Dansylsphingolipids for labelling and probing membranes

Richard T.C. Huang

Institut für Virologie, Justus-Liebig-Universität Giessen, D-6300 Giessen, FRG

Received 7 June 1982

Three fluorescent sphingolipids have been prepared, which contain dansyl sphingosine in the hydrophobic portion and various head groups (F-phosphorylcholine, galactose and lactose) in the hydrophilic end. These dansylsphingolipids are readily incorporated into membranes and show affinity toward several membrane proteins. Results of fluorescence measurements indicate, that these lipids may be useful as probes for studying protein–lipid interactions.

Dansylsphingolipid synthesis *Membrane label* *Membrane probe* *Protein–lipid interaction*

1. INTRODUCTION

Protein–lipid interaction is among the most important factors, which govern the structure and funtion of biomembranes. Thus, ionic as well as hydrophobic bindings take place between phospholipids and proteins, and in the case of glycolipids, the various glycosyl chains may serve as additional sites for protein–lipid recognition. In order to study these interactions, three fluorescent dansylsphingolipids have been synthesized, which are amphipathic like membrane lipids and contain head groups identical to those of the sphingolipids of animal cells. These dansylsphingolipids are readily incorporated into membranes and are unique among fluorescence probes because of their structural resemblance to natural lipids. Examples are given to illustrate possible use of these compounds for studying interactions associated with membranes.

2. MATERIALS AND METHODS

2.1. *Preparation of sphingolipids*

Sphingomyelin and cerebroside were isolated from human brain according to Klenk and Huang [1] and Klenk and Schuman [2]. Ceramide was prepared from cerebroside by the procedure of Carter et al. [3]. Gaver and Sweeley's method [4] was employed to obtain sphingosine from ceramide. Sphingosine-galactoside (psychosin) was prepared by hydrolysis of cerebroside with barium hydroxide [5] and Kaller's procedure [6] was used for isolating sphingosine-phosphoryl-choline from sphingomyelin.

Dansylsphingosine, dansylsphingosine-phosphoryl-choline (ds-PC) and dansylsphingosine-galactoside (ds-G) were prepared from pure sphingosine, sphingosine-phosphoryl-choline and sphingosine-galactoside by reacting with dansyl chloride (Serva) in aqueous ethanol at pH 10 as described previously for dansylsphingosine-choline (ds-C) [7].

Dansylsphingosine-lactoside (ds-L) was synthesized by coupling dansylsphingosine to lactose employing the Königs–Knorr reaction [8]. Briefly, lactose was acetylated in pyridine and acetic anhydride [9], and converted into the acetobromo derivative by reacting with hydrobromide in acetic acid [10]. After deacetylation in 0.1 N methanolic sodium hydroxide, the product (ds-L) was partitioned into the Folch lower phase [11] together with unreacted dansylsphingosine and dansylsphingosine-monohexoside (by-products, glucoside and galactoside). The lower phase was evaporated to dryness and the residue was fractionated by a silicic acid column. Dansylsphingosine and dansylsphingosine-monohexoside were eluted with chloroform/methanol (4:1), and ds-L was recovered by eluting with chloroform/methanol (1:1).

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

2.2. *Enzyme reaction*

One mg of sphingolipids were finely suspended in water by brief sonication. For enzymic hydrolysis one unit of β-galactosidase (EC 3.2.1.23) of Jack bean purchased from Sigma or one unit of sphingomyelinase (EC 3.1.4.12) of human placenta purchased from Sigma was added and the mixtures were kept at 37°C for 1 h. Then, 1 ml of chloroform was added to extract the sphingolipids. Aliquots of the chloroform extract were spotted on silica gel thin-layer plates and analysed for the hydrolysis products using chloroform/methanol/water (65:25:4) as solvents.

2.3. *Cells, viruses and liposomes*

BHK-21 and HeLa cells were grown as monolayers in Dulbecco's or Lavit's medium as usual. Fowl plague virus, an influenza virus, was grown in embryonated chicken eggs and purified by sucrose gradient centrifugation [12]. Liposomes were prepared by dissolving lipids (lecithin/cholesterol or lecithin) in 20% octylglucoside and subsequently removing octylglucoside by dialysis [13].

2.4. *Proteins*

The hemagglutinin and the neuraminidase of fowl plague virus were isolated from virus using octylglucoside as detergent as described previously [14]. After solubilization of hemagglutinin and neuraminidase by octylglucoside, the matrix protein remained isoluble and could be sedimented at 20 000 rev./min (1 h, Sorvall, rotor SS-34). The matrix protein was essentially pure and free of the nucleocapsid protein after repeatedly resuspending the pellet (5 times) in PBS following recentrifugation. Glycophorin was purified from human erythrocytes by the method of Springer et al. [15].

