Hoppe-Seyler's Z. Physiol. Chem.
Bd. 357, S. 21 - 33, Januar 1976

Lipid-Lipid and Lipid-Protein Interactions as Studied with a Novel Type of Fluorescent Fatty Acid and Phospholipid Probes

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(Received 3 November 1975)

Summary: A novel fluorescent-labelled group of fatty acids and phospholipids has been applied to determine phase transitions in liposomes by fluorescence intensity and polarisation measurements. The chromophore of these amphiphilic lipids proved to be very suitable to demonstrate temperature-dependent lipid-lipid interactions. Liposomes from 1,2-dipalmitoyl-3-*sn*-glycerophosphocholine, 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine and from lipids isolated from membranes of *E. coli* K 1062 mutant grown on elaidic acid were used in these studies.

These probes also made it possible to observe conformational changes in membrane proteins in isolated plasma membranes from this mutant. The changes in protein conformation were dependent on structural changes in the lipid phase.

Untersuchung von Lipid-Lipid- und Lipid-Protein-Wechselwirkungen mit Hilfe neuer fluoreszenzmarkierter Fettsäuren und Phospholipide

Zusammenfassung: Mit Hilfe neuer fluoreszenzmarkierter Fettsäuren und Phospholipide wurden Phasenumwandlungen an Liposomen durch Fluoreszenzintensitäts- und Fluoreszenzpolarisationsmessungen bestimmt. Der Chromophor in diesen amphiphilen Lipiden erwies sich als sehr geeignet, temperaturabhängige Lipid-Lipid-Wechselwirkungen anzuzeigen. Sie wurden in Liposomen aus 1,2-Dipalmitoyl-3-*sn*-glycerophosphocholin, aus 1-Stearoyl-2-oleoyl-3-*sn*-glycerophosphoäthanolamin sowie an solchen, die aus Membranlipiden der auf Elaidinsäure gewachsenen *E. coli* Bakterienmutante K 1062 hergestellt wurden, bestimmt. Ferner wurden die Lipid-Protein-Wechselwirkungen in der Plasmamembran dieser Mutante mit den Fluorophoren untersucht.

Key words: Fluorescent fatty acids, phospholipids, lipid-lipid interactions, lipid-protein interactions, phase transition temperatures, cooperative conformational changes, membranes.

Enzymes:
Acyl-CoA synthetase, acid : CoA ligase (AMP-forming) (EC 6.2.1.3);
Glycerolphosphate acyltransferase, acyl-CoA : *sn*-glycerol-3-phosphate *O*-acyltransferase (EC 2.3.1.15).

Abbreviations:
PC, phosphatidylcholine; PE, phosphatidylethanolamine; examples for the short-hand nomenclature of the novel ω-anthracene-labelled and of the normal fatty acids are 16 : 0/16 : 0-PC = 1,2-dipalmitoyl-3-*sn*-glycerophosphocholine; $18{:}2^{9,12}$/A-$15{:}2^{10,13}$-PC = 1-linoleoyl-2-[15-(9-anthryl)-10*c*,13*c*-pentadecadienoyl]-3-*sn*-glycerophosphocholine.

Fluorescence technique has been applied for the study of the structure and dynamics of macromolecules and membranes. Valuable information has been obtained because of the sensitivity and versatility of this method[1,2]. The emission properties (wavelength, quantum yield, life time) of an excited fluorophore allow interpretations of the polarity, microviscosity and conformational changes of the domain structures[3,4]. Measurement of fluorescence polarisation determines the locus of the probe, the microviscosity, orientation and mobility of molecules in membranes[5-10]. The transfer of excitation energy from a donor to an acceptor chromophore indicates the distance between these groups and conformational changes which might occur[11-14]. Fluorescent chromophores of hydrophilic (polar) and hydrophobic (apolar) structures have been used to probe the hydrophilic and hydrophobic regions of amphiphilic macromolecular structures. 8-Anilino-1-naphthalene sulfonate is one of the most commonly used fluorescent probes. Changes in the polarity of its surroundings are accompanied by alterations in the fluorescence maximum and quantum yield. This reagent has been used to study the polarity of membranes[15-18], phase transitions in lipid dispersions and bilayers and *Escherichia coli* membranes[19]. Its fluorescence polarisation and lifetime have been measured to reveal changes in microviscosity in axonal membranes as a function of the action potential[20,21].

X-ray studies suggest that 8-anilino-1-naphthalenesulfonate is deposited between the polar head groups and the bordering apolar part of phospholipids[22]. *N*-Phenyl-1-naphthylamine proved to be a useful chromophore to probe phase transitions of phospholipid bilayers and natural membranes [23-25]. Other apolar fluorescent probes such as 2-methylanthracene, 9-vinylanthracene and perylene have been applied in comparative fluorescence polarisation studies on phase transitions[10,26-31]. The aforementioned probes and also pyrene, the eximer formation of which has been used to determine conformational changes in membranes[32-34], are freely diffusible molecules. Fluorescent probes linked covalently to amphiphilic molecules, which are expected to orient in membranes or on macromolecular surfaces such as proteins in a predictable way, were synthesised by Waggoner and Stryer[32], one with the probe at the hydrophobic site, 12-(9-anthryl carbonyloxy)stearic acid and two at the polar head groups, *N*-dansylphosphatidylethanolamine and octadecylnaphthylaminesulfonate. X-ray data[22] have shown that the anthracene ring of 12-(9-anthrylcarbonyloxy)stearic acid is located in the hydrocarbon phase, whereas the naphthalene ring of octadecylnaphthylaminesulfonate is outside the hydrocarbon core. 12-(9-Anthrylcarbonyloxy)stearic acid has been recently applied to the study of lipid-protein interactions in the virus envelope[33] and the influence of cholesterol on membranes[34].

