Incorporation of Ganglioside Analogues into Fibroblast Cell Membranes. A Spin-Label Study†

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ABSTRACT: The uptake of ganglioside analogues by a permanent mouse fibroblast cell line has been studied by radiotracer techniques and ESR spectroscopy with ^3H- and nitroxide-labeled compounds. Analogues of G_{M1}, G_{M2}, and G_{M3} monosialogangliosides and of G_{D1a} and G_{D3} disialogangliosides were synthesized. The spin-label group was situated on the 5-, 9-, or 13-carbon atom of the C_{18} fatty acid chain, and the ^3H label was in the carbohydrate moiety. Part of the ganglioside associated with the cells could be removed by trypsin treatment and was shown to consist of ganglioside micelles attached to the cell surface. The trypsin-resistant component displayed characteristic anisotropic ESR spectra which closely resembled those of the same spin-labeled analogues at low dilution in liposomes prepared from the extracted cell lipids. The flexibility gradient, polarity profile, and temperature dependence displayed by the spectra were similar to those found for fluid phospholipid bilayer model membranes, and the high effective order parameters suggested a location in the cell plasma membrane. Similar results were obtained for all the different ganglioside analogues, indicating a common anchoring region in the hydrophobic interior of the membrane. Under the incubation conditions used the amount of trypsin-resistant ganglioside analogue taken up by the cells was about 15 nmol/mg of cellular protein, irrespective of the nature of the oligosaccharide moiety. By use of the natural ganglioside [^3H]G_{M3}, the trypsin-resistant uptake was about 19 nmol/mg of cellular protein. Although these amounts are quite similar, the uptake kinetics differed between the true ganglioside G_{M3} and the ganglioside analogues.

Gangliosides are characteristic constituents of the outer surface of animal cells, in which they are located with their ceramide portion embedded in the lipid layer and their sialooligosaccharide residue facing the extracellular environment. Gangliosides of mammalian cells have long been assumed to be involved in cell–cell recognition and cell–cell adhesion, as well as in cell contact inhibition of growth and possibly tumorigenesis [for review see Hakomori (1981)]. In addition, cell surface gangliosides interact with hormones, lectins, viruses, interferon, and bacterial toxins [for review see Fishman & Brady (1976)].

One approach to studying the role of gangliosides in these crucial cellular processes is to investigate the uptake of exogenously added gangliosides. Thus, in the study of (a) the regulation of ganglioside metabolism and ganglioside-metabolizing enzymes, (b) the intracellular transport of gangliosides and their metabolites, and (c) the functional effects of exogenous gangliosides on cultured cells, the incorporation of a defined labeled ganglioside into the outer leaflet of the plasma membrane can be of considerable importance. A critical question in such studies is whether the exogenous ganglioside is truly inserted into the membrane, rather than being simply adsorbed to its surface or alternatively trapped within membrane vesicles. A related question is whether the incorporated ganglioside is uniformly distributed throughout the membrane, as opposed to being incorporated in undispersed patches.

Previously, Callies et al. (1977) and Radsak et al. (1982) have shown that exogenous gangliosides were taken up by cultured fibroblasts even under inhibition of endocytosis. In addition, it was found that a major portion of the ^3H-labeled gangliosides could be released by treatment of the cells with either trypsin or serum, but with a residual portion remaining tightly bound to the cells. However, it remained unclear whether the unremovable gangliosides were inserted into cell membranes as for the natural lipids or were firmly attached to trypsin-insensitive membrane proteins. Thus, when ^3H-labeled gangliosides were used, no distinction could be made between these modes of ganglioside–membrane association.