2.5. *Labelling cells, viruses and liposomes with dansylsphingolipids*

For labelling cells, micellar solutions of dansylsphingolipids were prepared by rapidly injecting 20–50 μl of an ethanolic solution of sphingolipids (1 mg/ml) into 1–2 ml of a serum-free culture medium, using a Hamilton syringe. Cells were labelled after incubating in this medium for 30–120 min at 37°C. For labelling enveloped viruses and liposomes, the same conditions were employed except that PBS (phosphate buffered saline) was used instead of a culture medium.

2.6. *Fluorescence measurements*

For fluorescence measurements, monolayers of fluorescence labelled cells were trypsinized as usual and suspended in PBS in a concentration of about 200 000 cells per ml. Labelled viruses and liposomes were pelleted by centrifugation at 20 000 rev./min for 30 min (Sorvall, rotor SS-34) and resuspended in PBS in a concentration which gave about the same fluorescence intensity as the cell suspension. The Hitachi–Perkin–Elmer fluorescence spectrophotometer 204 was used for fluorescence measurements. The emission anisotropies were calculated from the equation:

$$(I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ refer to the fluorescence intensities parallel and perpendicular to the excitation polarization plane, respectively [17].

2.7. *Fluorescence energy transfer measurements*

First, fluorescence intensity was measured in the absence of protein – 10 μl of an ethanolic solution of dansylsphingolipids (1 mg/ml) was rapidly injected into a 2 ml cuvette and the emission intensity at 500 nm was measured at the excitation wavelength of tryptophan (290 nm). Then, proteins were added in increasing 50 μg portions (from 50 μg to 500 μg of protein dissolved in 10 μl to 100 μl of PBS). The emission intensity was measured after each addition of proteins until the highest plateau of emission intensity was reached (usually with about 200–250 μg of protein). The ratio of highest emission in the presence of protein to lowest emission in the absence of protein was calculated to indicate efficiency of fluorescence energy transfer from the tryptophan of protein to dansyl group.

3. RESULTS AND DISCUSSION

Cerebroside and sphingomyelin, the major lipids of the myelin sheath, were used as starting materials for the preparation of dansylsphingolipids. With the hydrolysis procedure employed, pure psychosin (sphingosine-galactoside) and sphingosine-phosphoryl-choline were obtained from cerebroside and sphingomyelin respectively. Dansylsphingosine-galactoside (ds-G) and dansylsphingosine-phosphoryl-choline (ds-PC) were obtained in quantitative yield by dansylation of these

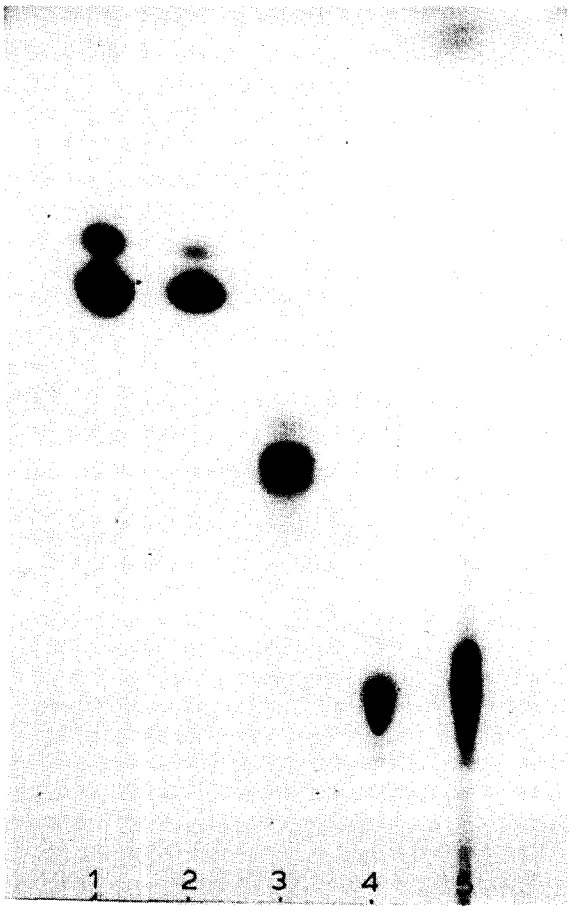

Fig.1. Thin-layer chromatogram of sphingolipids. Precoated silicic acid thin-layer plates (Kieselgel 60, Merck). Developed by chloroform/methanol/water (65:25:4). (1) cerebroside of human brain, (2) dansylsphingosine-galactoside (ds-G), (3) dansylsphingosine-lactoside (ds-L), (4) dansylsphingosine-phosphoryl-choline (ds-PC) and (5) sphingomyelin of human brain.