The aim of this publication is to present the application of a group of novel fluorescent probes, namely of ω-anthracene-labelled fatty acids, free or incorporated into lecithin molecules as acyl groups. Their synthesis and properties have been described in the preceding paper[35]. Results of studies of the following systems with these probes are reported: phase transitions of liposomes of 1) dipalmitoyl-3-*sn*-glycerophosphocholine, 2) 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine, 3) lipids obtained from the *E. coli* mutant K 1062 grown on elaidic acid, 4) plasma membranes of the same mutant grown on this acid.

Experimental

1,2-Dipalmitoyl-3-*sn*-glycerophosphocholine and 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine were synthesised in this laboratory according to established procedures[36,37]. The ω-anthracene labelled fatty acids, phosphatidylcholines and cholesterol esters were synthesised as described in the preceding paper[35].

Fluorescence spectra, intensity and polarisation measurements were recorded with the Hitachi Perkin Elmer spectrofluorimeter model MPF III equipped with the polarisation filter and temperature-adjustable cuvettes. Time dependence of fluorescence was measured with the ORTEC photon-counting fluorescence lifetime instrument. Filters in the wavelength regions 325 to 380 nm for excitation and 415 nm maximum for emission with a bandwidth of 8 nm were used.

A Philips electron microscope model 300 was used for the control of vesicle size by the negative staining procedure (2% buffered phosphotungstic acid).

Liposomes were prepared according to Huang[38] in a temperature-controlled vessel under an atmosphere of nitrogen with a Branson sonifier model B 12 at the following temperatures: 16:0/16:0-PC, 45 °C; 18:0/18:1-PE, 40 °C; *E. coli* lipid extract, 45 °C. Stand-

ard procedures were used for the determination of protein[39], phosphorus[40] and cholesterol[41]. Concentrations of fluorophores in individual lipid preparations are given under results.

The *E. coli* mutant K 1062 was grown according to Overath et al.[42] in the medium described, supplemented with elaidic acid (100 mg/*l*, 0.1% in Brij 35). Lipids were extracted from whole lyophilised bacteria by chloroform/methanol 2:1 (v/v) and the phospholipid fraction freed from free fatty acids by silicic acid chromatography. *E. coli* plasma membrane preparations of the mutant strain K 1062 were kindly provided by Professor P. Overath, Max-Planck-Institut für Biologie, Tübingen.

Results

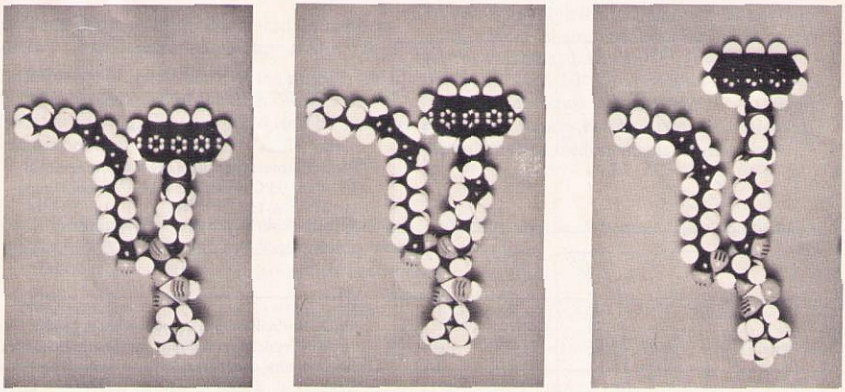
Fluorescence intensity and polarisation were measured in liposomes composed of the different phospholipids (Tables 1 and 2) and phosphatidylcholines labelled in the second position with different ω-anthracene-labelled fatty acids of different chain lengths, thus locating the fluorescent probe at distinct positions in the hydrophobic region of the bilayer. The excitation wavelength was 390 nm; the emission was recorded at 416 nm. The latter was plotted against temperature (increasing and decreasing; maximal temperature change 1 °C/min). The temperature of the sample cuvette was lowered after the complete phase transition.

The phase transition process visualized by recording the fluorescence intensity versus temperature was compared with a similar recording of a) the fluorescence polarisation of the same sample and b) the 90° light scattering at 400 nm. Liposomes without the anthracene-labelled probes were used for the determination of the phase transition temperature by 90° light scattering.

Fluorescence polarisation measurements require clear sample solutions. Vesicle suspensions of 1 mg phospholipid/m*l* form suitable samples and are not subject to depolarisation by fluorescent light scattering.

Fluorescence depolarisation can result from an intrinsic rotation of the chromophore within the bilayer and rotational diffusion of the whole vesicle. The latter effect can, however, be neglected, since vesicles with diameters between 250 to 500 Å possess rotation times of up to 50 μs.

Internal rotation of the probe contributes exclusively to the depolarisation if the lifetime of the probe is below 15 ns[43]. Therefore polarisa-



a) b) c)

Fig. 1. Corey-Pauling-Koltun models of a) 18:$2^{9,12}$/A-8:1^{6}-PC, b) 18:$2^{9,12}$/A-11:$2^{6,9}$-PC, c) 18:$2^{9,12}$/A-15:$2^{10,13}$-PC.

tion and depolarisation directly express the influence of the surrounding medium on the probe. We measured the lifetimes of our fluorescent probes with an ORTEC instrument to be approximately 1 - 3.5 ns. These approximate values agree well with those of related probes e.g. 2-methylanthracene in egg lecithin (lifetime 2.96 ns[29].

The degree of polarisation P of our probes in the vesicle suspension was measured as a function of temperature. The chromophore was excited at 390 nm, the longest absorption wavelength at which the absorption and emission oscillator are in parallel arrangement. Fig. 1 resembles the Corey-Pauling-Koltun models of the three phosphatidylcholines substituted in the 1-position with linoleic acid and in the 2-position with the ω-anthracene-labelled fatty acids and used in the subsequent studies.

1) 16:0/16:0-PC vesicles

1,2-Dipalmitoyl-3-*sn*-glycerophosphocholine (16:0/16:0-PC) vesicles are by far the most extensively studied artificial membrane preparations. Therefore they appeared to be particularly suitable to test the properties and capabilities of our fluorescent probes. Fluorescence intensity measurements were carried out on 2×10^{-4}M 16:0/16:0-PC vesicle suspensions containing 3×10^{-6}M $18{:}2^{9,12}$/A-fatty-acid-PC, Fig. 2.