On the other hand, electron spin resonance spectroscopy (ESR)[1] is a diagnostic method for studying the incorporation of lipids into cell membranes (Marsh et al., 1982). The spin-label ESR spectra are sensitive both to the state of lipid aggregation and to the characteristic dynamic environments of lipids in membranes. Thus, the spectra can be used to distinguish cell-associated lipids which have been truly integrated and dispersed within the membrane from those which are more peripherally associated. For its application the ESR method requires the synthesis of appropriately spin-labeled gangliosides. Labels with the nitroxide in the polar part of the ganglioside are unlikely to be ideally suited for such studies, since the mobility of the labeled groups is extremely high (Sharon & Grant, 1977; Lee et al., 1980). In a preliminary communication (Schwarzmann et al., 1981), we have shown that synthetic ganglioside analogues with a nitroxide group in their hydrophobic part are well suited to studying the incorporation of gangliosides into the membranes of cultured cells. Recently the incorporation of a ganglioside analogue into liposomes and erythrocytes has also been demonstrated

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[1] Abbreviations: ESR, electron spin resonance; G_{M1}, II^{3}Neu5Ac-GgOse$_4$Cer; G_{M2}, II^{3}Neu5Ac-GgOse$_3$Cer; G_{M3}, II^{3}Neu5Ac-LacCer; G_{D1a}, IV^{3}Neu5Ac,II^{3}Neu5Ac-GgOse$_4$Cer; G_{D3}, II^3(Neu5Ac)$_2$-LacCer; 5-G_{M1}II/-G_{M3}II, 1-deoxy-1-[2′-butanoylamino[4″-(4‴,4‴-dimethyl-2‴-tridecyl-3‴-oxyoxazolidin-2‴-yl)]]octadecanoylaminomonosialogangliotetraitol/-monosialolactitol; 9-G_{M1}II/-G_{M2}II/-G_{M3}II, 1-deoxy-1-[2′-octanoylamino[8″-(4‴,4‴-dimethyl-2‴-nonyl-3‴-oxyoxazolidin-2‴-yl)]]octadecanoylaminomonosialogangliotetraitol/-monosialogangliotriaitol/-monosialolactitol; 13-G_{M1}II/-G_{M2}II/-G_{M3}II/-G_{D1a}II/-G_{D3}II, [1-^3H]-1-deoxy-1-[2′-dodecanoylamino[12″-(4‴,4‴-dimethyl-2‴-pentyl-3‴-oxyoxazolidin-2‴-yl)]]octadecanoylaminomonosialogangliotetraitol/-monosialogangliotriaitol/-monosialolactitol/-disialogangliotetraitol/-disialolactitol; PBS, phosphate-buffered saline.

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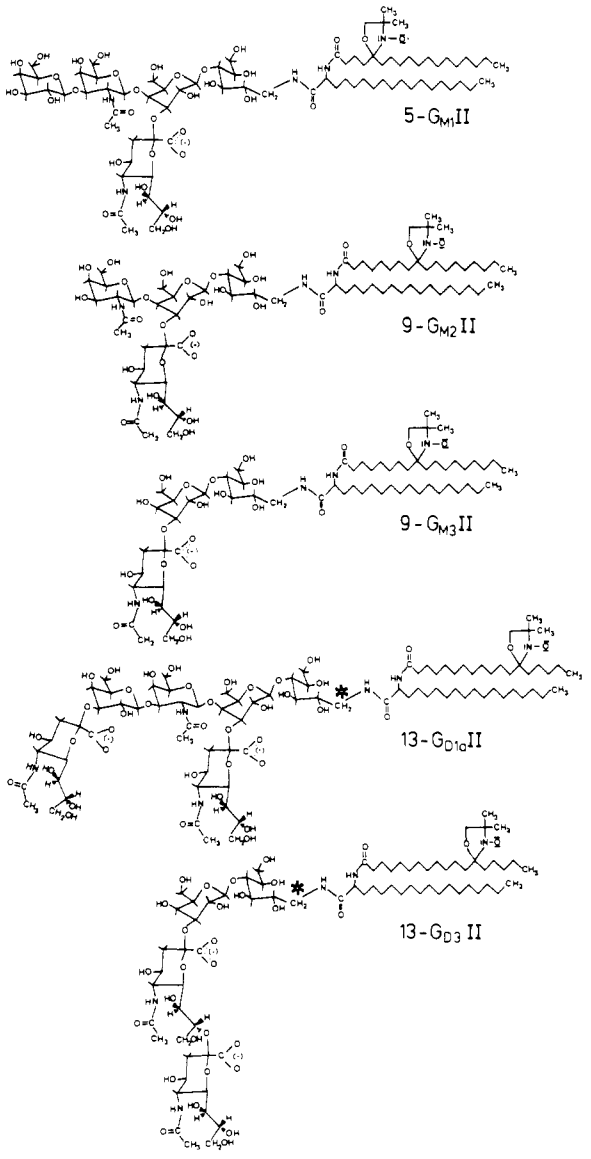
Chart I[a]



[a] Asterisk indicates position of the ^3H label.

