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A. S. C. Lawrence [a] & M. P. McDonald [a]
[a] The University of Sheffield and Sheffield College of Technology

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The Investigation of Lipid–Water Systems (Part I) by Classical and N.M.R. Methods

A. S. C. LAWRENCE and M. P. McDONALD
The University of Sheffield and Sheffield College of Technology

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Certain lipids form binary liquid crystalline (l.c.) phases with water. This is only one case of a general high degree of interaction between the polar group of the lipid and water which is observed also as unduly large solubilities of water in lipids which are themselves insoluble in water. In the solid state, crystalline hydrates are formed in some cases. These systems are not only of great biological interest but the lipid–water interaction involves a little considered aspect of hydrogen bonding. The nature of these systems is being examined by the study not only of the lipids themselves but also their simple lower homologues.

The lower and intermediate chain length alkanols, fatty acids and alkylamines may be regarded as the simplest amphiphilic prototypes of their higher homologues and of the important diols, phosphatides and cerebrosides which contain two or more polar groups, either non-ionic or ionic. Generally, these substances containing one or two long hydrocarbon chains and terminal polar groups associate by hydrogen bonding or interionic forces between the latter and crystallize in bimolecular layers. Normally however the property of amphiphily describes their behaviour in a solvent; in the commonest case, water, their polar groups are hydrophilic and the hydrocarbon part hydrophobic. In hydrocarbon medium, the reverse holds; the polar groups are oleophobic and the hydrocarbon oleophilic. In this paper we are concerned with the amphiphile water systems and the phases in which they exist especially the binary liquid crystalline one.

Liquid Systems

Information about the solubilities of the intermediate alkanols and fatty acids is scanty and that of water in them even more so; but the general picture is definite for the partially miscible systems, i.e., n-butanol to octanol. Figure 1 shows the phase diagram for the n-butanol–water system *vs* temperature;[1] it is binodal because there is a small decrease of solubility in water with temperature up to about 100° after which there is a slow increase which becomes rapid as the upper consolute temperature of 125.15° is reached. The initial fall has been recorded for a number of isomeric hexanols and heptanols;[2] it rarely appears on the water in alkanol side of

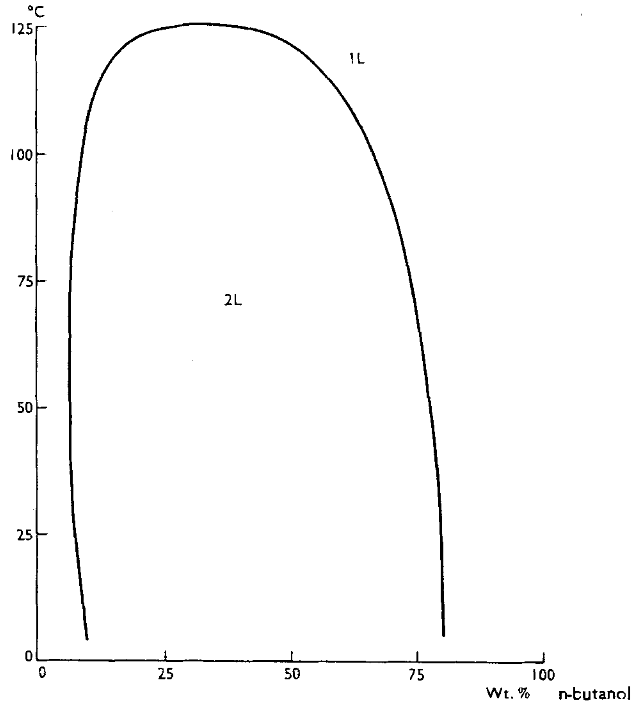


Figure 1. Butanol–water–liquid consolation curve.