The influence of the fluorescent-labelled fatty acids of different chain lengths on the phase transition temperature of these bilayers was compared with the light-scattering curves, Fig. 3. For this reason, the curves were normalised to transition degree curves (Θ), defined as $\Theta = \frac{a}{a+b}$. They were plotted as a function of temperature. Θ increases or decreases between 0 and 1 with respec-

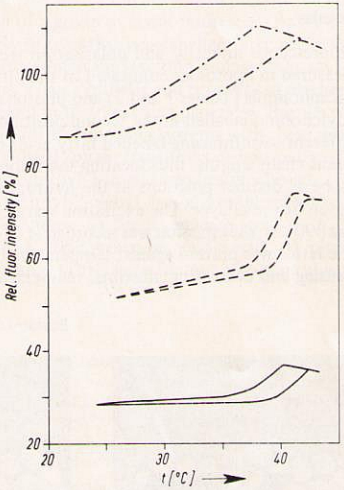


Fig. 2. Fluorescence intensity measurements of 16:0/16:0-PC vesicles (2×10^{-4}M) containing $18{:}2^{9,12}$/A-15:$2^{10,13}$-PC ——, $18{:}2^{9,12}$/A-11:$2^{6,9}$-PC – – –, $18{:}2^{9,12}$/A-8:1^{6}-PC –·–·–.

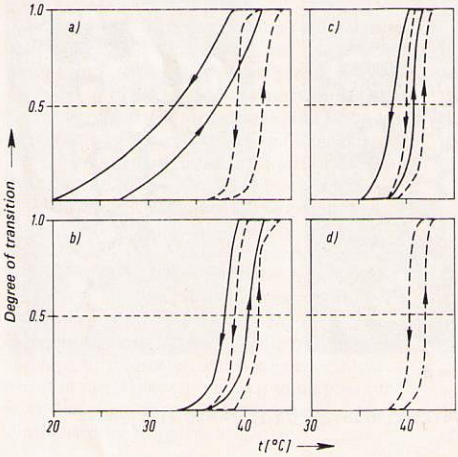


Fig. 3. Normalised phase transitions in 16:0/16:0-PC vesicles containing phosphatidylcholines labelled with ω-anthracene fatty acids as probes. ——, Measurement by fluorescence; – – –, measurement by light scattering. a) $18{:}2^{9,12}$/A-8:1^{6}-PC; b) $18{:}2^{9,12}$/A-11:$2^{6,9}$-PC; c) $18{:}2^{9,12}$/A-15^{10,13}-PC; d) without probe; Θ = degree of transition.

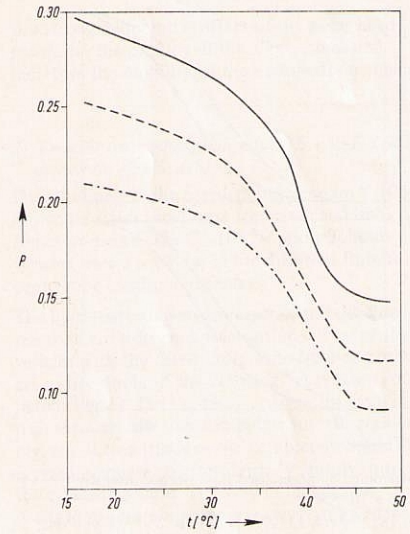


Fig. 4. Polarisation of fluorophores in 16:0/16:0-PC vesicles with $18:2^{9,12}$/A-$15:2^{10,13}$-PC ——, $18:2^{9,12}$/A-$11:2^{6,9}$-PC – – –, $18:2^{9,12}$/A-$8:1^{6}$-PC –·–·– as probes.

tive temperature changes. The transition temperature t_t is defined as the temperature at $\Theta = 0.5$.

The influence of the distance of the probe from the glycerol backbone of the PC-molecules on the phase transition of the same 16:0/16:0-PC vesicles becomes apparent from Table 1. Table 1 also presents the fluorescence polarisation values of the liposomes with the same PC species, substituted with ω-anthracene-labelled fatty acids, in 16:0/16:0-PC vesicles (16:0/16:0-PC concentration 1×10^{-3}M; anthracene-labelled PC, 3×10^{-6}M). The polarisation values of all three anthracene lecithins decrease rapidly in the temperature interval of the phase transition, Fig. 4. Before the phase transition is initiated and after it is completed, the polarisation curves are linear. The transition curves can therefore be normalised as shown for the fluorescence intensity and the degree of polarisation P at the transition temperature t_t determined (Table 1). t_t deduced from the normalised polarisation curves differs only very slightly when the lecithins with the anthracene residue group at the methyl terminal end of the longer chain acyl residues $18:2^{9,12}$/A-$15:2^{10,13}$- and $18:2^{9,12}$/A-$11:2^{6,9}$-PC were used as probes. However a lower t_t is obtained with the $18:2^{9,12}$/A-$8:1^{6}$-PC as probe in the fluorescence intensity measurements.

All phase-transition temperatures obtained from fluorescence intensity and polarisation measurements are shifted towards lower temperatures as compared to the light scattering data (Table 1).

2) 1-Stearoyl-2-oleoyl-3-sn-glycerophosphoethanolamine (18:0/18:1-PE) vesicles

This phosphatidylethanolamine species has been studied for its phase transitions established in previous monolayer studies[37]. It was used at 10^{-4}M with 3×10^{-6}M anthracene-labelled PCs for the fluorescence intensity and 10^{-3}M PE and 3×10^{-6}M fluorescent probe concentrations for the polarisation measurements, Fig. 5. Fig. 5

Table 1. Transition temperature t_t of dipalmitoyllecithin (16:0/16:0-PC) vesicles determined from normalised light scattering, fluorescence intensity and fluorescence polarisation curves.
→: derived from heating curve; ←: derived from cooling curve.

