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THE EFFECT OF THE DETERGENT CETYLTRIMETHYLAMMONIUMCHLORIDE ON THE PHASE BEHAVIOUR OF DIPALMITOYLPHOSPHATIDYLCHOLINE. A HIGH PRECISION CALORIMETRIC STUDY.

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ABSTRACT

The influence of the linear, positively charged detergent cetyltrimethylammoniumchloride (CTACl) on the well known phase behaviour of fully hydrated dipalmitoylphosphatidylcholine (DPPC) has been studied along fourteen isopleths of the phase diagram using high sensitivity scanning microcalorimetry. Particular interest was focused towards the effects of very small amounts of detergent down to the region of 1 CTACl per 10^4 DPPC-molecules.

Even starting with such low concentrations distinct changes of the thermograms are detectable by high sensitivity DSC. Essentially, the phase diagram is cut into four domains between the two limiting cases of pure phospholipid multilamellar dispersion and isotropic solution of detergent micelles, respectively. At the present stage these four regions in the phase diagram are identified as follows:

1. 0-1 Mol % CTACl: a modified lamellar phase
2. 1-50 Mol % CTACl: a two-phase region with this modified lamellar phase coexisting with a newly formed detergent-rich phase.
3. 50-90 Mol % CTACl: large mixed micelles.
4. >90 Mol % CTACl: an isotropic solution of small mixed phospholipid/detergent micelles.

INTRODUCTION

The phase behaviour of phospholipids as main constituents of the two dimensional matrix of biological cell membranes has been widely studied and a great number of investigations has been dedicated to the influence of other substances of biological origin as well as of model character on this particular system. At the present stage it seems very likely that there exist various mechanisms of mutual influencing of structural and functional properties, as for example in the important case of protein/phospholipid interactions. However, little is known about the influence of very low concentrations of effectors, say in the range down to 10^{-3} Mol % and below. Stimulated by observations like the stupendous effect of the bee venom melittin on DPPC (ref.1), with one single molecule changing the packing density of thousands of phospholipid molecules to a similar extent as the main chain melting transition, and also other observations with δ-lysin (ref.2), cyclosporin (ref.3) etc., it is attempted in the present study

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to do a first step in the direction of a systematic examination of the effects of small concentrations of molecules on the physicochemical properties of phospholipids. As a first model substance the linear detergent cetyltrimethylammoniumchloride was chosen, however, this study will be followed by others using different types of amphiphiles with varying hydrophilic/hydrophobic balance, molecular geometry and polarity distribution (head/tail e. g. or front/back etc.). In other words, representatives will be chosen in order to explain the general principles of interaction with phospholipids at high dilution.

Two requirements are imposed by such measurements: first one certainly needs sufficiently sensitive detection methods according to the low effector concentrations used and secondly there are extreme requirements with respect to the purity of the phospholipid. If for example the impurities of the material are in the same order of magnitude in concentration as the substance added for study it is clear that the observed effect could be obscured by the presence of equal amounts of unknown impurity.

Whereas the DSC-instrument used meets this requirement almost perfectly, the purity of the DPPC used was only in the range of 99,5 %. Nevertheless the observed phenomena are clearly not caused by impurities but by the added detergent.

METHODS AND MATERIALS

DSC

The instrument was the new DASM-4 differential scanning calorimeter produced by the Academy of Sciences of the USSR according to Privalov (ref.4). Its platinum cells, each constructed as a tube with 1 mm in diameter wound up to a helix contain ~0,5 ml. This results in a high surface/volume ratio and an accordingly small lag in heat conduction. The noise level is fairly low (~0,2 μW) so that e. g. for a 1 mg/ml DPPC dispersion the signal-to-noise ratio for the main transition is better than 1000. The sensitivity is ~4 V/mW which is roughly 10^5 times more than for those instruments using metal pans as sample container. The thermograms were put on an absolute scale by internal electrical calibration. The scanrate was $0,25^{\circ}$ K/min.

Samples

Synthetic, crystalline 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), was obtained commercially from Sigma Chemical Company and used without further purification. The purity estimated from TLC was about 99,5 %. Highly purified CTACl was obtained from the laboratory of C. A. Bunton (University of California, Santa Barbara). Samples were prepared by dispersing 1 mg DPPC/ml in a CTACl solution in H_2O of appropriate concentration to give the desired molar mixing ratio, supported by 1 minute vortexing above the main phase transition of DPPC ($41,5^{\circ}$C). In order to reach full equilibrium the dispersions were stored for

24 h at 45° and redispersed by several minutes of vortexing directly before use.