the curve. It will be observed that the solubility of water in alkanol is more than twice that of the alkanol in water, a property which is common to all these amphiphiles and which persists in the higher-homologues which, although insoluble in water, still dissolve notable amounts of it. The upper consolute temperature of n-pentanol is 184.5°;[3] its two mutual solubilities are smaller than those of butanol so that the phase diagram is also a flattened inverted U with little change of these solubilities up to about 150° C. This very small change of solubility over a wider temperature is characteristic of the alkanols and fatty acids. Log of solubility in water gives a linear plot against C_n from butanol to octanol but the reverse solubility does not fall off so rapidly. Figure 2 gives the results which show the remarkable finite values for water in the higher homologues; these do not level off when shown on a molar scale. Such figures as are available show a similar picture for the fatty acids.[4]

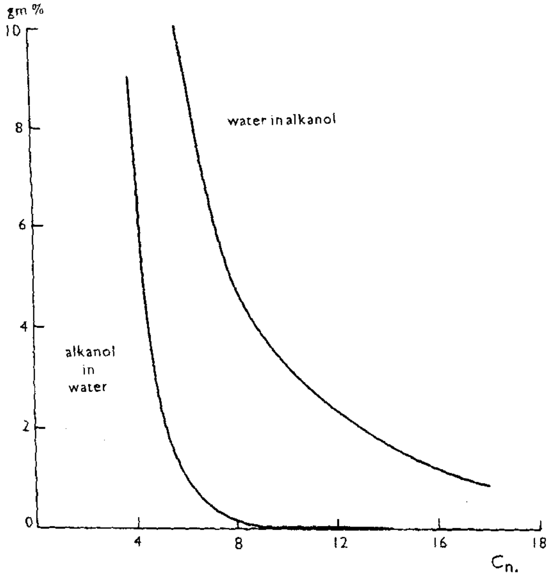


Figure 2. Solubilities of water in higher alcohols.

Solid Systems

It has been reported from this laboratory that penetration of water into the higher fatty acids and alcohols solid at room temperature sets in at a minimum temperature T_{pen} which is a few degrees below the melting point of the anhydrous substance. Freezing points plotted against amount of water show formation of a eutectic of the anhydrous and hydrated species; at saturation the fatty acid curve lies below the anhydrous melting point and for the alkanols above it. Clearly the eutectic minimum temperature T_E is T_{pen}.[5] These eutectic regions on the amphiphile-rich side of the T/C diagram are some 20 to 25° above the freezing point of ice for dodecanol and for decanoic acid; if completion of the diagram were possible and not limited by the small saturation limits, the curve could only fall down to 0° via another eutectic. The digram has been worked out for the t-butanol–water system[6] chosen as the most hydrophobic alkanol completely miscible with water and the results are shown in Fig. 3. The nearly flat-topped compound formation hump between the eutectic makes assignment of specific hydrates dubious. There are some cases where hydrates of unexpectedly high melting points have been observed. In 1859 Fittig reported the solid hexahydrate of pinacol;[7] Pushin and Glagoleva[1] examined this system and found two eutectics and a monohydrate as well as the hexahydrate, the former melting at 41.25° and the hexahydrate at 46.5°. Pinacol melts at 41.4°. Pentamethyl ethanol, which melts at 17°, forms a hemihydrate melting at 83°. Hatt,[8] in his review of the crystalline hydrates of alcohols and glycols, has drawn attention to the importance of steric factors; both in respect of the need for room for the water molecule and of the symmetry of the hydrate for crystal building.

Liquid Crystalline Systems

It has been reported already that the n-aliphatic amines[9] take up much larger amounts of water, although poorly soluble in it, and from C_6 upwards form a binary l.c. phase over a certain concentra-