(Kanda et al., 1982a,b), thus confirming the suitability of ESR spectroscopy in the study of incorporation into cell membranes of glycolipids labeled in their hydrophobic tail.

In the present work we describe the synthesis of a variety of different mono- and disialoganglioside analogues with the nitroxide group located at one of three different positions in the fatty acid chain (see Chart I) and with a ^3H label in the carbohydrate portion. These labeled analogues have been used to study the uptake of exogenous gangliosides by a permanent mouse fibroblast cell line and to investigate the membrane environment of the incorporated label.

Materials and Methods

Materials. Eagle's minimum essential medium with Hank salt and glutamine was from Flow Laboratories, U.K. Culture flasks (25 cm^2) were from Nunc, Denmark. Precoated thin-layer plates Kieselgel 60 and Kieselgel 60_{F254} (0.25 mm layer thickness) and silica gel Lichroprep Si 60 were obtained from E. Merck A.G., Darmstadt, FRG. 2-Methyl-2-amino-propan-1-ol and 3-chloroperbenzoic acid were from Fluka, Buchs, Switzerland. 2-Bromostearic acid was obtained from ICN Pharmaceuticals, Inc., Plainview, NY. All other chemicals were of the highest purity available.

Analytical Assays. Protein content of cells was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. Glycolipid-bound Neu5Ac was measured by the method of Svennerholm (1957) as modified by Miettinen & Takki-Luukkainen (1959) with α_1-acid glycoprotein (12.6% Neu5Ac) as reference.

Synthesis of Ganglioside Analogues. (*a*) *Preparation of N-Succinimidyl 2-Azidooctadecanoate.* A total of 1 mmol of 2-bromooctadecanoic acid (363 mg) and 5 nmol of sodium azide (315 mg) was stirred in 20 mL of a mixture of 1,4-dioxane/water (5:1 v/v) at 60 °C for 15 h. Following acidification with dilute hydrochloric acid and evaporation of the solvents, the 2-azidooctadecanoic acid was extracted into *n*-hexane, dried over solid sodium sulfate, and evaporated to dryness. Recrystallization from *n*-hexane gave crystals, mp 64–66 °C, in 90% yield. This compound was further characterized by its NMR and IR spectra and by elemental analysis and was found to be pure.

A total of 0.7 mmol of 2-azidooctadecanoic acid (228 mg), 0.7 mmol of *N*-hydroxysuccinimide (81 mg), and 0.7 mmol of dicyclohexylcarbodiimide (144 mg) was dissolved in dry ethyl acetate (20 mL) and stirred overnight at 20 °C. Dicyclohexylurea was removed by centrifugation and the supernatant dried in vacuo. *N*-Succinimidyl 2-azidooctadecanoate was crystallized from ethanol containing (6%, w/v) water and obtained in 80% yield.

(*b*) *Preparation of Nitroxide-Labeled Octadecanoic Acids and Their N-Succinimidyl Ester.* 4-(4′,4′-Dimethyl-2′-tridecyl-3′-oxyoxazolidin-2′-yl)butanoic acid, 8-(4′,4′-dimethyl-2′-nonyl-3′-oxyoxazolidin-2′-yl)octanoic acid, and 12-(4′,4′-dimethyl-2′-pentyl-3′-oxyoxazolidin-2′-yl)dodecanoic acid were prepared essentially as described (Hubbell & McConnell, 1971). The nitroxide-labeled stearic acids were obtained in 10% yield based on the starting dicarboxylic acid and were found to be pure on thin-layer chromatography by using two different solvent systems. These acids were esterified as follows: 200 μmol of spin-labeled acid (77 mg), 200 μmol of *N*-hydroxysuccinimide (23 mg), and 200 μmol of dicyclohexylcarbodiimide (41 mg) were dissolved in dry ethyl acetate (5 mL) and stirred overnight at 20 °C. Dicyclohexylurea was centrifuged off and the solvent evaporated under a stream of nitrogen. The residue was dissolved in dry tetrahydrofuran (0.8 mL) and stored at −20 °C in the dark until use.