RESULTS AND DISCUSSION

A typical DSC scan illustrating the quality of the measurements is shown in Fig. 1 in form of an original trace of the DASM-4 instrument.

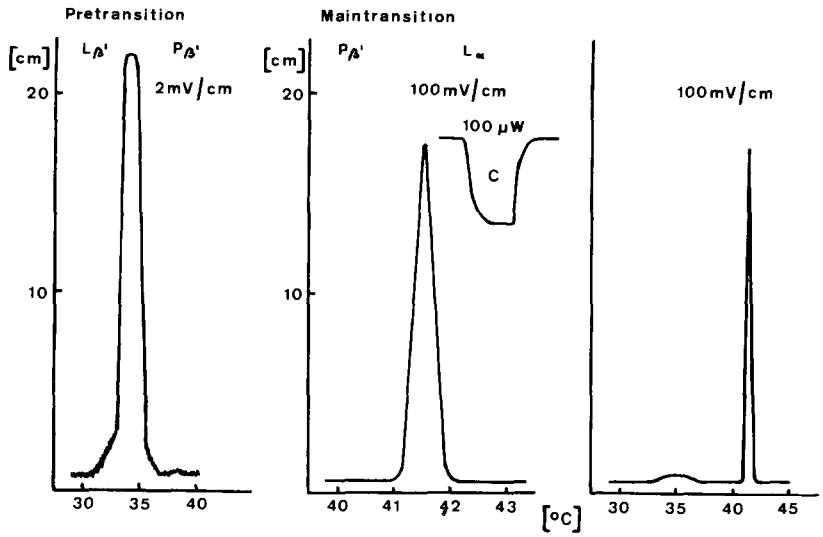

Fig. 1. Original scans of a 0,02 Mol % CTACl/DPPC mixture. Total amount of DPPC: 0,47 mg in a 1 mg/ml dispersion. The left curve shows the pretransition on a 2 mV/cm scale, the middle curve is the main transition recorded at 100 mV/cm and to the right the both curves are put together on a common scale.

The changes in the behaviour of DPPC are shown in Fig. 2, where a series of thermograms is plotted at various DPPC/CTACl mixing ratios. On the first view the following observations can be made.

1. Atypically, the excess heat capacity related to peak 2 i. e. the main transition first increases with increasing amounts of incorporated detergent up to concentrations of 0,05 Mol % before it starts to decrease.
2. The pretransition, usually completely abolished at more than about 5 % of foreign substance as observed for numerous systems persists up to 50 Mol % in this system.
3. At 2 Mol % and above, the main transition peak separates into two signals, with one of decreasing intensity and constant position at the original value

of T_2, i. e. ~41,4° C while the other one is continuously shifted upwards with increasing detergent concentration.

4. Complete abolishion of any calorimetric signals does not occur before 90 Mol % (!) detergent is present.

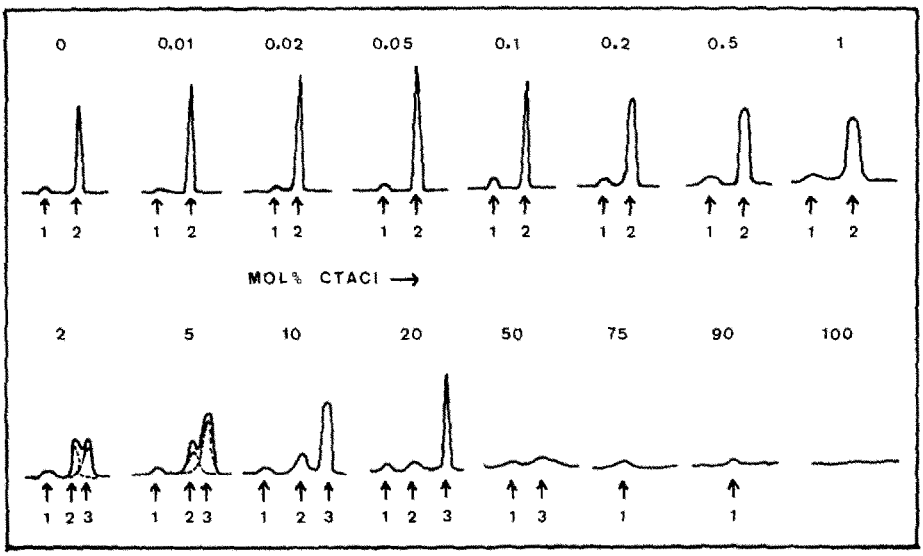

Fig. 2. Schematic view of the thermograms obtained for different CTACl/DPPC mixtures on arbitrary scales; index 1: pretransition, index 2: main transition and index 3: newly formed phase C.