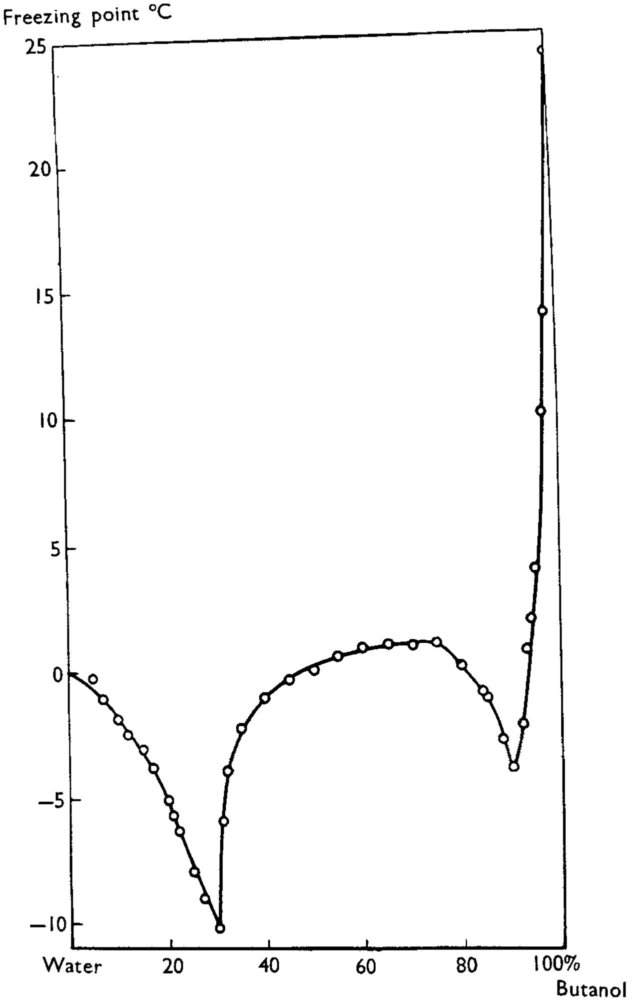


Figure 3. Binary liquid–solid diagram for t-butanol–water system.

tion range. For example, n-octylamine dissolves 27% and is l.c. from 27% to 79% water at 25°; these substances also show a lower consolute temperature change to two liquids on heating.[10] Diols such as the 1-monoglycerides and hexadecane 1,2-diol show similar behaviour with a T_{pen} below the melting point of the pure substance; α-hydroxy-hexadecanoic acid is penetrated by water at its melting point to form a binary l.c. phase.

The Monolaurin–Water System

Monolaurin was chosen for detailed phase equilibrium study; the solid–l.c. and the l.c.–liquid phase boundaries were established; the saturation value by water was determined and for the higher water concentrations separation into two isotropic phases was observed (Fig. 4). The crystallization of the solid was retarded by serious supercooling so that this transition had to be measured by heating the solid. The l.c. phase was extremely viscous until just below its melting point where it is seen to appear in the liquid as batonnets; the liquid phase however is fluid and excess of water above saturation is seen easily. An unusual feature is the broken line showing the metastable l.c. phase appearing below the freezing point of solid; this is yet another case in which supercooling of the solid but not for the l.c. phase has been observed. All specimens were examined in sealed tubes to prevent evaporation of water and this was obligatory for the transitions above 100°. Some observations were made on very small amounts beneath a $\frac{5}{8}$ in. diameter coverglass sealed down on to a standard microscope slide by Sellotape and observed on the heating stage of the polarizing microscope: no indication of more than one l.c. phase was observed.

The solid β modification of monolaurin melts at 61° and addition of water lowers this to the eutectic minimum of 41°, previously recorded as the T_{pen},[5] after which the phase boundary rises to a peak indicating the formation of a monohydrate and then falls again and levels off up to saturation. There is no indication of formation of a higher hydrate, such as the hexahydrate of pinacol, but neither is there apparent the free water which should be present if it is not bound as a higher hydrate. On heating, the solid passes to a binary l.c. phase which at a higher temperature melts to isotropic liquid. The temperature at which this transition occurs rises steeply and linearly with increase of water at first but above about 100°, the rate of increase falls off rapidly and finally the curve levels off as the saturation value of 47% water is approached. Here the limit of existence of the l.c. phase is 55° above the melting point of the anhydrous substance. When more than 39% of water is present, the liquid phase separates into two liquids on further heating; the temperature at which this transition occurs rises very