(*c*) *Isolation of Ganglioside Sugars.* Monosialoganglio-tetraose $II^3Neu5AcGgOse_4$, monosialogangliotriaose $II^3Neu5AcGgOse_3$, monosialolactose $II^3Neu5AcLac$, disialolactose $II^3(Neu5Ac)_2Lac$, and disialogangliotetraose $IV^3Neu5Ac,II^3Neu5AcGgOse_4$ were prepared from their parent gangliosides G_{M1}, G_{M2}, G_{M3}, G_{D3}, and G_{D1a}, respectively, in 60% yield by ozonolysis and alkaline fragmentation (Wiegandt & Baschang, 1965). Mono- and disialolactose were also isolated from bovine colostrum and purified (von Nicolai et al., 1978).

(*d*) *Reductive Amination of Ganglioside Sugars.* The appropriate sialooligosaccharide (15 μmol) was dissolved in liquid ammonia (about 5 mL) and heated overnight at 50 °C in a screw-capped vial. After cooling to −40 °C, solid sodium borohydride (50 μmol) was added. The reduction by borohydride of the osimin formed was performed at 20 °C over a period of 6 h. Following evaporation of ammonia, the residue was dissolved in water (1 mL) and acidified with acetic acid (20 μL) to destroy the excess borohydride. The solution was then freed of sodium ions by passage through SP-Sephadex C-25 (H^+ form; 1 mL) in a small column. The column was washed with water (10 mL), and the effluent was fractionated.

All fractions containing the reductaminated oligosaccharide were pooled and dried. Boric acid in the product was removed as trimethyl ester. The product was then freeze-dried and the yield determined by its Neu5Ac content. About 12 μmol (80%) each of 1-amino-1-deoxymonosialogangliotetraitol, 1-amino-1-deoxymonosialogangliotriaitol, 1-amino-1-deoxymonosialolactitol, 1-amino-1-deoxydisialogangliotetraitol, and 1-amino-1-deoxydisialolactitol was obtained and used in the next step without further purification. When ^{3}H-labeled sialooligosaccharides were needed, borotritiide was used instead of borohydride for reductive amination of the ganglioside sugars.

(*e*) *Preparation of 1-[(2-Azidooctadecanoyl)amino]-1-deoxymonosialogangliotetraitol, -monosialogangliotriaitol, -monosialolactitol, -disialogangliotetraitol, and -disialolactitol, Respectively.* The appropriate reductaminated ganglioside sugar (12 μmol) and sodium hydrogen carbonate (50 μmol) were dissolved in tetrahydrofuran/water (1:1 v/v; 1 mL). Solid *N*-succinimidyl 2-azidooctadecanoate (25 μmol) was added over a period of 4 h at 20 °C. The reactants were kept at 20 °C for an additional 8 h until all ninhydrin-positive material had disappeared. The solvents were then evaporated, and the 1-[(2-azidooctadecanoyl)amino]-1-deoxymonosialogangliotetraitol, -monosialogangliotriaitol, -monosialolactitol, -disialogangliotetraitol, and -disialolactitol were purified by chromatography on silica gel Lichroprep Si 60 with mixtures of chloroform, methanol, and water of increasing polarity. The fractions containing the products were pooled and the latter quantified by the Neu5Ac content. In general the yield was about 7.2 μmol (60%).

(*f*) *Hydrogenation of the Azido Compounds.* The appropriate azido compounds (7.2 μmol) dissolved in ethanol/water (7:3 v/v; 3 mL) were hydrogenated (3 bars) at 20 °C for 4 h following the addition of platinum dioxide (10 mg). Platinum was removed by centrifugation and the solvent evaporated under nitrogen. 1-[(2-Aminooctadecanoyl)amino]-1-deoxymonosialogangliotetraitol, -monosialogangliotriaitol, -monosialolactitol, -disialogangliotetraitol, and -disialolactitol were obtained almost quantitatively.