Probe in 16:0/16:0-PC vesicles	Light scattering				Fluorescence intensity				Polarisation		
	t_t →	Δt	t_t ←	Δt	t_t →	Δt	t_t ←	Δt	t_t →	P_t	Δt
$18:2^{9,12}$/A-$15:2^{10,13}$-PC	42.0	0.4	40.2	0.7	40.9	0.8	38.6	2.8	39.7	0.197	5.1
$18:2^{9,12}$/A-$11:2^{6,9}$-PC	41.7	0.8	39.0	0.9	40.8	1.9	38.0	2.4	39.5	0.160	7.8
$18:2^{9,12}$/A-$8:1^{6}$-PC	42.0	1.0	39.1	1.2	37.4	9.8	32.9	14.4	39.5	0.139	10.0
Without probe	42.0	0.4	40.2	0.4							

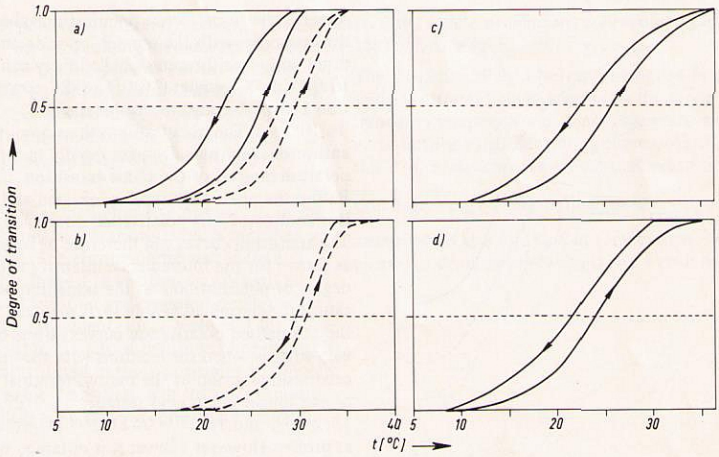


Fig. 5. Phase transitions of 18:0/18:1-PE (10^{-3}M) vesicles determined by normalised fluorescence intensity (——) and light-scattering curves (– – –) with anthracene-labelled PC (3 x 10^{-6}M). 18:0/18:1^9-PE vesicles with a) 18:$2^{9,12}$/A-15:$2^{10,13}$-PC, b) 18:$2^{9,12}$/A-11:$2^{6,9}$-PC, c) 18:$2^{9,12}$/A-8:1^6-PC, d) without fluorescence probe.

again compares the fluorescence intensity, the polarisation and light scattering data of Table 2. The fluorescent lecithins with the anthracene-labelled fatty acids of different chain lengths exert effects on the phase transition of this PE-species similar to those demonstrated for the 16:0/16:0-PC vesicles with respect to phase transition temperature t_t, transition temperature interval Δt and the cooperativity, indicated in hysteresis of the "heating" and "cooling" areas.

Again the degree of polarisation decreases from highest values with 18:$2^{9,12}$/A-15:$2^{10,13}$-PC via 18:$2^{9,12}$/A-11:$2^{7,10}$-PC to 18:$2^{9,12}$/A-8:1^6-PC with lowest polarisation. The polarisation values in vesicles of this 18:0/18:1^9-PE species and 16:0/16:0-PC vesicles prove that the chromophore possesses a reduced mobility in the PE bilayer compared with the 16:0/16:0-PC vesicles, although the PE-molecules contain one monounsaturated fatty acid. We interpret this finding

Table 2. Transition temperature t_t of 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine (18:0/18:1^9-PE) determined from normalised light scattering, fluorescence intensity and polarisation curves.

Probe in 18:0/18:1^9-PE vesicles	Light scattering				Fluorescence intensity				Polarisation		
	t_t $\rightarrow$	Δt	t_t $\leftarrow$	Δt	t_t $\rightarrow$	Δt	t_t $\leftarrow$	Δt	t_t $\rightarrow$	P_t	Δt
18:$2^{9,12}$/A-15:$2^{10,13}$-PC	30.4	7.3	28.8	7.1	26.8	7.5	22.6	10.7	30.2	0.303	7.2
18:$2^{9,12}$/A-11:$2^{6,9}$-PC					25.7	10.4	22.4	14.2	31.1	0.251	9.0
18:$2^{9,12}$/A-8:1^6-PC					24.6	13.0	21.6	14.6	26.0	0.141	14.0
Without anthracene	31.4	7.2	30.4	7.9							

as a strong cooperative effect of the polar head groups on the packing of the alkyl chains and therefore the mobility of the hydrocarbon chains.

3) Vesicles from phospholipids of E. coli K 1062 grown on elaidic acid

Phospholipids of the *E. coli* double mutant K 1062 grown on elaidic acid were sonicated and liposomes prepared. The 2×10^{-4}M phospholipid vesicles were 3×10^{-6}M in the different fluorescent probe lecithin molecules.

The light-scattering measurements and the fluorescence intensity and polarisation curves of the vesicles with the three anthracene-labelled probes are rather similar if the "heating" curves are compared (Fig. 6). The "cooling" curves, however, pass through two transition intervals. On cooling, about two thirds of the completely liquid crystalline phase changes, initially rapidly, into the crystalline state. The system remains unchanged over a temperature interval of more than 10 °C until, on further cooling below 18 °C, the rest of the lipid phase becomes crystalline. The biphasic transition can only be observed in the fluorescence analysis. Table 3 summarises the results of the fluorescence intensity and light scattering measurements of phospholipids from *E. coli* K 1062 grown on elaidic acid.

The transition temperature of phospholipid liposomes from *E. coli* K 1062 phospholipids grown on elaidic acid is determined by fluorescence intensity and light scattering measurements.

