Hoppe-Seyler's Z. Physiol. Chem.
Bd. 357, S. 917 - 924, Juli 1976

Biosynthetic Incorporation of Fatty Acids with Photosensitive Groups into Membrane Lipids of Cells in Tissue Culture

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(Received 3 May 1976)

Summary: The physical methods (^{13}C-NMR-spectroscopy and fluorescence spectroscopy) hitherto used for the elucidation of lipid-lipid and lipid-protein interactions in artificial and simple natural membranes were extended to the application of fatty acids, phospholipids and sphingolipids with photochemical labels (azide group) in defined positions, which on photolysis generate nitrenes. These highly reactive groups react with neighbouring molecules, either lipids or polypeptide chains, with insertion or addition. Highly radioactive 12-azido[9,10-$^3H_2$]stearic acid, 12-azido[12-^{3}H]oleic acid and 18-azido-[9,10,12,13-$^3H_4$]linoleic acid were added to the growth medium of eukaryotic cell lines in tissue culture (BHK 21 cells and Chang liver cells). They were incorporated into neutral, phospho- and sphingolipids in amounts comparable with the unsubstituted parent fatty acids.

The distribution of the azido fatty acids in the phospholipids has been determined by enzymatic hydrolysis (phospholipase A_2) on the basis of the distribution of their radioactivity. Radio gas chromatography and combined gas chromatography and mass spectroscopy revealed that the azide group of the radioactive fatty acids remained unaltered.

Biosynthetischer Einbau von photoaktivierten Fettsäuren in die Membranlipide von Zellen in Gewebekultur

Zusammenfassung: Die bisher verwendeten physikalischen Methoden ^{13}C-, ^{31}P-NMR-Spektroskopie und Fluoreszenzspektroskopie zum Studium der Lipid-Lipid- und Lipid-Protein-Wechselwirkungen erfassen die Dynamik der Lipide in künstlichen und natürlichen Membranen. Diese Methoden wurden nun zur Erfassung und chemischen Definition der hydrophoben Wechselwirkungen erweitert durch den Einsatz von Fettsäuren, Phospholipiden und Sphingolipiden, die durch Azidogruppen in bestimmten Positionen markiert sind und die durch Photolyse Nitrene generieren, die durch Einschiebungen und/oder Addition mit dem nächsten Nachbarn (Lipide, Proteine) reagieren.

Hoch radioaktiv markierte 12-Azido-[9,10-$^3H_2$]-stearinsäure, 12-Azido-[12-^{3}H]ölsäure und 18-

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Enzymes:
Acyl-CoA synthetase, acid:CoA ligase (AMP-forming) (EC 6.2.1.3);
Glycerophosphate acyltransferase, acyl-CoA:*sn*-glycerol-3-phosphate *O*-acyltransferase (EC 2.3.1.15);
Sphingosine acyltransferase, acyl-CoA: sphingosine *N*-acyltransferase (EC 2.3.1.?).

Azido-[9,10,12,13-$^3H_4$]linolsäure wurden dem Medium von verschiedenen Zellklonen in der Gewebekultur (BHK 21 und Chang-Leberzellen) angeboten. Diese Azidofettsäuren werden von den eukaryoten Zellen in Ausbeuten, die mit denen der entsprechenden unsubstituierten Säuren vergleichbar sind, aufgenommen und in die Neutrallipide, Phospholipide und Sphingolipide mit hoher Effizienz eingebaut. Die Verteilung der Azidofettsäuren in den Phospholipiden wurde nach Phospholipase-A_2-Hydrolyse aufgrund der Radioaktivitätsverteilung bestimmt. Radiogaschromatographische und gekoppelte gaschromatographmassenspektroskopische Analysen zeigen, daß die Azidogruppe in den radioaktiven Fettsäuren unverändert erhalten ist.

Key words: Photosensitive fatty acids, eukaryotic cell lines in tissue culture, biosynthetically labelled membrane lipids, characterization of complex lipids, distribution of photosensitive fatty acids in membrane lipids.