(*g*) *Preparation of the Nitroxide-Labeled Ganglioside Analogues 5-G_{M1}II, 5-G_{M3}II, 9-G_{M1}II, 9-G_{M2}II, and 9-G_{M3}II and of the Nitroxide-^{3}H-Labeled Ganglioside Analogues 13-G_{M1}II, 13-G_{M2}II, 13-G_{M3}II, 13-G_{D1a}II, and 13-G_{D3}II.* The appropriate amino compound of step f (5 μmol) was dissolved in tetrahydrofuran/0.1 M $NaHCO_3$ (1:1 v/v; 0.4 mL). The nitroxide-labeled *N*-succinimidyl octadecanoate of step b (10 μmol) in tetrahydrofuran was added over a period of 3 h with stirring. The reactants were kept at 20 °C until all ninhydrin-positive material had disappeared. The solvents were then evaporated under nitrogen and the nitroxide-labeled ganglioside analogues purified on silica gel Lichroprep with mixtures of chloroform, methanol, and water of increasing polarity. Fractions containing the pure products were pooled and quantified by the Neu5Ac content. The yield of 5-G_{M1}II and 5-G_{M3}II was about 3.5 μmol (70%), which is typical for all the different analogues. For the double-labeled ganglioside analogues, the tritium-labeled amino compounds were used.

Preparation of Ganglioside [^{3}H]G_{M3}. Ganglioside G_{M3} was tritium labeled essentially as described (Schwarzmann, 1978) and freed of any radioactive impurities by chromatography on silica gel Lichroprep Si 60 with mixtures of chloroform, methanol, and water of increasing polarity.

Cell Culturing. A permanent mouse fibroblast cell line (Clone-1D) was used for all experiments (Clayton & Teplitz, 1972). For incorporation studies with labeled exogenous ganglioside G_{M3} and ganglioside analogues, cells were grown in monolayers to near confluency in a humidified 5% CO_2–95% air atmosphere at 37 °C in 25 cm^2 plastic flasks containing 5 mL of growth medium. This medium consisted of Eagle's minimum essential medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), 5-bromodeoxyuridine (10 μg/mL), and 5% (v/v) heat-inactivated newborn calf serum. Routine examinations for mycoplasma contamination using Hoechst dye 33258 (Chen, 1977) were found to be negative. Prior to incubation of the monolayer cells with glycolipid-containing media, it was essential to remove the residual calf serum from the growth medium by extensive washing with Eagle's minimum essential medium without calf serum, since serum has previously been observed to interfere with ganglioside accumulation by the cells (Callies et al., 1977).

Incorporation of Ganglioside [^{3}H]G_{M3} and Ganglioside Analogues into Cells. An aliquot of the required glycolipid in chloroform/methanol/water (60:35:8 v/v/v) was dried in vacuo in a glass vial overnight. The dry residue was taken up in Eagle's minimum essential medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 5-bromodeoxyuridine (10 μg/mL), and the suspension was sonified for 5 min in the cuphorn of a Branson sonifier B-12 (Branson Sonic Power Co.) at a power setting of 60 W.

Washed monolayer cells in cell-culture flasks (25 cm^2) were incubated with the glycolipid-containing medium (2.5 mL) at 37 °C in a humidified 5% CO_2–95% air atmosphere for various lengths of time as indicated under Results. Following removal of the glycolipid-containing medium after incubation, the monolayer cells were washed 4 times with PBS (1 mL). The cells were then detached by incubation with 0.25% trypsin in PBS (2 mL) for 15 min at 37 °C. The cells were isolated by centrifugation at 4 °C at 1000g_{av} and washed 3 times with PBS (1 mL). The cells were then resuspended in PBS (40 μL) and transferred to a glass capillary (1-mm diameter). The cells were concentrated at one end of the sealed capillary by centrifugation for 10 min at 4 °C and 4000g_{av} and then subjected to ESR measurements.