The degree of polarisation of $18:2^{9,12}$/-A-$15:2^{10,13}$-PC in *E. coli* phospholipid vesicles is largest; that of $18:2^{9,12}$/A-8:1-PC is smallest. For all three probes, it decreases continuously with increasing temperature, and reaches a constant value over a small temperature range (Fig. 6), but no sharp phase transition as shown with 16:0/16:0-PC and 18:0/18:1-PE could be observed. The narrow range (with increasing temperature) of the phase transition of the elaidic acid-enriched phospholipid extract of *E. coli*

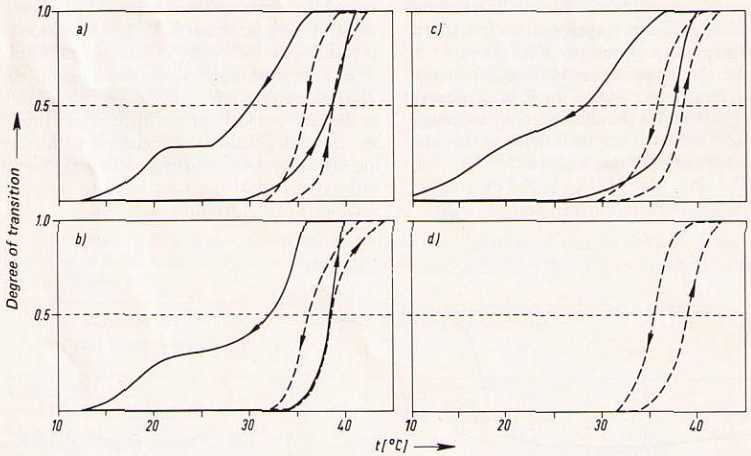


Fig. 6. Transition temperature determined by normalised fluorescence intensity (——) and light-scattering curves (– – –) *of E. coli mutant K 1062 phospholipids (2×10^{-4}M) in vesicles with fluorescence probes (3×10^{-6}M). The* mutant was grown on elaidic acid. $18:1^{9t}$ phospholipids with a) $18:2^{9,12}$/A-$15:2^{10,13}$-PC, b) $18:2^{9,12}$/A-$11:2^{6,9}$-PC, c) $18:2^{9,12}$/A-$8:1^{6}$-PC, d) without probe.

Table 3. Transition temperatures of liposomes formed from phospholipids from *E. coli* 1062 grown on elaidic acid. The transitions were determined by fluorescence intensity and light scattering measurements.

Liposomes *E. coli* K 1062 Probes added	Light scattering				Fluorescence intensity			
	t_t $\rightarrow$	Δt	t_t $\leftarrow$	Δt	t_t $\rightarrow$	Δt	t_t $\leftarrow$	Δt
18:$2^{9,12}$/A-15:$2^{10,13}$-PC	38.6	2.3	35.8	4.0	38.4	6.0	30.0	16.0
18:$2^{9,12}$/A-11:$2^{6,9}$-PC	38.6	3.8	36.0	5.5	38.4	1.2	31.8	18.0
18:$2^{9,12}$/A-8:1^{6}-PC	38.4	3.5	35.6	5.0	37.4	3.7	27.8	21.0
Without fluorescent probe	39.4	5.4	35.2	4.8				

K 1062 is in the light-scattering and fluorescence intensity curves. It is indicative of high cooperativity, and is very different from the two phase transitions observed with continuously decreasing temperature. The combination produces a broad fluorescence hysteresis curve.

4) Fluorescence studies on membranes of E. coli K 1062 grown on elaidic acid

Lipid-protein interactions in the cytoplasmic membranes of *E. coli* K 1062 grown on elaidic acid, with a phospholipid/protein ratio of 0.82, were studied using our novel fluorescent probes.

The three fluorescent-labelled lecithins were sonicated with the membrane preparation in a short burst. The final concentration of label was 3×10^{-6} M; the temperature, 44 °C. Fig. 7 shows the broad hysteresis curve of the light-scattering recording at 400 nm and the high cooperativity. This recording is identical with those of the fluorescence intensity changes with 18:$2^{9,12}$/A-15:$2^{10,13}$-PC, 18:$2^{9,12}$/A-11:$2^{6,9}$-PC and 18:$2^{9,12}$/A-8:1^{6}-PC in the membrane (Fig. 8).

The light-scattering curves of the *E. coli* membrane (Fig. 7) and of the 16:0/16:0-PC vesicles (Fig. 3) have an inverse direction. The intensity of light scattering by the liposomes decreases as the phase transition progresses, whereas that of the membrane increases. Also the free ω-anthracene-labelled fatty acids A-15:$2^{10,13}$ and A-11:$2^{6,9}$ and A-8:1^{6} were used as probes to signal the phase transition in the membrane of *E. coli* K 1062 grown on elaidic acid.

Table 4 summarises the results and indicates that the fluorescent-labelled fatty acids and phosphatidylcholines are equally useful. The long chain chromophore changes the transition temperature only by about 2 °C. The transition temperature increases with the shorter-chain anthracene-labelled phosphatidylcholines incorporated. The polarisation values of the fluorescent lecithins in the phase transition are higher than those of the free fatty acids. They increase with increasing distance of the anthracene moiety from the carboxy group of the ester bond to the glycerol backbone of the lecithin molecule.

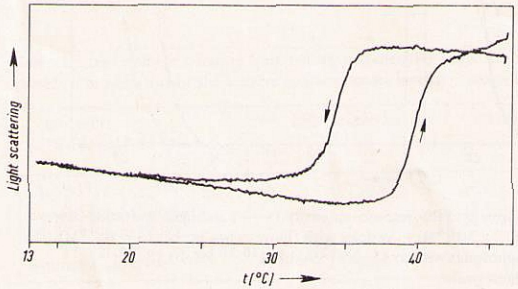


Fig. 7. Transition temperature of membrane isolated from *E. coli* mutant K 1062 grown on elaidic acid. Light-scattering curve.