Recent studies in this laboratory on the interactions of lipids, particularly phospho- and sphingolipids, in artificial membranes (liposomes), human high density lipoproteins and simple natural membranes (vesicular stomatitis virus envelope) have utilized ^{13}C-NMR[1-5] and fluorescence spectroscopy[6-8]. For this purpose chemical synthesis was used to enrich fatty acids specifically, in particular carbon atoms along the alkyl chain. In addition phosphatidylcholine and sphingomyelin labelled with ^{13}C in the choline moiety were used. This isotope enrichment made a measurement of spin lattice relaxation times feasible and allowed conclusions to be drawn regarding ionic or hydrophobic interactions between lipids and apoproteins on the basis of physical parameters. On this background we developed a novel type of fluorescent labelled fatty acids, ω-anthryl fatty acids[6] and demonstrated their useful properties, e.g. for monitoring phase transitions of membranes[7]. Finally, we observed that this type of fluorescent fatty acids effectively passes the plasma membrane of eukaryotic cells in tissue culture, is activated and transferred to the glycerol and sphingosine backbone of neutral phospholipids and sphingolipids[8]. The bulky anthracene group in the terminal position of the fatty acid, of which the distance to the backbone of the phospholipid molecule is determined by the alkyl chain linked to the aromatic tricyclic systems, does not interfere with cell growth and division. The aforementioned chemical and biochemical results challenged us to extend these biophysical studies to a chemical approach in defining the molecular basis of hydrophobic interactions between lipids and lipids and proteins. It is well known that energy-rich uncharged molecular species with an electron sextet, such as carbenes or nitrenes (imenes), can be generated by photolytic release of N_2 from diazoalkanes or azides[9,10], respectively. Nitrenes react with insertion into C-H-bonds and are therefore capable of reacting with their nearest neighbour. Although the azide group is also a bulky group, we were encouraged by the biosynthetic incorporation of the ω-anthryl fatty acids to use fatty acids labelled with photochemically reactive groups for studies in vivo in order to label the lipid phase of subcellular membranes of eukaryotic cells. We synthesized the azide derivatives of the three fatty acids most prominently present in membrane phospholipids. These were 12-azidostearic, 12-azidooleic and 18-azidolinoleic acid. Each fatty acid was labelled with tritium to high specific radioactivity.

This paper describes the effective resorption of these azido fatty acids by the eukaryotic cell lines BHK 21 (baby hamster kidney) and Chang liver cells from their growth medium and the incorporation into neutral lipids, phospholipids and sphingolipids. The distribution of the azidoacids in these lipid classes has been analyzed. Evidence is provided that these fatty acids with their photosensitive groups are unaltered by the cell metabolism.

Photochemical labelling of the surface protein of the erythrocyte membranes by *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate has been reported by Staros and Richards[11] and membrane components of sarcoplasmic reticulum by photolysis of 1-azidonaphthalene and 1-azido-4-iodobenzene by Klip and Gitler[12].

While this work was in progress, we got notice of a report by Chakrabarti and Khorana[13] on an approach similar to that chosen in this laboratory. These authors also use fatty acids and phospholipids with photosensitive groups for the study of lipid-protein interactions.

Experimental

The chemical synthesis of the acids used in studies described here, 12-azido-[9,10-$^3H_2$]stearic acid (spec. act. 13.2 μCi/μmol) 12-azido-[12-^{3}H]oleic acid (spec. act. 2.0 μCi/μmol) and 18-azido-[9,10,12,13-$^3H_4$]linoleic acid (spec. act. 100 μCi/μmol) will be outlined together with other photosensitive derivatives of lipids in a subsequent paper.*

The acids were chemically and radiochemically pure as shown by radio thin-layer chromatography of their esters (solvent system: methylene chloride) and combined gas chromatography/mass spectroscopy (3% SE 30, 2 m, 200 °C, electron energy 70 eV, filament current 300 μA, Varian MAT model CH5).

BHK 21 cells and Chang liver cells were grown in Roux flasks (growth area 250 cm^2) under the same conditions. Dulbecco's medium[14] supplemented with 5% fetal calf serum was used. 4 μmol of the acids in 100 μl ethanol was added to 60 ml medium per flask, the monolayers being in a preconfluent state. Cells were grown to confluency at 37 °C under an atmosphere of 95% air, 5% CO_2. 24 h after addition of the labelled acid, the medium was decanted and the monolayer washed twice with phosphate-buffered saline (PBS). The cells were scraped from the glass surface and harvested by centrifugation (600 × *g*, 10 min). The pellet was suspended in water and sonicated for 5 min at 0 °C to allow the protein determination[15]. About 15 mg of protein was obtained from the five flasks of each experiment. The sonicate was lyophylized and extracted with 5 ml chloroform/methanol 1:2 for 15 min. Lipids were separated into neutral lipids, glycosphingolipids and phospholipids by silicic acid column chromatography (10 g)[16]. Neutral lipids were eluted with 200 ml chloroform, glycolipids with 200 ml acetone/methanol (9:1) and phospholipids with 300 ml methanol. The following solvent systems were used for radio thin-layer chromatography: light petroleum/ether/acetic acid 70:30:1 (I) for the separation of neutral lipids; chloroform/methanol/water 100:42:8 (II) for glycosphingolipids; and chloroform/methanol/water 65:25:4 (III) or chloroform/methanol/acetic acid/water 50:25:8:4 (IV) for phospholipids.