In the case of the tritium-labeled glycolipids the washed cell pellet was resuspended in PBS (1 mL), mixed with Rialuma (10 mL), and subjected to scintillation counting by using a Packard Tri-Carb 460 C liquid scintillation system.

Preparation of Clone-1D Cell-Derived Lipid Extract and Liposomes. Approximately 50 mg of pelleted wet Clone-1D cells were disrupted in a Potter–Elvehjem homogenizer at 0 °C with five strokes at 1100 rpm. The suspension of disrupted cells was immediately centrifuged at 4 °C for 30 min at 10000g_{av} to remove nuclei and mitochondria. The supernatant was then centrifuged at 4 °C for 60 min at 100000g_{av}. The pellet obtained was extracted twice with $CHCl_3/CH_3OH$ (2:1 v/v, 2 mL) at 40 °C for 2 h, and the extracts were combined. Following the addition of water (1.2 mL), the lipids were partitioned according to Folch et al. (1957). The lower phase was dried in a stream of nitrogen, and the lipids were redissolved in $CHCl_3/CH_3OH$ (2:1 v/v) to a final concentration of 2 mg/mL. This solution was kept under nitrogen in the cold until use.

The solution of Cl-1D cell-derived lipids (1 mg) in chloroform/methanol (2:1 v/v) containing a nitroxide-labeled ganglioside analogue (10 μg) was thoroughly dried in vacuo. The dried residue was dispersed in PBS (40 μL) with vigorous shaking for 1 min on a Vortex mixer. The dispersion was transferred to a glass capillary (1-mm diameter) for ESR measurements.

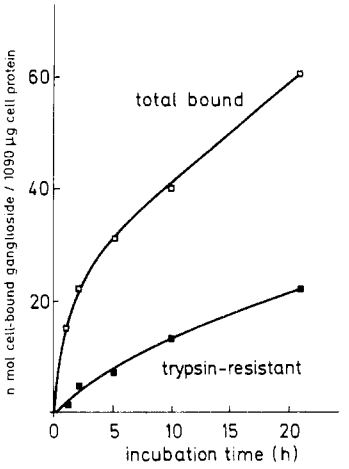


FIGURE 1: Uptake kinetics of ^{3}H-labeled G_{M3} into Clone-1D cells. Cl-1D monolayer cells (ca. 1 mg of total cellular protein) were incubated at 37 °C for various lengths of time with 10^{-4} M labeled ganglioside in 2.5 mL of medium: (□) total radioactivity associated with cells after extensive washing; (■) activity remaining associated with cells after trypsin treatment.

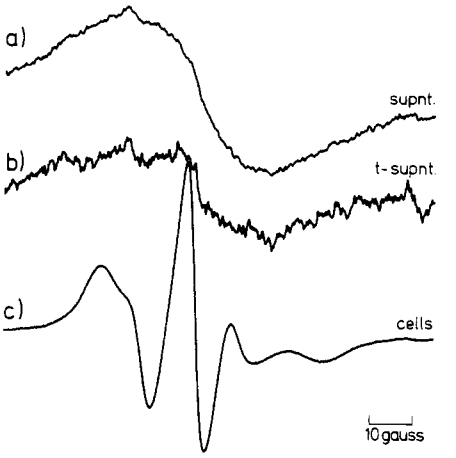


FIGURE 2: Uptake of spin-labeled G_{M2} analogue, 9-G_{M2}II, by Cl-1D monolayer cells. Cells (ca. 1 mg of total cellular protein) were incubated with 2.5×10^{-4} M 9-G_{M2}II in 2.5 mL of medium for 23 h at 37 °C. ESR spectra at 37 °C of (a) supernatant from incubation medium, (b) supernatant from washed, trypsin-treated cells after incubation, and (c) washed cells after trypsin treatment.