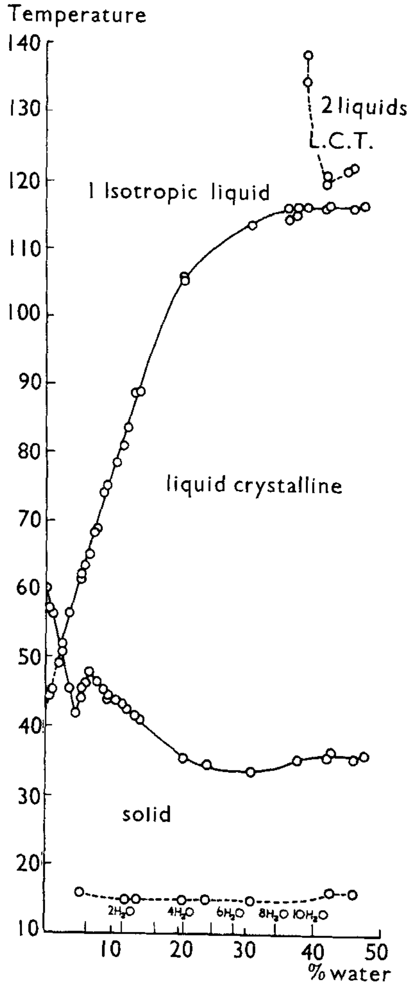


Figure 4. Monolaurin–water phase diagram.

rapidly with decrease of water content and the mixture containing 30% remained clear at 200°. The lower consolute temperature is 119 and the composition 42% of water; with more, the curve rises again but this arm of the classical U curve is terminated by the 47% saturation limit.

If the l.c. phase is chilled in cold running water, it solidifies after about 15 min and this solid, when heated slowly, passes to l.c. at the temperatures indicated by the lower horizontal broken line; i.e. at 15 to 16°. It would appear that super-cooling of the stable solid allows this metastable one to form and it is to be observed that the phase change sub-α_L to α_L in the anhydrous monolaurin occurs at 15°. Volume changes are notable in the monolaurin–water mixtures in this temperature region. There are indications of a eutectic at the lower water concentrations but observation of the phase change from solid to liquid is extremely difficult because of the extremely high viscosity of the l.c. phase at this low temperature.

N.M.R. Spectra

(a) *Monolaurin–Water*

Measurements were made on the A.E.I. R.S.2 dual purpose n.m.r. spectrometer at a temperature of 30° C except where otherwise stated.

The values of the second moment given are each the mean of six spectra corrected for the effect of modulation together with the standard mean deviation. A narrow line is observed, superimposed on the broad line in the spectra of the monolaurin–water and pinacol–water systems. It probably includes both free water and some uncrystallized organic material since the line is still present in the diol–deuterium oxide systems and the measurements are all within 15° C of the melting points of these materials.

The narrow line was ignored in the second moment calculations and the broad line shape in that region obtained by extrapolation from the broad peaks through the origin.

Table 1 shows the second moments obtained of monolaurin anhydrous and with one and six molecules of water. Dr. Chapman of Unilever Research (U.K.) has studied the broad line spectra of a number of glycerides including monomyristin and monopalmitin.[11] Our sample of monolaurin was in the β form, m.p. 61° C and the spectrum and second moment are very similar to those of the corresponding forms of monomyristin and monopalmitin observed by Chapman. For a rigid lattice he estimated a second moment of *c.* 25 G^2 for these types of material. The smaller values obtained in room temperature measurements by ourselves and Chapman are probably due to reorientation of the end methyl group at a rate sufficient to cause narrowing and also torsional oscillation of the molecule as a whole about the long chain axis.

TABLE 1 Second Moments Monolaurin–Water System (in G^2)

	Monolaurin	Monolaurin $+ H_2O$	Monolaurin $+ 6H_2O$	Monolaurin $+ 6D_2O$
Solid	17.62 ± 0.47	19.41 ± 0.31	19.17 ± 0.43	17.10 ± 0.71
Liquid crystalline		1.29 ± 0.05	0.77 ± 0.04	

The solids formed on crystallization of mixtures of monolaurin with one and six molecules of water have similar line widths to that of the anhydrous monolaurin.

Table 1 shows that the second moments of hydrated monolaurin are identical within experimental error and are both significantly higher than that of anhydrous monolaurin. The second moment of monolaurin $+ 6D_2O$ is within experimental error of the anhydrous monolaurin value.

