cm).

spacings and intensities but its bilayer repeat decreased from 51.0 Å to 45.2 Å. Any further increase of the CTC concentration decreased DMPE intensities but did not affect the spacings and bilayer repeat any further. However, when these specimens were X-ray diffracted 12 days later, all of them showed again the pattern of DMPE in pure water, including its 51.0 Å bilayer period, with only minor variations in some intensities (Fig. 4(b)).

Finally, Fig. 5 shows the patterns of DMPC samples in contact with water and aqueous solutions of CTC. They were obtained 2 and 14 days after preparation without showing any significant change with time. It was observed that DMPC expanded its bilayer period from about 56 Å when dry to 64 Å when it was in water changing, at the same time, from a crystalline phase to the lamellar  $L\beta'$ . The observed reflections were reduced to only the first three orders of the 64 Å repeat in the low angle region and one at 4.2 Å in the high angle. About the same pattern was observed when DMPC was immersed in  $10^{-5}$  M and  $10^{-4}$  M CTC solutions, although the intensities of the reflections

became considerably weakened. The  $10^{-3}$  M CTC solution produced the vanishing of the low angle reflections, which were replaced by a central diffuse scattering, and a very weak 4.2 Å reflection. When the CTC concentration was raised to  $10^{-2}$  M no reflections from DMPC were observed. The central diffuse scattering made the only difference of this pattern with respect to that of pure water. These results clearly show that CTC in solution produced a deep perturbation on the DMPC bilayer structure.

## Discussion

Model systems consisting of synthetic phospholipids have been a valuable tool for obtaining structural information on membrane phospholipid bilayers. As a function of hydration and temperature the bilayers of diacylphosphatidylethanolamines and diacylphosphatidylcholines undergo the phase transitions  $L_c \rightleftharpoons L\beta' \rightleftharpoons P\beta' \rightleftharpoons L\alpha$ , where  $L_c$  denotes the crystalline phase,  $L\beta'$  the gel phase,  $P\beta'$  the rippled gel phase and  $L\alpha$  the liquid-crystalline phase [24]. The latter is generally present at high temperatures [20]. In the present study, the amphiphilic antibiotic CTC was made to interact with DMPE and DMPC, phospholipids which have been extensively studied in our laboratory [17–19].

These interactions, carried-out below the phospholipid main transition temperatures, were made either in hydrophobic solutions ( $\text{CHCl}_3:\text{CH}_3\text{OH}$  3:1) from which the resulting products were recrystallized, or by mixing each phospholipid in their  $L_c$  phases with CTC aqueous solutions. In this way, the fluidization effect of CTC on DMPE and DMPC bilayers could be followed by X-ray diffraction.

The results obtained indicate that CTC was able to perturb to different degrees both phospholipid bilayers. In the case of DMPE, the observed effects were rather mild. In fact, no structural alteration was observed in dry samples after being allowed to interact with CTC in the  $\text{CHCl}_3:\text{CH}_3\text{OH}$  3:1 solution. On the other hand, aqueous solutions of the antibiotic produced a change in the X-ray pattern of DMPE which was related to a shortening of its bilayer repeat from 51.0 Å to 45.2 Å. However, after a few days DMPE showed again the 51.0 Å repeat pattern. The explanation lies in that DMPE presents two polymorphic phases. In one of them

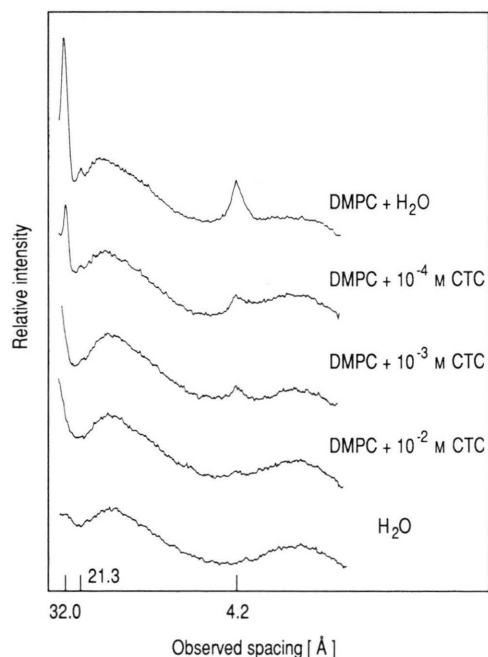


Fig. 5. Microdensitograms from X-ray diffraction diagrams of aqueous mixtures of DMPC. These patterns were the same two days and two weeks after preparation. Flat-plate cameras ( $D = 8$  cm).