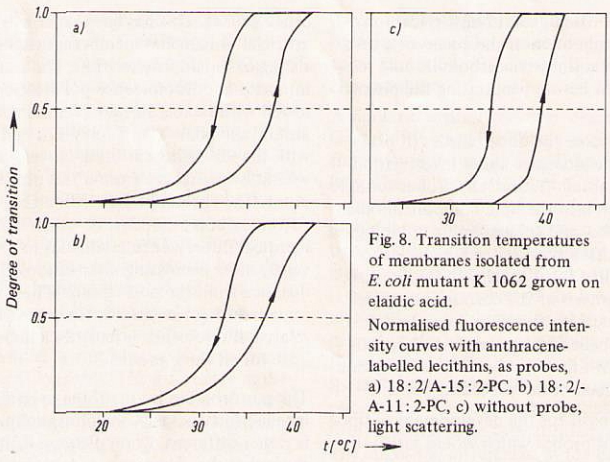


Fig. 8. Transition temperatures of membranes isolated from *E. coli* mutant K 1062 grown on elaidic acid.
Normalised fluorescence intensity curves with anthracene-labelled lecithins, as probes, a) 18:2/A-15:2-PC, b) 18:2/-A-11:2-PC, c) without probe, light scattering.

The fluorescence polarisation of the probes in the membrane as compared to their incorporation in the bacterial phospholipid liposomes also differ markedly. Both the fluorescence intensity on the cooling branch of the hysteresis curve (see e.g. Fig. 4, 5 and 8) and the polarisation curves of the anthracene-labelled compounds in the membrane indicate that lipid-protein interactions do occur. Particularly the increased degree of polarisation, which is even enhanced for the free anthracene-labelled fatty acids, is due to the influence of membrane proteins, as will be discussed later.

Discussion

Most of the fluorescence probes used hitherto have obvious disadvantages for probing artificial and natural macromolecules and molecular arrangements such as liposomes, lipoproteins and membranes. Their association with or incorporation into these structures has in many cases only been deduced from their physical properties (hydrophobicity, hydrophilicity). None of them possesses a chemical structure which is suitable for biosynthetic incorporation into natural mem-

Table 4. Determination of phase transition temperatures by degree of polarisation (P_t), fluorescence intensity und light scattering in *E. coli* K 1062 plasma membrane cosonicated with ω-anthracene-labelled free fatty acids and PC labelled with these acids.

Probes added to membrane preparation	Fluorescence intensity				P_t
	t_t →	Δt	t_t ←	Δt	
A-15:$2^{10,13}$	37.0	6.0	32.8	5.0	0.158
A-8:1^{6}	32.0	21.4	28.4	24.4	0.087
18:$2^{9,12}$/A-15:$2^{10,13}$-PC	37.1	5.8	33.7	6.0	0.241
18:$2^{9,12}$/A-11:$2^{6,9}$-PC	36.1	10.8	33.0	10.4	–
18:$2^{9,12}$/A-8:1^{6}-PC	35.5	7.0	31.8	8.4	0.116
Light scattering Without probes	39.4	2.4	34.0	2.4	–

branes. Also, the often used 12-anthrylcarbonyl-oxystearic acid cannot enter the eucaryotic cells because its bulky anthracenecarboxylic acid residue is hydrolysed before penetrating the plasma membrane*.

In order to overcome the uncertainties of probe localisation in the structure under investigation and possibly to obtain fluorescent probes suitable for incorporation into the lipid components such as glycerophospho- and sphingolipids of biological membranes, we have synthesised a new class of fluorescent-labelled fatty acids, which were also used for the synthesis of the corresponding lecithins. The chemical syntheses of a number of ω-anthracene-labelled fatty acids and phospholipids labelled with these probes in the 2-position have been described in the preceding paper[35].

The theoretical basis for the development of lipophilic fluorescent probes which would satisfy the requirements of a suitable probe was as follows: the tricyclic anthracene moiety would have suitable fluorescence properties. This ring system should be attached to the terminal methyl group of fatty acids with different chain lengths and the desired unsaturation. The known chain length would allow determination of the distance from the glycerophosphate or sphingosine backbone and therefore, probe-defined regions of the lipid phase. Furthermore, there was some hope that the bulky anthracene group at the distant end of the fatty acid molecule would not interfere with the activating thiokinases and acyl-CoA transferases operative in the synthesis of complex phospholipids and sphingolipids. Knoop[44] had derived the principles of fatty acid β-oxidation some seventy years ago with the less bulky phenyl substituted even and odd-numbered fatty acids. Therefore our approach appeared to be worthwhile to pursue. Indeed the ω-anthracene-labelled fatty acids are effectively incorporated biosynthetically into phospholipids of membranes in cells in tissue culture. The results of these applications will be reported in a subsequent paper**.

In this paper we have demonstrated that the ω-anthracene-labelled fatty acids and phospholipids used here are very satisfactory as fluorescence probes. This has been demonstrated in artificial unilamellar membranes (liposomes) consisting of dipalmitoyllecithin. The fluorescence intensity and fluorescence polarisation were followed with increasing and decreasing temperature around the phase transition point and compared with the 90° light scattering curves, with their well known changes around this phase transition point. The chromophores with eight, eleven and fifteen carbon atom chains, which exhibit the identical fluorescence intensities in organic solvents, show decreasing intensities with increasing distance from the polar group. These differences remain during transitions. However the change in relative fluorescence intensities amounts to 20 to 25% for all three probes.

The perturbation by the three ω-anthracene labelled fatty acids in the phospholipid bilayers is rather different. Corey-Pauling-Koltun models show that the chromophore is closer to the polar group in $18:2^{9,12}$/A-$8:1^{6}$-PC than in $18:2^{9,12}$/-A-$11:2^{6,9}$-PC and $18:2^{9,12}$/A-$15:2^{10,13}$-PC. This leads to a wider spacing of the alkane chains, reducing the van der Waals' forces at the carboxy terminal end of the fatty acids and therefore rendering the whole chain more mobile at lower temperatures. This is clearly expressed in broad transition temperature intervals (Δt = 0.8 - 9.8 °C) in the normalised fluorescence intensity. The long-chain anthracene-labelled probe ($18:2^{9,12}$/-A-$15:2^{10,13}$-PC) shows the same sharp transition temperature (Δt = 0.8 °C) as the 90° scattering curve (Δt = 0.4 °C). Also, the fluorescence polarisation of the three probes in lipid bilayers, e.g. dipalmitoyllecithin liposomes, responds very sensitively to changes in the microviscosity of the lipid phase surrounding the probe. The degree of fluorescence polarisation increases with the distance of the chromophore from the polar group. Using the normalised polarisation curves or the normalised fluorescence intensity curves, one can determine the phase transition temperature.