Figure 5 shows the spectra obtained from the l.c. phases of monolaurin $+ H_2O$ and monolaurin $+ 6H_2O$ at 30° C. The second moments were respectively 1.29 ± 0.05 and 0.77 ± 0.04 G^2 as shown in Table 1.

Although these systems are metastable at 20° C the solid takes so long to crystallize that the l.c. phase sometimes remains for days at this temperature of 10° C below its freezing point.

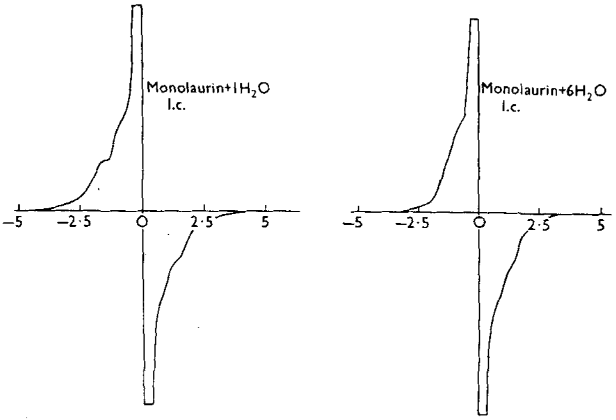


Figure 5. N.m.r. spectra of l.c. phase.

The spectra both appear to have four component lines: the very narrow liquid line and three others symmetrically about the centre with $\Delta H = 1.5$, 2.9, and 4.2 G in the case of the monohydrate and 1.5, 2.1, and 3.1 G in monolaurin + $6H_2O$.

Unfortunately the sensitivity of the variable temperature probe at the time of these measurements was much lower than that of the normal probe and therefore a higher modulation had to be used to bring up the broad line to a useful amplitude. The extent of this modulation—1 G—is such as to destroy the fine structure of the line and so this was not visible in these spectra. When the smaller modulation was used—0.3 G—the broad line was barely discernible.

The effect of increase in temperature was to reduce the amplitude of the broad lines gradually. No sharp changes were seen under these conditions until the melting point when a residual broad line 1 G wide disappeared at the same moment as the fine structure of the high resolution spectrum appeared. On cooling again the small broad line—1 G wide—reappeared as soon as the fine structure disappeared.

The melting point agreed with that obtained visually in contrast with the results of Dunell[12] on sodium stearate where he found the narrow line of the liquid state appearing some 20° C below the visual melting point.

It is hoped to follow the spectrum in more detail at higher temperatures when a more sensitive variable temperature probe is available.

(b) *Pinacol–Water*

The structures of the simple diol, pinacol and its hydrates are being studied by n.m.r. methods and it is hoped that the information obtained will help in the investigation of the solid and l.c. phases of the monolaurin–water system.

Table 2 shows the second moments of pinacol, its mono and hexahydrate and hexadeuterio hydrate.

TABLE 2 Second Moments Pinacol–Water System (in G^2)

Pinacol	7.16 ± 0.21
Pinacol. H_2O	3.67 ± 0.05
Pinacol. $6H_2O$	8.81 ± 0.22
Pinacol. $6D_2O$	7.04 ± 0.38

Discussion of N.M.R. Results

(a) *Monolaurin–Water*

The values of the second moment obtained give some support to the phase diagram in suggesting that monolaurin forms a definite monohydrate. If the water is located rigidly we would expect an additional 1.5 G^2 on the second moment due to the interaction of the two water protons with each other. Since the water is almost certain to be located near the two OH groups of the glyceride, hydrogen bonding will take place and the second moment will increase further from this interaction. Larsson in his X-ray work on the structure of glycerides was not able to say exactly how the end groups of monoglycerides are located in the lattice although there are indications that only the OH groups are involved in the hydrogen bonding and not the ester oxygen atoms.[13]

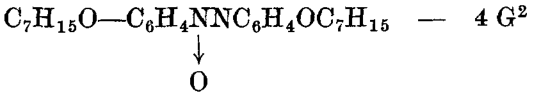
We find it difficult to understand the way in which the water is held in the solid state above concentrations of 1 H_2O : 1 monolaurin.