For two-dimensional thin-layer chromatography of total lipids, the following solvent systems were used: 1) chloroform/methanol/10% ammonia 60:30:6 (V); 2) chloroform/methanol/acetone/acetic acid/water 75:15:30:15:7.5 (VI). Phosphorous-containing bands and spots were visualized with the reagent of Dittmer and Lester[17], the rest by spraying with 50% sulfuric acid and charring. For phosphorous determination the procedure of Rouser[18] et al. was followed. Radioscans of thin-layer plates were performed with the Berthold scanner.

Radioactive bands were scraped off the thin-layer plates and eluted with chloroform/methanol 1:1. The solvent was then evaporated for scintillation counting.

Results

Two eukaryotic cell lines BHK 21 (baby hamster kidney cells) and Chang liver cells were grown in tissue culture. Their nutritient medium was supplemented by three long-chain fatty acids labelled with the photosensitive azide group and in addition with tritium: 12-azido-[9,10-$^3H_2$]stearic acid, 12-azido-[12-^{3}H]oleic acid and 18-azido-[9,10,12,13-$^3H_4$]linoleic acid. The three acids were taken up by these two cell lines in yields comparable with their unsubstituted fatty acid analogous stearic, oleic and linoleic acid. 23% of the 12-azidostearic, 28% of the added 12-azidooleic, and 32% of the 18-azidolinoleic acid were recovered in the total lipid extract.

Distribution of azido fatty acids in lipid classes BHK 21 cells

In general five Roux flasks were sufficient for a complete lipid analysis and the determination of the distribution of the radioactive azido fatty acid. The lipid extract was separated by silicic acid chromatography into the neutral lipid fraction (eluent: chloroform), glycosphingolipids (eluent: acetone/methanol 9:1) and phospholipid fraction (eluent: methanol) (Table 1). The radioactivity of the neutral lipid fraction was mainly concentrated in the diglyceride, free fatty acid and triglyceride fractions. This is exemplified in Fig. 1, presenting the one-dimensional radio thin-layer scans of neutral lipid fractions of BHK 21 cells grown e.g. on 18-azidolinoleic acid. Among the glycosphingolipids, hematoside, ceramide tri-, di- and monohexoside, and ceramide as well were labelled with the corresponding azido fatty acids, as demonstrated in Fig. 2.

* Stoffel, W., Salm, K. & Körkemeier, U. manuscript in preparation.

Table 1. Distribution of ^{3}H-labelled 12-azidostearic-, 12-azidooleic and 18-azidolinoleic acid in the lipid classes of BHK 21 cells.

Total lipids	12-Azidostearic		12-Azidooleic		18-Azidolinoleic	
Radioact. [dpm]	1.36×10^8		1.14×10^7		1.4×10^9	
% of applied acid incorporated	23		28		32	
	Radioact. [dpm]	% of incorp. radioact.	Radioact. [dpm]	% of incorp. radioact.	Radioact. [dpm]	% of incorp. radioact.
Neutral lipids	6.25×10^7	46	5.7×10^6	50	6.5×10^8	47
Glycosphingolipids	7.8×10^6	6	0.58×10^6	5	0.7×10^8	5
Phospholipids	6.5×10^7	48	5.12×10^6	45	6.7×10^8	48

Most important, the azido fatty acids are incorporated into the main phospholipid classes. Four phospholipid classes (Fig. 3a, b) were labelled: sphingomyelin, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. Table 2 summarizes the distribution of 12-azodostearic acid and 18-azidolinoleic acid in phospholipid classes.