ESR Spectroscopy. ESR spectra were recorded on a Varian E-12 Century Line 9-GHz spectrometer equipped with nitrogen gas-flow, temperature regulation system. The sealed sample capillaries (1-mm diameter) were accommodated within standard 4-mm diameter quartz tubes containing light silicon oil for thermal stability. Temperatures were measured with a thermocouple placed just at the top of the cavity, within the quartz tube. Spectra were digitized by using a Digital Equipment Corp. LPS system with PDP 11/10 dedicated computer. Spectral subtractions and integrations were performed with a VT-11 display by using interactive software written by Dr. W. Möller of the Max-Planck-Institut, Göttingen. Order parameters were calculated from the expression

$$S = \frac{A_{\parallel} - A_{\perp}}{\mathbf{A}_{zz} - \frac{1}{2}(\mathbf{A}_{xx} + \mathbf{A}_{yy})} \frac{a_0'}{a_0} \quad (1)$$

where $2A_{\parallel}$ is equal to the outer, maximum hyperfine splitting ($2A_{max}$) and $A_{\perp}$ is obtained from the inner, minimum hyperfine splitting ($2A_{min}$) according to

$$A_{\perp}(G) = A_{min}(G) + 1.4\left[1 - (A_{\parallel} - A_{min}) \Big/ \left[\mathbf{A}_{zz} - \frac{1}{2}(\mathbf{A}_{xx} + \mathbf{A}_{yy})\right]\right] \quad (2)$$

The isotropic hyperfine splitting constant is given by

$$a_0 = \frac{1}{3}(A_{\parallel} + 2A_{\perp}) \quad (3)$$

and the isotropic hyperfine splitting constant corresponding to the single crystal environment in which the principal values of the hyperfine tensors $\mathbf{A}_{xx}$, $\mathbf{A}_{yy}$, and $\mathbf{A}_{zz}$ were measured is $a_0' = {}^1/_3(\mathbf{A}_{xx} + \mathbf{A}_{yy} + \mathbf{A}_{zz})$. The values for $\mathbf{A}_{xx}$, $\mathbf{A}_{yy}$, and $\mathbf{A}_{zz}$ for doxylpropane were taken from Jost et al. (1971). Further details of the ESR methods can be found in Marsh (1982).

Results

The uptake kinetics of the ^{3}H-labeled ganglioside G_{M3}, on incubation with Clone-1D cells, are indicated in Figure 1. A time-dependent increase in the total amount of radioactivity associated with the cells is observed after washing, although the mode of association is not clear. Trypsin treatment of the cells after incubation considerably reduces the amount of associated radioactivity, indicating that at least part of the exogenous ganglioside is attached to the surface proteins and not fully incorporated into the cell membrane. Again, the exact nature of the association of this trypsin-resistant component is not known.

The mode of uptake of exogenous ganglioside by the cells has been studied by using the spin-labeled ganglioside analogues (see Chart I). The ESR spectrum of the washed and trypsinized cells after incubation with the 9-G_{M2}II spin-labeled analogue is given in Figure 2, along with the spectra of the supernatants obtained immediately after incubation and after trypsinization, respectively. The ESR spectrum of the supernatant from the incubation medium consists of a single spin–spin broadened line, characteristic of the unincorporated ganglioside spin-label micelles (cf. Marsh et al., 1982). The spectrum obtained from the supernatant after trypsinization also consists essentially of a single spin–spin broadened line, demonstrating that ganglioside removed from the cells by trypsin treatment was attached to the cell surface in micellar form and not specifically bound to individual proteins or carbohydrate moieties. The spectrum associated with the cells contains an anisotropic component which is characteristic of this type of spin-label located in a fluid lipid membrane environment [see, e.g., Marsh (1981)]. This clearly indicates that a substantial proportion of the ganglioside analogue remaining associated with the cells after trypsin treatment is fully integrated in the cell membrane.