compounds. Similarly, dansylsphingosine-lactoside (ds-L) could be synthesized in a high yield (50%) by the Königs–Knorr procedure. All these fluorescence lipids moved to the same positions as their natural analogues on silica gel thin-layer plates, using chloroform/methanol/water (65:25:4) as the developing solvents (fig.1). By the same chromatography system it was found that dansylsphingolipids were degraded by hydrolytic enzymes. Thus, ds-G and ds-L were quantitatively transformed into dansylsphingosine and dansylsphingosine glucoside by the β-galactosidase of Jack beans, and ds-PC was completely degraded to dansylsphingosine by sphingomyelinase of human placenta.

The maximal excitation and emission wavelengths of dansylsphingolipids were found to be 340 nm and 495 nm. Organic solvents enhanced the emission intensity of these lipids, as observed for all other fluorescence probes. However, in contrast to other fluorescence probes, were the emission is shifted to the blue in organic solvents, the same solvents shifted the emission towards longer wavelengths in the case of dansylsphingolipids (fig.2).

Dansylsphingolipids are readily incorporated into cellular or artificial membranes (fig.3). The uptake of lipids into cellular membrane is dependent on incubation time and concentration of lipids. Normally, concentrations of 20–50 μg of lipids per ml in an incubation time of 1 to 2 h are adequate to label cells in culture. Under these conditions, dansylsphingolipids were not hazardous to

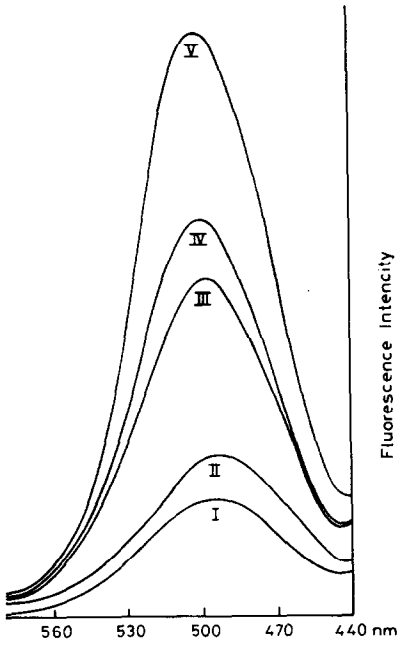

Fig.2. Fluorescence emission (excitation at 340 nm) of dansylsphingosine-galactoside in aqueous, aqueous ethanolic, and ethanolic solutions. I: water, II: water/ethanol (75:25), III: water/ethanol (50:50), IV: water/ethanol (25:75) and V: ethanol.