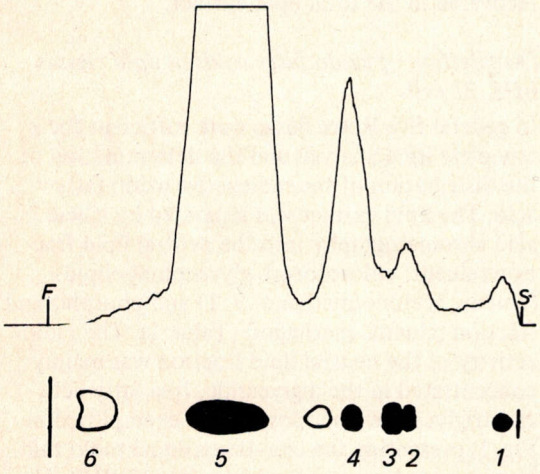


Fig. 1. Radioscan of neutral lipids isolated from BHK 21 cells grown on 18-azido[9,10,12,13-$^3H_4$]linoleic acid.

Solvent system I.

1, monoglyceride; 2, diglyceride; 3, cholesterol; 4, fatty acid; 5, triglyceride; 6, cholesterol ester.

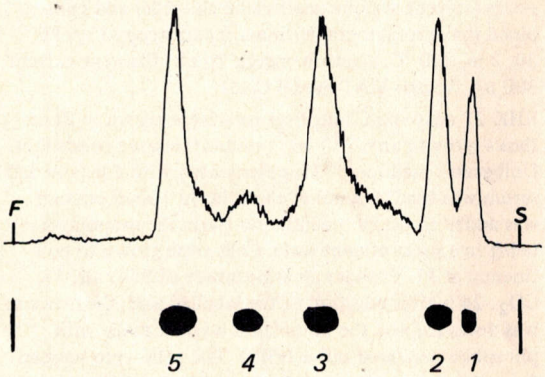


Fig. 2. Radioscan of glycosphingolipids isolated from BHK 21 cells grown on 18-azido[9,10,12,13-$^3H_4$]linoleic acid.

Solvent system II.

1, hematoside; 2, diglycosylceramide; 3, triglycosylceramide; 4; not identified; 5, monoglycosylceramide.

In order to prove that the BHK 21 cells have not altered the azide substituent group and that it is still present in the radioactive acyl residues, the total phospholipid fraction was dissolved in chloroform for IR spectroscopy. The absorption band at 2100 cm^{-1}, characteristic of aliphatic azides, was strong.

Another attempt to prove the integrity of the azido fatty acid in the phospholipids consisted of

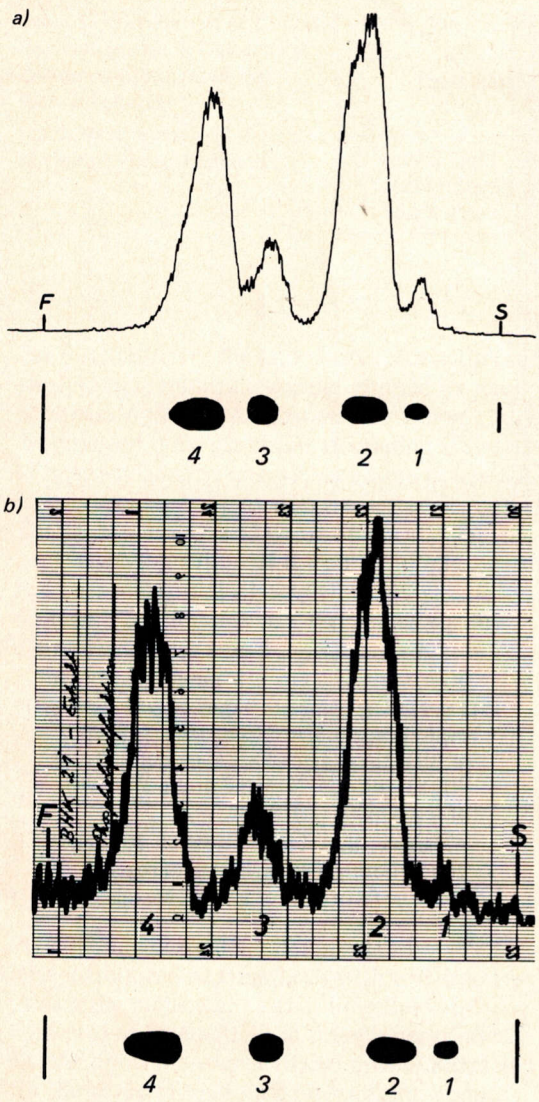


Fig. 3. Radioscan of phospholipids isolated from BHK 21 cells grown on a) 18-azido-[9,10,12,13-$^3H_4$]linoleic acid or b) 12-azido-[9,10-$^3H_2$]stearic acid.
Solvent system III.
1, sphingomyelin; 2, phosphatidylcholine; 3, phosphatidylserine; 4, phosphatidylethanolamine.