For a more detailed description of this complex system, the common parameters accessible by DSC-analysis have been evaluated and are listed in Table 1 under the same indices 1, 2 and 3 as shown in Fig. 2.

An additional observation, which helps to interpret these data are systematic changes of the turbidity when CTACl is incorporated into DPPC lamellar dispersions. Presently no quantitative light scattering measurements have been done, so only the optical appearance of the samples is described.

Interestingly, the original turbidity of a DPPC dispersion decreases considerably when small amounts of CTACl are present and reaches a minimum between 1 and 2 Mol % detergent. At higher CTACl concentrations the dispersions again become more and more turbid with a maximum turbidity around 10 Mol % detergent. At 50 Mol % the samples are not more than slightly opaque, and clear solutions are

TABLE 1

Mol % CTACl	T			$\Delta T_{1/2}$		$\Delta C_{p,max}$		ΔH		w_2	N_2	P
	1	2	3	2	3	2	3	2	3			
0	34,4	41,36		0,24		38,9		7,3			420	A
0,01	35,0	41,45		0,24		39,7		7,4			420	
0,02	34,8	41,46		0,25		40,9		7,7			400	
0,05	34,7	41,46		0,24		65,3		8,3			380	
0,1	34,6	41,52		0,27		37,8		7,9			350	B
0,2	34,5	41,49		0,29		32,1		7,6			320	
0,5	34,4	41,56		0,31		32,0		7,6			320	
1	33,7	41,48		0,32		30,1		7,4			320	
2	34,1	41,52	41,80	0,32	0,26	30,1	17,6	7,4	3,5	0,30	320	B
5	34,5	41,50	41,92	0,32	0,30	30,1	33,0	7,4	7,7	0,15	320	✝
10	35,5	41,50	42,36	0,32	0,30	30,1	29,3	7,4	6,3	0,08	320	C
20	36,8	41,54	42,62	0,32	0,30	30,1	19,7	7,4	4,5	0,03	320	
50	36,5	41,50	43,60	0,32	8	30,1	0,5	7,4	3,0	0,01	320	
75	35,8											C
90	35,0											
100												D

Temperatures are in ^{o}C. Values for $\Delta C_{p,max}$ are given in cal/K^{o},g and in Kcal/Mol for ΔH, respectively. w_2 is the weight fraction of phase 2 in the two phase region, N_2 the average number of molecules forming a cooperative unit and P indicates the various phases: A: DPPC-liposomes; B: modified lamellar phase; C:mixed micelles; D: globular detergent micelles. The values for ΔC_p and ΔH in the B+C region have been calculated. For explanation see text.

obtained between 80 % and 90 % CTACl. Concomitantly the stability of the dispersions estimated from the sedimentation time, changes inversely with turbidity.

To interpret the phase behaviour of this system, one first has to state, that on the basis of these thermodynamic data only a phenomenological description can be given. X-ray measurements for a structural characterization of the various states are in work, however, with some preliminary results and some general experience an interpretation is given in the following which rigorously seen goes somewhat beyond that information extractable from the presented data.

Only the limiting cases of 0 % CTACl and 100 % CTACl, respectively, are well defined: on the one hand, large, multilamellar vesicles of DPPC and small, globular micelles of CTACl on the other hand. Incorporation of small amounts of detergent leads to an increase of all the thermodynamic parameters listed up in

Table 1. As also seen from Figs. 3, 4 and 5, there is a maximum effect at 0,05 Mol % CTACl for $\Delta C_{p,max}$ and ΔH of the main chain melting transition, while the transition temperature T_2 is continuously shifted upwards. T_1, i. e. the temperature of the pretransition also shows a maximum, however, already at 0,01 Mol %.