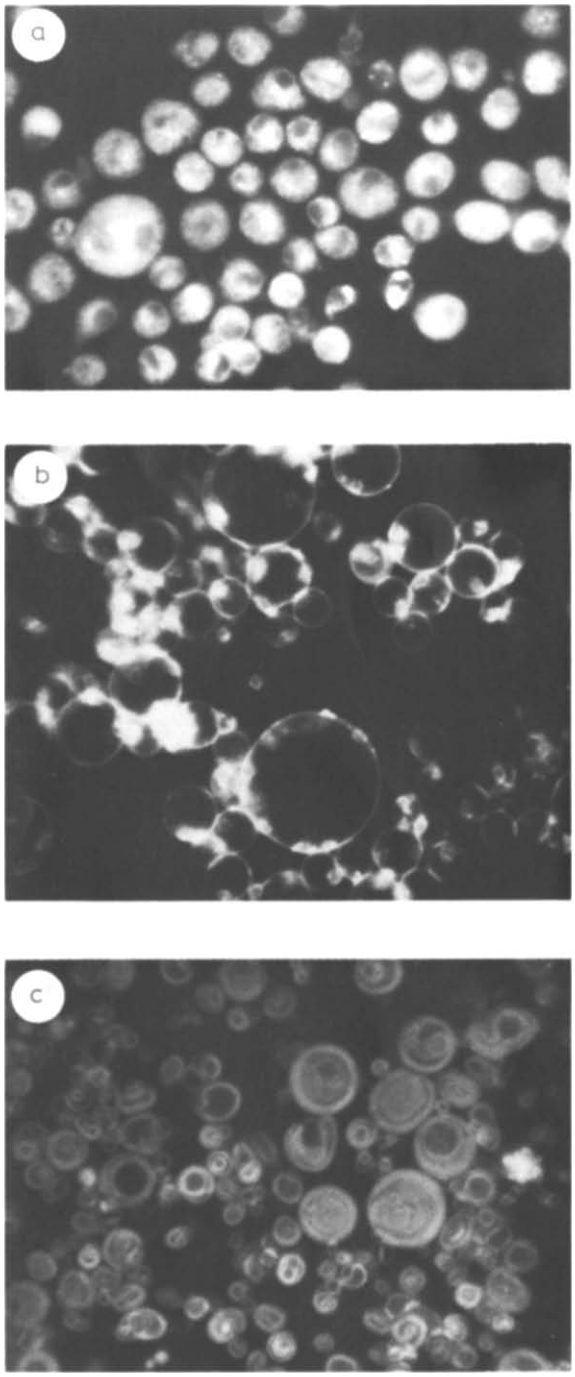

Fig.3. Labelling of membranes with dansylsphingosine-lactoside. a: chick fibroblasts. b: chick erythrocytes during fusion by influenza virus [19]. c: liposomes made of lecithin/cholesterol (4:1).

Table 1

Anisotropies of dansylsphingolipids[a] from various membranes

	Anisotropies		
	ds-PC	ds-G	ds-L
Liposomes			
lecithin	0.035	0.048	0.047
lecithin + cholesterol (4 + 1)	0.035	0.048	0.048
Fowl plague virus			
virus	0.150	0.148	0.157
virus + glutaraldehyde (1%)	0.167	0.165	0.173
virus + concanavalin A (0.1%)	0.280	0.288	0.301
Erythrocyte (chick)	0.140	0.150	0.155
BHK-21 cell	0.122	0.128	0.128
HeLa cell	0.120	0.124	0.133

[a] Dansylsphingosine-phosphoryl-choline (ds-PC), dansylsphingosine-galactoside (ds-G) and dansylsphingosine-lactoside (ds-L) were used. Anisotropies were obtained as described in Materials and Methods section.

cells. After labelling, cells remained healthy and could pass the incorporated lipids to several generations of progeny cells. Incorporated sphingolipids were slowly metabolized by cells – after the labelled cells were incubated for 24 h, most of the sphingolipids remained as the original compounds, but about 10% were converted into dansylsphingosine as identified by silicic acid thin-layer chromatography using chloroform/methanol/water (65:25:4) as developing solvents.

Information regarding the mode of insertion of these dansylsphingolipids into membranes was obtained by emission anisotropy measurements (table 1). Comparable values were obtained with ds-PC, ds-G and ds-L. The anisotropies obtained from liposomes were the same, regardless of whether cholesterol was present or absent in the liposomes. This indicates that the dansyl portion was anchored in a region exterior to the cholesterol domain of the lipid bilayer. This is to be expected from the structure of dansylsphingolipids, but the property is in striking contrast to other fluorescence probes which are incorporated in the interior of the lipid bilayer [16], and therefore yield aniso-

Table 2

Fluorescence energy transfer from the tryptophan molecules of proteins to dansylsphingolipids [a]

Proteins added	Fluorescence enhancement[b]		
	ds-PC[c]	ds-G[c]	ds-L[c]
None	1	1	1
Hemagglutinin	1.6	1.1	3.2
Neuraminidase	1.2	1.1	2.1
Matrix protein	5.1	3.1	9.8
Glycophorin	3.2	1.1	5.0

[a] The excitation wavelength was that of tryptophan (290 nm) and the fluorescence was measured at the emission wavelength of dansyl group (500 nm). Experimental procedure was as described in the Materials and Methods section.

[b] Ratio of highest emission intensity in the presence of protein to lowest emission intensity in the absence of protein.

[c] As in the legend of table 1.

tropies subject to the influence of cholesterol. Higher anisotropies were obtained when membranes contained proteins. Thus, the anisotropy from the membrane of fowl plague virus was higher, and that from tissue culture cells (BHK and HeLa cells) was somewhat lower. The anisotropies were elevated when membrane of the labelled virus was fixed by treating with glutaraldehyde or concanavalin A. The effect was especially dramatic with concanavalin A which immobilized the viral glycoprotein by crosslinking the glycosyl chains. This suggests that dansylsphingolipids were tightly bound to viral glycoproteins in such a way, that they could be immobilized with the glycoproteins by concanavalin A.