Microscopic examination of the 6:1 mixture showed no trace of free water. The sample showed very little birefringence and behaved more like a wax when pressure was applied to the coverslip of the microscope slide.

The fact that the second moment remains stationary above one molecule of water per molecule of monolaurin also suggests that the water is not entering into the lattice structure of a higher hydrate. This could be confirmed by looking at the deuterium resonance in a mixture monolaurin + $6D_2O$ to see whether it is a narrow line or a broad line.

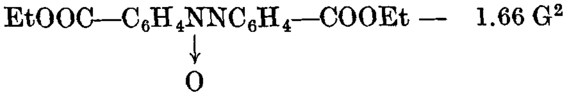
No conclusions can be drawn from the n.m.r. spectra of the l.c. phase until the more detailed variable temperature measurements have been made.

It is interesting that the line shape of these spectra resembles those obtained by Weber[14] who together with Lippmann studied the smectic and nematic phases of *p*-azoxy anisole and a number of substituted anisoles. Our second moments are lower than those reported by Weber for the smectic phases of



$$C_7H_{15}O\text{—}C_6H_4\underset{\overset{\downarrow}{O}}{N}NC_6H_4OC_7H_{15} \quad \text{—} \quad 4\ G^2$$

and



$$EtOOC\text{—}C_6H_4\underset{\overset{\downarrow}{O}}{N}NC_6H_4\text{—}COOEt \quad \text{—} \quad 1.66\ G^2$$

Since the l.c. sample of monolaurin + $6D_2O$ gives a similar line shape and second moment to monolaurin + $6H_2O$ it is assumed that none of the wider lines arise from the protons of the water molecules.

(b) *Pinacol–Water*

The structural units of pinacol contributing to the second moment are four methyl groups and two OH groups. The second moment of a rigid methyl group is 21.7 G^2 and if it is rotating 5.42. It is quite clear that then the methyl groups in pinacol are rotating freely about their C3 axes at this temperature. If we subtract the proportional second moment ($\frac{6}{7} \times 5.42$) from 7.16 we are left with 2.51 G^2 to account for.

External interaction of the methyl groups with each other will add up to not more than 0.5 G^2. The interaction of the OH groups with the methyl groups will only be small if they are rotating freely. The remaining second moment will therefore be mainly due to the interaction of the OH protons with each other and/or the OH groups of neighbouring molecules.

Unfortunately there are no fully worked out crystal structures for any aliphatic diols and so we have little notion of the conformation of the OH groups. It seems certain however that there will be some hydrogen bonding in these structures and this should be sufficient to account for the remaining 2 G^2 second moment.

Rushworth[15] found the second moment of ethylene glycol to be 21 G^2 with $\Delta H = 14$ G at 100°K but this decreased to a much lower value at 60°C below the melting point corresponding to $\Delta H = 0.7$ G. The second moment of an isolated $-CH_2-$ group (rigid) is only 11 G^2 so in this molecule there are obviously appreciable hydrogen bonding interactions in the rigid lattice which probably account for most of the extra 10 G^2 second moment although there will be larger interactions of the OH groups with methylene than with methyl groups because the methylene groups are not able to rotate.

In chloral hydrate which Bishop and Richards[15] showed to be a gem-diol the theoretical second moment for a single molecule was 3.8 G^2 and the experimental value was 9 G^2. The remaining 5.2 G^2 was here attributed to intermolecular hydrogen bonding interactions.

We consider that one of the reasons for the smaller excess value in this case of pinacol is because of the very small interactions of rotating methyl groups.

The second moment of pinacol monohydrate is much lower than that of the anhydrous pinacol and also appreciably lower than $\frac{6}{8}$ of 5.42 G^2—the proportional contribution of the freely rotating methyl groups. This means that additional modes of motion must be possible in this structure at these temperatures.