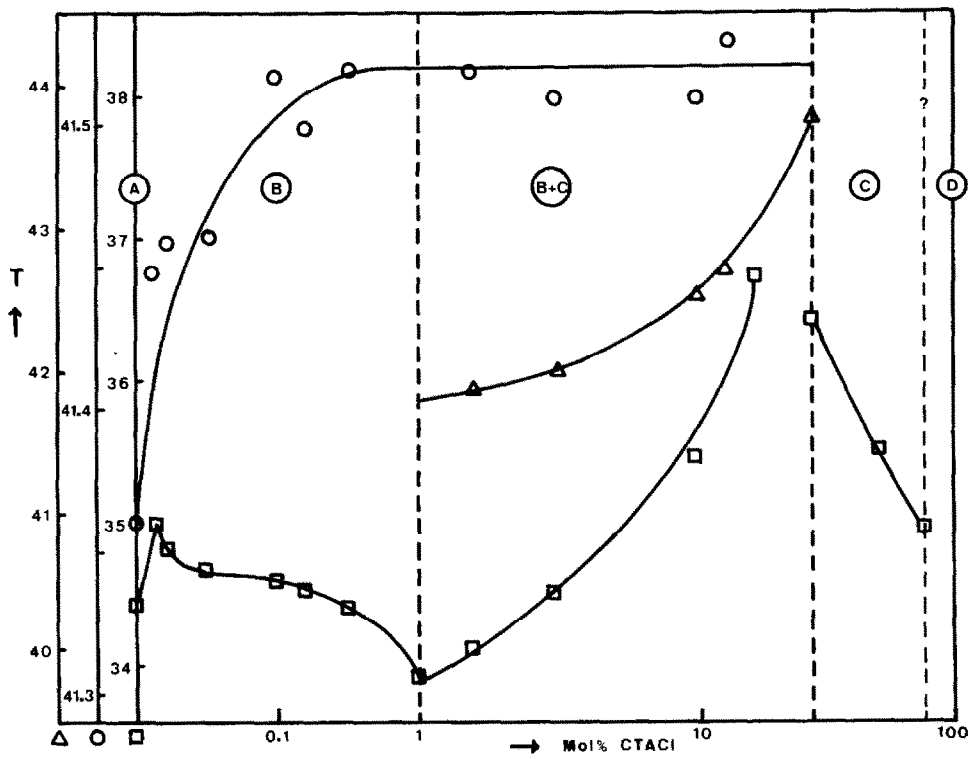

Fig. 3. Dependence of the peak temperatures (^{o}C) on the mixing ratio. □ = pretransition, ○ = main transition, △ = transition of the newly formed, detergent-rich phase. The dotted line at 90 Mol % drawn into only this diagram indicates the possible change from mixed disc to globular mixed micelles.

It is certainly not easy to interpret these data in a way that fits into the common picture, i. e. one would expect the phospholipid liposomes to decrease in size with some increase in hydration leading to a somewhat longer lamellar repeat distance (ref.5,6). Both, the smaller size and the higher hydration,

could be understood as the reason for the decrease of turbidity mentioned above. However, from numerous studies (ref.7,8) we know, that the transition temperature is lowered in such a case, if only to a small degree, and not increased, as observed with the system described here. Another surprising effect is the increase of ΔH from 7,3 to 8,3 Kcal/Mol, which is a change of 14 % caused by only 1 single detergent molecule for 2000 phospholipid molecules.