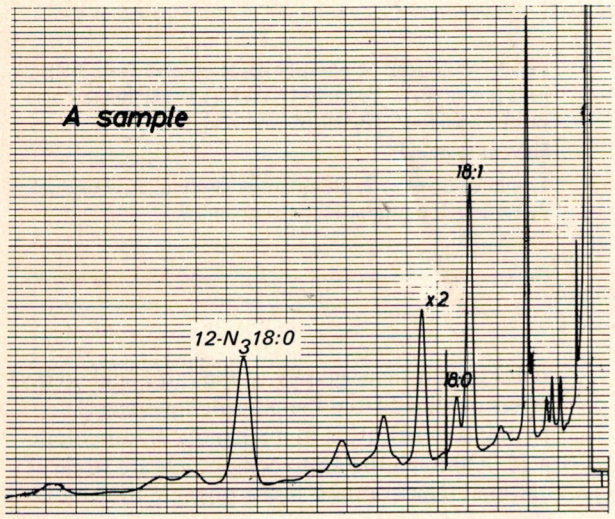
radio gas chromatography of the fatty acid methyl esters obtained from the phospholipid fraction by transesterification in methanolic hydrochloric acid. Although the azido compounds are labile at elevated temperatures, it was possible to demonstrate their presence in the phospholipid/fatty acid mixture. Combined gas chromatography/mass spectroscopy (Fig. 4), e.g. of the fatty acid methyl ester mixture obtained from the phospholipids of the 12-azidostearic acid experiment, clearly gave the additional proof of the unaltered azido acid.

Chang liver cells

Similar experiments to those described for BHK 21 cells were performed with Chang liver cells applying 12-azido-[9,10-$^3H_2$]oleic and 18-azido-[9,10,12,13-$^3H_4$]linoleic acids as substrates. This cell line also utilized the fatty acids labelled with the photosensitive azido group for the biosynthesis of ester and amido lipids. Table 3 summarizes the distribution of the labelled fatty acids in the neutral, glycosphingo- and phospholipid fractions.

Table 2. Distribution of the radioactive azido fatty acids in phospholipid classes isolated from BHK 21 cells grown on 12-azidostearic and 18-azido-[^{3}H]linoleic acid.

Phospholipid classes	12-N_3-18:0		18-N_3-18:2	
	[%]	Spec. act. [dpm/μmol]	[%]	Spec. act. [dpm/μmol]
Sphingomyelin	5.6	1.52×10^6	3.6	2.3×10^7
Phosphatidylcholine	48.3	2.08×10^6	57.5	9.7×10^7
Phosphatidylserine	8.8	6.5×10^5	5.4	3.2×10^7
Phosphatidylethanolamine	37.3	1.69×10^6	33.5	7.0×10^7



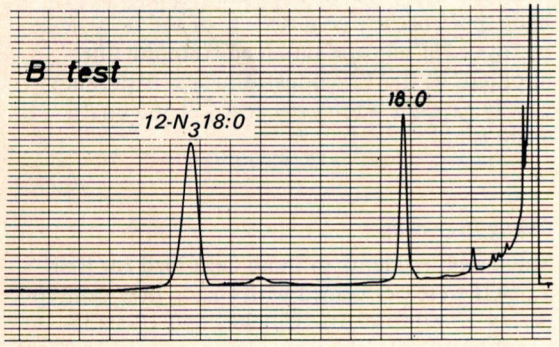
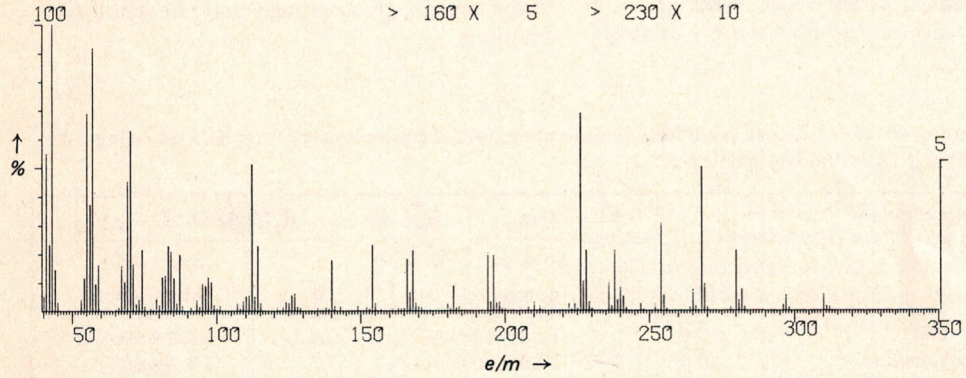