If the water molecule was rigidly fixed in the lattice it would itself give a contribution of 3.0 G^2 to the second moment. Hatt has proposed a structure for pinacol monohydrate which shows the molecules linked in infinite chains by water molecules—the OH groups of the pinacol being disposed in the *trans* configuration. This might be expected to be a fairly rigid structure and the low value of second moment could be attributed to large scale torsional oscillation or restricted rotation of the whole polymeric chain.

Pentamethyl ethanol hemihydrate has been shown by the X-ray work of Pachler and Stackelberg to have a similar structure to that proposed for pinacol monohydrate. We intend to calculate the theoretical second moment of this alcohol from the X-ray data and compare it with the experimental value 3.23 ± 0.19 G^2—which we have seen is of the same order as that of pinacol monohydrate at room temperature in order to elucidate the nature of the molecular motion taking place.

The second moment of pinacol hexahydrate shows an appreciable increase which does not occur in the hexadeuteriohydrate. X-ray pictures by Dr. A. J. Smith of Sheffield University, Chemistry Department show that the structures of the hexahydrate and anhydrous pinacol are fairly similar but both appreciably different from that of the monohydrate.

We consider it very probable that in anhydrous pinacol and the hexahydrate the OH groups are in the *cis* configuration so that an intramolecular hydrogen bond is formed. It may be possible for the pinacol molecules to link up in the anhydrous state by hydrogen bonding between pairs of OH groups and it seems more likely that larger amounts of water sufficient to form a hexahydrate may be contained in a sandwich structure between alternate layers of pinacol molecules in this configuration. The unchanged second moment of pinacol hexadeuteriohydrate also supports this argument.

The higher second moments of anhydrous pinacol and the hexahydrate suggest in general a more rigid structure than in the monohydrate and this is borne out to some extent in the values of the melting points of the three substances quoted earlier.

Discussion

Lyotropic binary l.c. phases are formed by the pentration of water into a variety of amphiphilic substances; this penetration leads to extrusion of myelin forms which are strongly birefringent.[18] These appear to be in the true smectic layer state with the layers bent into coaxial cylinders which flow telescopically; the viscous resistance to flow is small and occasionally ephemeral focal ionic structure has been seen in the extruding cylinders. The unit of structure is the bimolecular sandwich of water in amphiphile, the polar groups of the latter being orientated inwards as described earlier by one of us (Lawrence).[19] For those who wish to examine or demonstrate myelin extrusion, it should be noted that the cross-section is circular and the width of the forms seen microscopically is therefore determined by the thickness of the specimen; the cover glass should therefore not be pressed down. The conditions for the formation of a l.c. water in amphiphilic lipid phase are simple in many cases; firstly, that chain length is such that solution of the whole molecule has become thermodynamically unfavourable, i.e. at a minimum of C_5 to C_6 which is also a length such that van der Waals forces bind the chains in a two-dimensional layer lattice; and, secondly, that above T_{pen} entry of a considerable amount of water between the polar groups is thermodynamically favourable. We do not know what is the minimum amount of water required to allow slip in our bimolecular sandwich though clearly this would need to be of polymolecular thickness which is not approached by the alkanols and acids. Figure 6 gives a diagrammatic picture approximately to scale of the degree of swelling in the alkanols and fatty acids, the diols (and alkylamines) and the increased effect by the ionic soap upon the swelling of the alkanols and fatty acids. In the phosphatides and cerebrosides we have ionic binding by the P^- and a residue of choline, ethanolamine or serine with N^+ or the non-ionic inositol which is strongly hydrophilic; the inositol is strictly similar to the polyethylene oxide non-ionic soaps which also form a binary l.c. phase with water. The soaps also form a binary l.c. phase with water but they are soluble in it as isotropic solution up to the point at which middle soap l.c. phase appears; this concentration falls with increasing hydrocarbon chain length and is in fact a saturation point. Below C_8 the l.c. phase is not formed.[20] The phosphatides and cerebrosides have very high melting points[21]