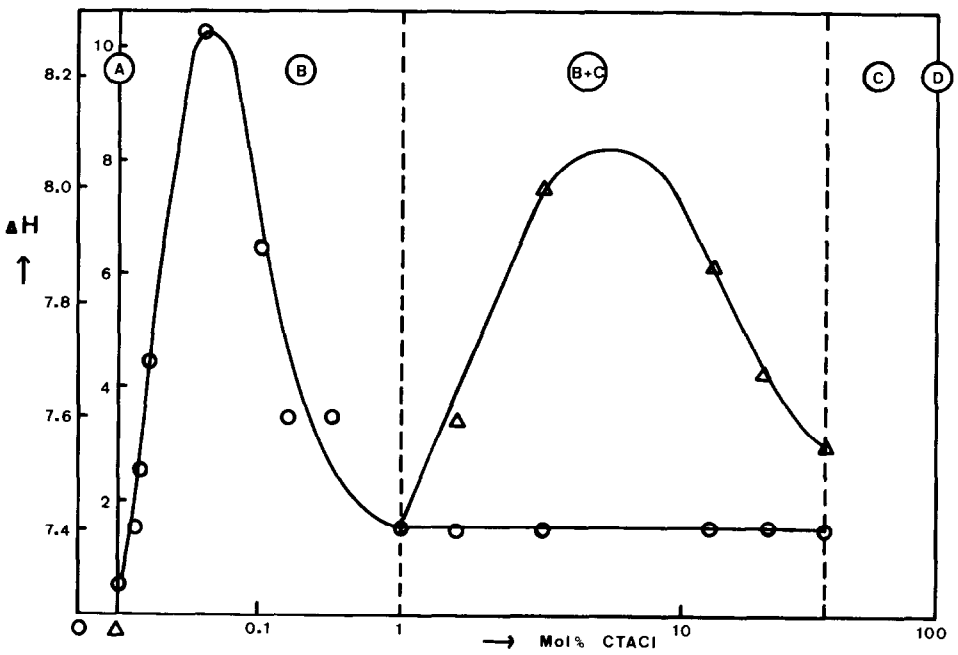

Fig.4. Transition enthalpies (Kcal/Mol) of the lamellar (O) and micellar (Δ) phases.

We are unable to give any explanation for this certainly enormous effect at the present, which is even stronger than the one described for the peptide melittin (ref.1), which in any case, is a much bulkier molecule, and has only the pronounced head-tail amphiphilicity in common with the detergent CTACl.

Another point for discussion is the low ΔH value of 7,3 Kcal/Mol for pure DPPC. Although ΔH values are known to scatter considerably for different preparations this is a remarkably low value, and certainly a reliable quantitative assessment of this part of the phase diagram cannot be given before more comprehensive data

are collected, calorimetrically and with X-ray methods etc., taking special care for the purity of the phospholipid preparation.

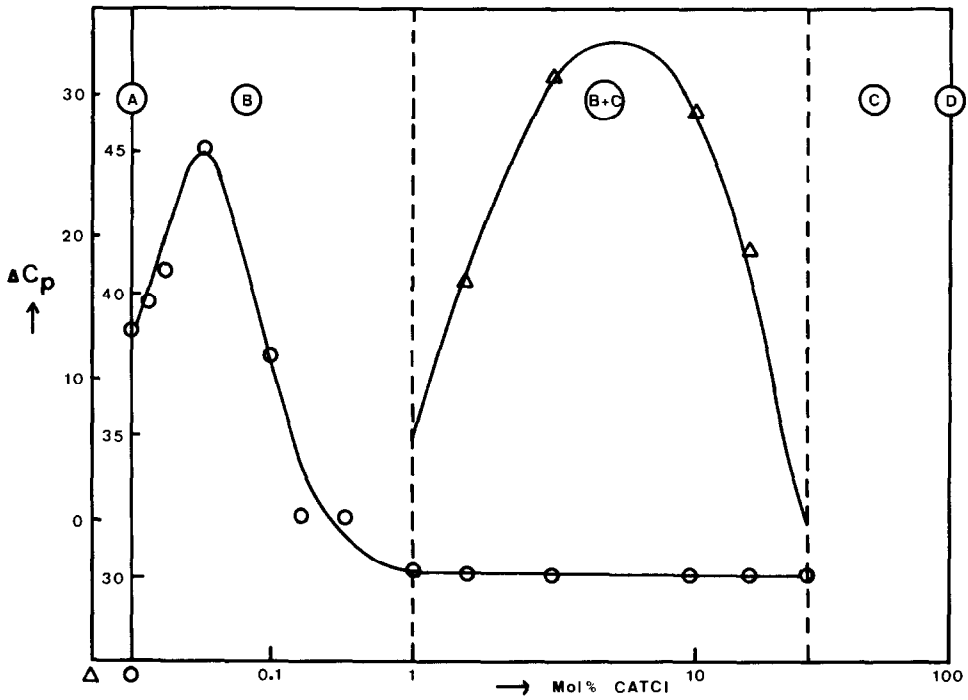

Fig. 5. Maximum excess heat capacities (cal/°K,g) for the lamellar (O) and micellar (Δ) transitions.

Finishing the discussion of this part of the phase diagram we can clearly state, that even smallest amounts of detergent show considerable effects on the thermodynamic parameters of DPPC-multilamellar dispersions. Without understanding at present the molecular details we call the result of detergent incorporation in this range of mixing ratios a "modified lamellar phase" assigned as phase B in Figs. 3, 4 and 5.