The good agreement between these and the 90° light-scattering results of DPL-vesicles not perturbed by fluorescence probes therefore shows that these new probes can be used to indicate phase transitions in phospholipid bilayers.

The same probes were also tested when integrated in liposomes of a phosphatidylethanolamine spe-

* W. Stoffel, unpublished observation

** W. Stoffel and G. Michaelis, manuscript in preparation.

cies (1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine) with a t_t of 30 °C as derived from monolayer and light scattering studies. Fatty acyl chains in phosphatidylethanolamine are more closely packed than in phosphatidylcholine molecules. Monolayer studies[37] have indicated that this is due to the combination of intermolecular ionic interactions between the polar head groups and the reduced hydration of the phosphorylethanolamine group. As a consequence, a reduced diffusion of ions and small molecules in phosphatidylethanolamine vesicles has also been described[28]. The higher degree of order due to the closer packing of the acyl residues also explains why the degree of polarisation of the anthracene residue is much higher than in the corresponding PC-vesicles. The difference in the degree of polarisation in the two types of vesicles is also observed above the transition temperature. The close packing of PE acyl chains in bilayers also explains the enhanced perturbation by the anthracene probe, which is expressed in the shift of t_t to lower temperature.

The third system studied with our new fluorescent probes was the phospholipid extract of the fatty acid auxotrophic *E. coli* mutant K 1062[42] grown on elaidic acid. This extract, which consisted of more than 70% phosphatidylethanolamine and about 20% phosphatidylglycerol and 10% cardiolipin containing elaidic acid, was sonicated together with the fluorescent probes (A-15 : $2^{10,13}$-PC, A-11 : $2^{6,9}$-PC and A-8 : 1^{6}-PC) and the vesicles were used for temperature-dependent light-scattering fluorescence intensity and polarisation measurements. The light-scattering curves indicated only a cooperative monophasic transition, whereas the three fluorescence probes yield normalised fluorescence intensity curves in which the transition point of the warming up branch coincided well with the respective light-scattering curve, but the cooling branch showed a biphasic transition. About two thirds of the lipid molecules crystallise at approx. 30 °C, whereas the rest passes into the crystalline order below 10 °C.

The sharp transition shown for 16 : 0/16 : 0-PC liposomes is missing. The same was observed for the fluorescence polarisation of the three chromophores. Here too, a broad hysteresis of the fluorescence occurs during the complete transition cycle. The strong broadening of the fluorescence hysteresis caused by the cooling process is due to an inhibition of the crystallisation around the chromophore.

A similar transition has been described by Overath[23] using 8-anilino-1-naphthalenesulfonate as probe in the *E. coli* lipid extract. A lateral phase separation and cluster formation of components which pass over into higher order at higher temperature within the bilayer is suggestive. Whether lipids with different polar head groups are responsible for this process or whether it occurs in both halves of the bilayer or asymmetrically, in only one side of the sandwich, cannot be decided, since nothing is known on the structure of the liposomes formed. Due to the fatty acid composition, it is unlikely that phospholipid species with identical polar groups form clusters, since they form ideal mixtures. The larger the differences in the polar head groups are, and the broader the crystallisation process, the more likely cluster formation will be.

The fluorescence probes were also applied in studies of the lipid-protein interactions in the cytoplasmic membrane of the *E. coli* mutant K 1062 grown on elaidic acid. The phase transition behaviour of the lipid extract of these membranes has been described before[23,42].

The change with temperature in light-scattering intensity of the membranes is the reverse of that observed with 16 : 0/16 : 0-PC or membrane lipid bilayers. Whereas the light-scattering intensity of the lipid bilayer decreases, that of the membrane increases as the phase transition progresses.

The transition of the membrane and the hysteresis of this process is convincingly monitored by the anthracene-labelled fluorescent lecithins, which were integrated into the membrane preparation by short ultrasonication. The long-chain probe (18 : $2^{9,12}$/A-15 : $2^{10,13}$-PC) changes t_t by only 2 °C, compared to the light-scattering results. The transition interval Δt is greater, the closer the anthracene residue is located to the carboxylic group.

The fluorescence polarisation monitors a distinct change in the mobility of the chromophore in the membrane. It is very different from that of the lipid extract, particularly during the cooling process. Therefore an interaction between membrane

lipids which include the chromophore with membrane proteins must be responsible for these changes. The enhanced polarisation of the chromophore in the lipid phase of the membrane is due to the membrane proteins. They apparently prohibit phase separation with cluster formation during crystallisation of the hydrophobic region. Denaturation (heating at 65 °C for 2 min) again reveals the biphasic transition of the fluorescence intensity on cooling of the membrane preparation.

This interpretation is in agreement with electron microscopy of freeze-etched *E. coli* membranes below and above the phase transition temperature of the membrane lipid. The micrographs show a separation of proteins and lipids in the crystalline state and a randomisation of the proteins in the liquid state of the lipid phase[45].

The results obtained in the four systems described, liposomes of dipalmitoyllecithin, 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphoethanolamine, of membrane lipids from the fatty acid auxotroph *E. coli* mutant K 1062 grown on elaidic acid, and of plasma membranes from this mutant indicate that the new class of fluorescent-labelled ω-anthracene fatty acids incorporated in phospholipids are very useful probes, particularly because they allow the prediction of the localisation of the chromophoric group.

The subsequent paper will show the usefulness and application of these compounds in probing eucaryotic cell membranes after biosynthetic incorporation of the ω-anthracene-labelled fatty acids by cells in tissue culture.