Further evidence for the close association between dansylsphingolipids and membrane proteins was obtained using fluorescence energy transfer techniques described by Stryer [17]. As the fluorescence emission from aromatic amino acids of proteins can excite dansyl groups in their immediate neighbourhood (20–50 Å), this technique can be used to estimate how near the proteins and dansylsphingolipids are associated with each other. For this purpose, dansylsphingolipids were reacted with four membrane proteins, three isolated from fowl plague virus (hemagglutinin, neuraminidase and matrix protein) and one obtained from human erythrocytes (glycophorin). Table 2 shows the results of such studies. The highest enhancement of fluorescence was observed when dansylsphingolipids were mixed with the matrix protein, being about 10-fold with ds-L, 5-fold with ds-PC and 3-fold with ds-G. Other proteins (hemagglutinin, neuraminidase and glycophorin) also caused varying enhancement of fluorescence with ds-L (2- to 5-fold) and with ds-PC (1- to 3-fold). With ds-G, no enhancement of fluorescence was caused by membrane proteins except by the matrix protein. Generally, with all dansylsphingolipids used, the potency of membrane proteins to enhance fluorescence was in the order of matrix protein > glycophorin > hemagglutinin > neuraminidase. In the case of viral proteins, this compares favorably with previous findings, that matrix protein and hemagglutinin are more efficiently incorporated into artificial membranes than neuraminidase [13,18]. It is interesting to note, that ds-L, which contains a disaccharide portion, caused the highest fluorescence enhancement. This may mean, that ds-L is either more strongly bound to membrane proteins than other dansylsphingolipids, or that it is bound nearest to protein regions subject to fluorescence energy transfer. The results of fluorescence energy transfer suggest, that there may be varying types of protein–lipid binding determinable by both proteins and lipids.

In conclusion, dansylsphingolipids described above possess properties unique among other fluorescence probes. The conventional probes are suitable for testing membrane fluidity within the lipid bilayer, because most of them bear no resemblance to natural lipids, and are incorporated into membranes due to their lipophilic nature. In comparison, the dansylsphingolipids are structurally related to natural sphingolipids, and the fluorescence measurements obtained with these probes may yield useful information with regard to protein–lipid interactions vital for the integrity and function of biomembranes.

ACKNOWLEDGEMENT

This work was supported by the Sonderforschungsbereich 47 of the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Klenk, E. and Huang, R.C.T. (1969) Hoppe-Seyler's Z. Physiol. Chemie 350, 373–378.
[2] Klenk, E. and Schumann (1942) Hoppe-Seyler's Z. Physiol. Chemie 272, 177–188.
[3] Carter, H.E., Rotfus, J.A. and Gigg, R. (1961) J. Lipid Res. 2, 228–239.
[4] Gaver, R.C. and Sweeley, C.C. (1965) J. Am. Chem. Soc. 42, 294–304.
[5] Klenk, E. (1926) Hoppe-Seyler's Z. Physiol. Chem. 153, 74–82.
[6] Kaller, H. (1961) Biochem. Z. 334, 451–458.
[7] Huang, R.T.C. (1976) Z. Naturforsch. 31, 737–740.
[8] Talley, E.A. (1963) in: Methods in Carbohydrate Chemistry (Whistler, R.L. and Wolfrom, M.L. eds) pp, 337–340, Academic Press, New York.
[9] Wolfrom, M.L. and Thompson, A. (1963) in: Methods in Carbohydrate Chemistry (Whistler, R.L. and Wolfrom, M.L. eds) pp. 211–215, Academic Press, New York.
[10] Fletcher, H.G., Jr. (1963) in: Methods in Carbohydrate Chemistry (Whistler, R.L. and Wolfrom, M.L. eds), pp. 226–228, Academic Press, New York.
[11] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 485–497.
[12] Chucholowius, H.W. and Rott, R. (1972) Proc. Soc. Exp. Biol. Med. 140, 245–247.
[13] Huang, R.T.C., Wahn, K., Klenk, H.-D. and Rott, R. (1979) Virology 97, 212–217.
[14] Bosch, F.X., Mayer, A. and Huang, R.T.C. (1980) Med. Microbiol. Immunol. 168, 249–259.
[15] Springer, G.F., Nagai, Y. and Tegtmeyer, H. (1971) Biochemistry 5, 3254–3272.
[16] Petri, W.A. Jr., Pal, R., Barenholz, Y. and Wagner, R.R. (1981) Biochemistry 20, 2796–2800.
[17] Stryer, L. (1968) Science 162, 526–533.
[18] Bucher, D.J., Kharitonenkov, I.G., Zakmirdin, J.A., Grigoriev, V., Klimenko, S.M. and Davis, J.F. (1980) J. Virol. 36, 586–580.
[19] Huang, R.T.C., Rott, R. and Klenk, H.-D. (1981) Virology 110, 243–247.