Increasing the detergent concentration above 1 Mol % results in an increase in turbidity and a separation of the main transition peak into two components, the one staying at constant temperature (41,52° C) and steadily decreasing in peak area and the other one continuously shifted upwards reaching 43,7° C at 50 Mol % (see Figs. 2 and 3). This newly formed signal, which is only present between 1

and 50 Mol % CTACl is assigned by the index 3 and goes through an intensity maximum around 10 Mol % detergent, which coincides approximately with the second turbidity maximum.

Considering the temperature as the most sensitive indicator for compositional changes of a mixed system we conclude from the invariance of T_2 in this part of the phase diagram, that a phase separation occurs and the system passes through a two-phase region consisting of phase B, i. e. the modified lamellar phase and a new phase formed when 1 % CTACl concentration is exceeded. This new phase is called phase C and is thought to take up all the increasing amounts of detergent while phase B remains at constant composition as reflected by its constant value T_2. Under this assumption it is possible to calculate the amounts of phase B and C being in equilibrium with each other since we consider its molar ΔH constant and consequently take the area under peak 2 as a quantitative measure for the molar amount of phase C present in the mixture.

This is quite easily done in these cases where peaks 2 and 3 are already fully separated. For 2 and 5 Mol % it is first necessary to calculate the individual contributions of peaks 2 and 3 by a decomposition routine. Assuming two state processes the individual components are approximated by van't Hoff curves which are multiplied by solution coefficients chosen by an iterative procedure so that the sum of the components shows minimum deviation from the experimental data. This method has been used and described in an earlier communication (ref.9).

It is now possible to calculate a weight fraction w_2 (listed in Table 1), and consequently the difference of total material minus that part coexisting in form of phase B is then the amount of phase C, for which ΔH and $\Delta C_{p,max}$ now can be determined. Figs. 3, 4 and 5 demonstrate the fact that again, like in region B of the diagrams, T_3 increases continuously with increasing detergent concentration whereas ΔH and $\Delta C_{p,max}$ go through a maximum.

Phase B, which obviously is saturated at 1 Mol % CTACl, decreases in amount and seems to completely disappear around 50 Mol % detergent. This is evident from the disappearence of peak 2 characteristic for the chain melting in a cooperative multilamellar phospholipid arrangement. Instead, phase C has to increase in amount. The nature of phase C, in structural terms, is presently unclear and necessitates further studies. The most straight forward explanation could well be a continuous breaking-up process of the liposomes resulting in large mixed micelles of also decreasing size as the detergent concentration raises. How this expected behaviour parallels with the thermodynamic data in detail is unknown at the present stage. The most confusing fact is that the pretransition is still observed up to about 50 Mol % with its temperature T_1 shifted continuously upwards as also observed for T_2. This is quite unusual since the pretransition is known to be very sensitive for even small amounts of

impurities and is normally broadened and abolished much earlier than in this system.

Above 50 Mol % there is only one single thermal signal left at temperatures varying from 36,5° C to 35° C with intensities varying from approximately five times higher to almost equal that of the pretransition. This signal is not completely abolished before ~90 Mol % CTACl is present, i. e. the point where the samples become finally absolutely clear.

Although it seems impossible that this thermal event has anything to do with the pretransition, the index 1 has been left in the diagrams and in the Table for simplicity. Again it is unknown what happens structurally at this temperature and so we also here do not attempt a quantitative evaluation of this peak and omit ΔH and ΔC_p values. The structures present at such high detergent concentrations are presumably mixed disc micelles with some unperturbed phospholipid domains left capable to undergo some order-disorder transformation. It is also possible that the thermogram reflects some transition from single mixed discs to stacked, multilamellar discs. Anyway, pure phase C is thought to consist of mixed disc micelles which decrease in their lateral dimension with increasing detergent concentration to give finally, above 90 Mol % CTACl, an isotropic solution at small globular mixed micelles analogous to those reported for bile salt/phospholipid mixed systems (ref.10). Pure phase D, i. e. a solution of pure, globular detergent micelles is the final state reached at the end of the Mol % scale.

An additional uncertainty of this purely thermodynamic work is the fact, that we do not know the distribution coefficients of CTACl between the dispersed phospholipid and the aqueous phase. In other words, 10 Mol % CTACl does not necessarily mean a mixing ratio of 90/10 Mol % phospholipid/detergent within a certain structural complex (whatever this is) formed by the two; however, it must be assumed that the actual phospholipid content will be higher than the stoichiometrical one, since parts of the detergent will coexist with the mixed phospholipid detergent complex in form of simple detergent micelles.

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