( $\beta_1$  phase), the hydrocarbon chains are parallel to the bilayer normal [18, 25] whereas in the other the hydrocarbon chains are tilted by about  $28^\circ$  ( $\beta_2$  phase) resulting in a shorter bilayer repeat. Each one of these two phases could also be obtained simply by recrystallizing DMPE from  $\text{CHCl}_3:\text{CH}_3\text{OH}$  solutions: the  $\beta_1$  phase from a 3:1 mixture and the  $\beta_2$  phase from that in the ratio of 1:3, as can be observed in Fig. 6. This polymorphism of DMPE is somewhat equivalent to that reported for dilauroylphosphatidylethanolamine [25]. The physicochemical conditions of the CTC solutions were such that favored the tilted phase of DMPE. However, it could not be maintained for too long due to the instability of CTC in aqueous solutions. As reported, this antibiotic undergoes epimerization at carbon 4 in solutions of intermediate pH, loosing its activity [3, 26]. Under this new condition, DMPE bilayers rearranged again in the  $\beta_1$  phase.

The changes produced by CTC on DMPC bilayers were indeed more pronounced and permanent than those observed in DMPE. As it can be seen in Fig. 3, only one molecule of the antibiotic in ten molecules of DMPC in dry samples was enough to perturb in a significant extent the bilayer structure. This indicates a cooperative type of interaction as no other phases were present in the X-ray diagram. This structural alteration increased with higher

proportions of CTC in the mixtures producing a phase transition from the crystalline Lc to more fluid phases. The absence of reflections from CTC in all its dry mixtures with DMPC, except when it was present in a great excess (1:5), shows that the antibiotic was completely incorporated into the phospholipid bilayer. The fact that the bilayer repeat of  $55.8 \text{ \AA}$  remained unchanged despite the gradual incorporation of CTC points to a deep penetration of the antibiotic into the DMPC hydrophobic core, resulting in its fluidization. The appearance of the  $4.2 \text{ \AA}$  reflection in the 5:1 and 1:1 DMPC:CTC mixtures proves that the hydrocarbon chains became hexagonally arranged [20]. This is most remarkable as these perturbations were produced in the absence of water and below the main transition temperature of DMPC.

When water was present, the DMPC structure was somewhat perturbed by CTC  $10^{-4} \text{ M}$ , being completely destroyed when it was  $10^{-2} \text{ M}$ . The unreversibility of these changes indicates that, unlike what was observed in DMPE, CTC was incorporated into DMPC bilayers not being, therefore, affected by epimerization after a similarly long exposure to water. The disappearance of the low angle reflections of DMPC with  $10^{-3} \text{ M}$  and  $10^{-2} \text{ M}$  CTC solutions suggests that in this case the antibiotic molecules are mostly located in the polar region of the phospholipid.

The different type and degree of perturbation produced by CTC on DMPE and DMPC can be related to their respective packing arrangements and the effect of water upon them. As explained in the Introduction, DMPE molecules are so tightly packed in the crystalline and gel phases that neither water nor CTC is able to disrupt them in a significant extent. DMPC, on the contrary, presents large interbilayer spaces – which increase in size as water fills in – that allow CTC molecules to incorporate and, consequently, perturb its structure.

Finally, it should be mentioned that the results obtained in this study do not quite agree with those reported by Behn *et al.* [11–15]. In fact, they have claimed that the interactions of CTC with phospholipids do not depend on the type of their polar heads but rather on the concentration of calcium in each monolayer. There is no doubt that this is an important factor given the Ca-chelating properties of CTC [3]. However, the different type of interactions of CTC with phospholipids located

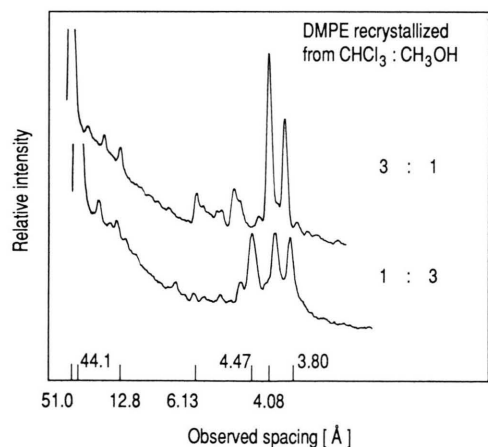


Fig. 6. Microdensitograms from X-ray diffraction diagrams of dry specimens of DMPE recrystallized from  $\text{CHCl}_3:\text{CH}_3\text{OH}$  3:1 and 1:1. Flat-plate cameras ( $D = 8 \text{ cm}$ ).

in either side of the erythrocyte membrane as determined in the present study certainly must play a relevant role in the cell deformation produced by this antibiotic.

#### Acknowledgements

Research grants from FONDECYT (0783/88) and from the University of Concepción (20. 13. 60) are gratefully acknowledged.

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