This study was supported by the *Deutsche Forschungsgemeinschaft.*

Literature

1 Radda, G.K. & Vanderkooi, J. (1972) *Biochim. Biophys. Acta* **265**, 509 - 549.
2 Brand, L. & Gohlke, J.R. (1972) *Annu. Rev. Biochem.* **41**, 843 - 868.
3 Stryer, L. (1968) *Science* **162**, 526 - 533.
4 Edelman, G.M. & McClure, W.O. (1968) *Acc. Chem. Res.* **1**, 65 - 71.
5 Weber, G. (1971) *J. Chem. Phys.* **55**, 2399 - 2407.
6 Weber, G. (1952) *Biochemistry* **51**, 145 - 167.
7 Yguerabide, J. (1970) *J. Mol. Biol.* **51**, 573 - 590.
8 Yguerabide, J. (1973) in Fluorescence Techniques in Cell Biology (Thaer, A.A., Sernatz, M., ed.) pp. 365 - 379, Springer-Verlag, Heidelberg, New York.
9 Yguerabide, J. & Stryer, L. (1971) *Proc. Nat. Acad. Sci. U.S.A.* 68, 1217 - 1221.
10 Shinitzky, M., Dianoux, A.C., Gitler, C. & Weber, G. (1971) *Biochemistry* **10**, 2106 - 2113.
11 Latt, S.A., Cheung, H.T. & Blout, E.R. (1965) *J. Am. Chem. Soc.* 87, 995 - 1003.
12 Galley, W.C. & Stryer, L. (1968) *Proc. Nat. Acad. Sci. U.S.A.*, **60**, 108 - 114.
13 Haugland, R.P., Yguerabide, J. & Stryer, L. (1969) *Proc. Nat. Acad. Sci. U.S.A.* 63, 23 - 30.
14 Förster, Th. (1965) in Modern Quantum Chemistry, Istanbul Lectures (Sinanoglu, O., ed.) pp. 93 - 137, Acad. Press, New York.
15 Stryer, L. (1965) *J. Mol. Biol.* **13**, 482 - 495.
16 Turner, D.C. & Brand, L. (1968) *Biochemistry* 7, 3381 - 3390.
17 Ainsworth, S. & Flanganan, M.F. (1969) *Biochim. Biophys. Acta* **194**, 213 - 221.
18 Azzi, A., Chance, B., Radda, G.K. & Lee, C.P. (1969) *Proc. Nat. Acad. Sci. U.S.A.* 62, 612 - 619.
19 Träuble, H. (1971) *Naturwissenschaften*, **20a**, 277 - 284.
20 Tasaki, I., Hallett, M. & Carbone, E. (1973) *J. Membr. Biol.* **11**, 353 - 376.
21 Patrick, J., Valeur, B., Monnerie, C. & Changeux, J. (1971) *J. Membr. Biol.* **5**, 102 - 114.
22 Lesslauer, W., Cain, J.E. & Blasie, J.K. (1972) *Proc. Nat. Acad. Sci. U.S.A.* 69, 1499 - 1503.
23 Overath, P. & Träuble, H. (1973) *Biochemistry* **12**, 2625 - 2694.
24 Träuble, H. & Overath, P. (1973) *Biochim. Biophys. Acta* **307**, 491 - 512.
25 Träuble, H. & Eibl, H.J. (1974) *Proc. Nat. Acad. Sci. U.S.A.* 71, 214 - 219.
26 Cogan, U., Shinitzky, M., Weber, G. & Nishida, T. (1973) *Biochemistry* **12**, 521 - 528.
27 Jacobson, K. & Wobschall, D. (1974) *Chem. Phys. Lipids* **12**, 117 - 131.
28 Papahadjopoulos, D., Jacobson, K., Nir, S. & Isac, T. (1973) *Biochim. Biophys. Acta* **311**, 330 - 348.
29 Grätzel, M. & Thomas, J.K. (1973) *J. Am. Chem. Soc.* **95**, 6885 - 6889.
30 Cheng, S., Thomas, J.K. & Kulpa, C.F. (1974) *Biochemistry* **13**, 1135 - 1139.
31 Faucon, J.F. & Lussan, C. (1973) *Biochim. Biophys. Acta* **307**, 459 - 466.
32 Waggoner, A.S. & Stryer, L. (1970) *Proc. Nat. Acad. Sci. U.S.A.* 67, 579 - 589.
33 Lenard, J., Wong, C.Y. & Compans, R.W. (1974) *Biochim. Biophys. Acta* **332**, 341 - 349.
34 Vanderkooi, J., Fishkoff, S., Chance, B. & Cooper, R.A. (1974) *Biochemistry* **13**, 1589 - 1595.
35 Stoffel, W. & Michaelis, G. (1976) *this J.* **357**, 7 - 19, preceding paper.
36 Baer, E. & Buchnea, D. (1959) *Can. J. Biochem. Physiol.* 37, 953 - 959.
37 Stoffel, W. & Pruss, D. (1969) *this J.* **350**, 1385 - 1395.
38 Huang, C. (1969) *Biochemistry* 8, 344 - 351.
39 Lowry, O.H., Roseborough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265 - 275.

40 Rouser, G., Siakotos, A.N. & Fleischer, S. (1966) *Lipids* 1, 85 - 86.
41 Zak, B., Dickenman, R.C., White, E.G., Burnett, H. & Cherney, P.J. (1954) *Am. J. Clin. Pathol.* 24, 1307 - 1315.
42 Overath, P., Schairer, H.U. & Stoffel, W. (1970) *Proc. Nat. Acad. Sci. U.S.A.* 67, 606 - 612.
43 Faucon, J.F. & Lussan, C. (1971) *C. R. Acad. Sci. Ser. D.* 273, 646 - 649.
44 Knoop, F. (1905) *Beitr. Chem. Physiol. Pathol.* 6, 150 - 156.
45 Haest, C.W.M., Verkleij, A.J., De Gier, J., Scheek, R., Ververgaert, P.H.J. & Van Deenen, L.L.M. (1974) *Biochim. Biophys. Acta* 356, 17 - 26.

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