Fig. 4a. Gas chromatography of fatty acid methyl esters isolated from phospholipids of BHK 21 cells grown on 12-azido[9,10-$^3H_2$]stearic acid. Note the strong mass peak of the substrate fatty acid.

A, sample; B, test mixture 3% SE 30, 2 m, 200 °C isothermal; carrier gas, nitrogen. Only the fatty acids of interest are marked.



4b) Mass spectrum of the azido ester peak.

Notice the characteristic peaks at *m/e* 310 [M-28 (N_2)] and 297 [M-42 (N_3)].

Table 3. Distribution of 12-azido[9,10-$^3H_2$]oleic and 18-azido[9,10,12,13-$^3H_4$]linoleic acid in lipid classes of Chang liver cells grown in Dulbecco's medium supplemented with these substrates.

Lipids were separated by silicic acid chromatography.

Lipid class	12-N_3-18:1		18-N_3-18:2	
	[dpm]	[%]	[dpm]	[%]
Neutral lipids	3.39×10^6	6	2.9×10^7	20.8
Glycosphingolipids	1.01×10^6	1.8	5.0×10^6	0.7
Phospholipids	5.20×10^7	92.2	1.10×10^8	78.5

The distribution of the azido fatty acids within the phospholipid molecule was determined by phospholipase A_2 (*Crotalus adamanteus* venom) hydrolysis and radio gas chromatographic analysis of fatty acid methyl esters released and isolated from lysophospholipids after transesterification. The 18-N_3-linoleic acid was the main acyl substituent in the 2-position of the phospholipids which had been submitted alltogether to enzymatic hydrolysis. 12-Azidooleic acid was almost equally distributed in the 1- and 2-position, whereas 12-azidostearic acid was isolated to more than 90% from the lysophospholipid fraction. This means that the saturated acid, despite the substitution by the azide group, which leads to the depression of the melting point, is transferred by the acyltransferase specifically to the 1-position.

Discussion

Previous and current studies in this laboratory have introduced new methods with which to approach the molecular basis of lipid-lipid and lipid-protein interactions in three types of models, the liposome as an artificial membrane model for the study of lipid-lipid interactions, apoproteins of high density lipoproteins and the viral envelope as a model of a well defined natural membrane. ^{13}C-NMR spectroscopy and fluorescence spectroscopy have been used in conjunction with suitable experimental design, namely introducing the respective ^{13}C-isotopic probe or the fluorescent label in well defined positions of the fatty acyl chains or hydrophilic groups of phospholipids required for liposome formation or recombination studies.

We have labelled the membrane lipids of host cells of enveloped viruses with either the ^{13}C-enriched fatty acid or choline moiety or the fluorescent labelled ω-anthryl fatty acids in order to obtain pure virus preparations as simple natural membrane models for physical investigations. Because of the rather high incorporation of the fluorescent fatty acid with the terminal bulky anthracene group, we were encouraged to study the incorporation of the fatty acids labelled with the photosensitive azide group either in the middle of the acyl chain (12-azidostearic acid) or at its terminal position (18-azidolinoleic acid). The highly radioactively labelled precursors indeed proved able to permeate the plasma membrane of the cell lines used in these studies. Like their unsubstituted parent acids, they are activated and utilized for ester and amide bond formation in glycero- and sphingolipids by the respective acyltransferases.

The labelling of membrane lipids of eukaryotic cells by precursors with photosensitive substituents on biosynthetic pathways allows the approach to a number of problems relevant to membrane structure. This chemical approach should valuably extend the scope of the physical approach to studies on lipid-protein interactions.

We gratefully acknowledge the support by the *Deutsche Forschungsgemeinschaft* (SFB 74).

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