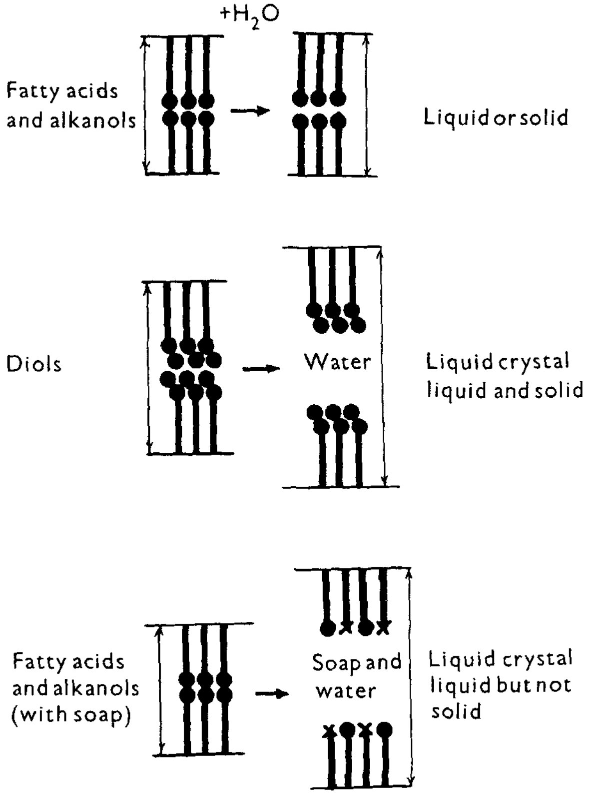


Figure 6. Penetration of water into amphiphiles.

due to their ionic polar groups but their two long hydrocarbon chains prevent them from dissolving in water. Their swelling to form an l.c. phase has long been known.[22]

The valuable method of examining a homologous series of amphiphiles has the defect that, at a fixed temperature, the members are not in corresponding state. However, values for T_{pen} do clearly run parallel with melting points. It is well known that increase of chain length in paraffin chain amphiphiles increases melting point and correspondingly reduces solubility; the Krafft point temperature of soaps increases linearly with chain length;[20] it is equally well known that chain branching increases solubility and that the resultant solubility depends upon the longest straight chain in the molecule. In the mono-ethanoid chain substances, the *cis* form has substantially lower melting point and higher solubility than its *trans* isomer. Most long chain amphiphiles exhibit polymorphism, the 2-monoglycerides being a conspicuous exception.

The existence of a T_{pen} below which water does not enter amphiphiles and its parallelism with melting point suggests strongly the need for a loosening of the solid lattice and we must ask whether there is in fact a sharp transition in the anhydrous amphiphile at T_{pen}. Bernal showed that water entered dodecanol at 16° and the hydrated form was hexagonal whereas the anhydrous substance is monoclinic,[23] we have repeated this and found the transition to be reversible; X-ray diffraction showed the hexagonal form above T_{pen} and the same specimen cooled below 16° gave the monoclinic spacings. Andrew[24] has used n.m.r. broad line spectroscopy upon anhydrous dodecanol and found the hexagonal form between the melting point of 24° and 16°; he concluded that the long chain molecules were rotating about their long axes as hydrocarbons do below their melting point. The anhydrous monoglycerides have also been examined by n.m.r., X-ray diffraction and other physical methods; D. Chapman has summarized the position in his recent book which is a mine of valuable information.[25]

Acknowledgments

We wish to express our thanks to Messrs. Unilever for samples of pure monoglycerides and for financial aid to one of us (A.S.C.L.); also to Mrs. J. Stevenson for assistance in the experimental work.

Note Added in Proof

Since this paper was written, we have examined 2-monolaurin and 2-monostearin and find that, like their isomers, they exist in two solid forms easily observed by the microscope. Again, the stable form has the higher melting point and the metastable one can be obtained only from the supercooled melt and it passes to the stable form very much more easily than do the 1-compounds; so much so that we have never succeeded in cooling it below 30°. This explains why previous workers have failed to observe it. By reheating before it has transformed, the melting points and T_{pen} have been observed and are shown in the table.

	Stable form		Metastable form	
Substance	m.p.	T_{pen}	m.p.	T_{pen}
2-monopalmitin	68	48.5	58	34
2-monostearin	73	53.5	55	

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