In Figure 3 the ESR spectrum of the trypsin-resistant ganglioside analogue in the cells is compared with the spectrum of pure micelles of the spin-labeled ganglioside analogue and of the same ganglioside analogue incorporated at low dilution (1%) in the total cell lipid extract dispersed in buffer. The similarity of the spectrum from the cells with that from the extracted lipids confirms that the ganglioside analogue is dispersed uniformly within the lipid environment of the cell membrane. Relatively little spin–spin broadened component is evident in the spectrum of the cells, and digital subtraction of the spectrum of the micelles (Figure 3a) from that of the trypsinized cells (Figure 3b) by using the spectrum of the extracted lipids (Figure 3c) to determine the subtraction end point (cf. Marsh et al., 1982) indicates that at least 70% of

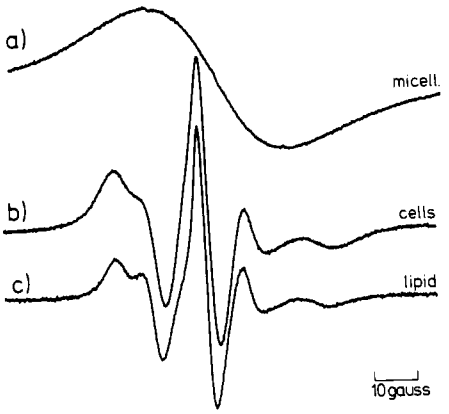


FIGURE 3: Incorporation of spin-labeled G_{M2} analogue, 9-G_{M2}II, into Clone-1D cell membranes (incubation conditions as for Figure 2). ESR spectra at 35 °C of (a) 9-G_{M2}II suspended in buffer, (b) washed, trypsin-treated cells after incubation, and (c) 9-G_{M2}II (1 mol %) in cell membrane lipid extract dispersed in buffer.

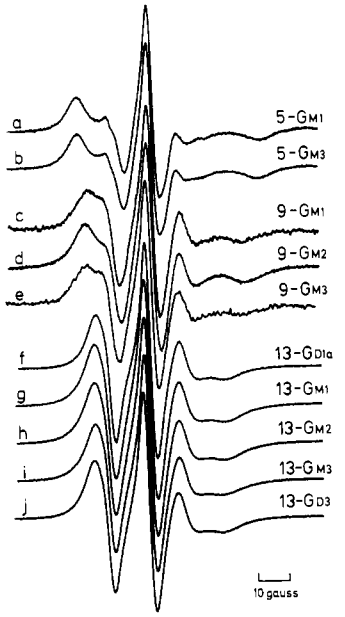


FIGURE 4: ESR spectra at 35 °C of spin-labeled ganglioside analogues incorporated into Cl-1D monolayer cells after incubation and trypsinization (incubation conditions as for Figure 2). (a) 5-G_{M1}II ganglioside analogue, (b) 5-G_{M3}II ganglioside analogue, (c) 9-G_{M1}II ganglioside analogue, (d) 9-G_{M2}II ganglioside analogue, (e) 9-G_{M3}II ganglioside analogue, (f) 13-G_{D1a}II ganglioside analogue, (g) 13-G_{M1}II ganglioside analogue, (h) 13-G_{M2}II ganglioside analogue, (i) 13-G_{M3}II ganglioside analogue, and (j) 13-G_{D3}II ganglioside analogue.

the ganglioside analogue is incorporated in the fluid lipid membrane environment.

The ESR spectra of a variety of different spin-label positional isomers of different ganglioside analogues in trypsin-treated cells are given in Figure 4. (Complementary data on the cellular uptake of the 13-positional analogues which were also ^{3}H-labeled are given below in Table II.) As in Figure 2, the spectra of Figure 4 indicate that in each case very little ganglioside micelles or other spin–spin broadened species are associated with the cells after trypsinization. The spectra of a given positional isomer in Figure 4 are rather similar, indicating that the different ganglioside analogues are incorporated into the cell membranes in a similar manner. The different positional isomers display a decreasing extent of spectral anisotropy, indicating an increasing amplitude of angular motion, on proceeding down the lipid chain toward the terminal methyl end. This "flexibility gradient" is a characteristic fingerprint of fluid lipid membranes (Marsh, 1981), again confirming the site of incorporation of the ganglioside analogues.

Table I: Effective Order Parameters, S^{eff}, and Effective Isotropic Hyperfine Splitting Constants, a_0^{eff}, of Ganglioside Spin-Labels Incorporated into Cl-1D Cells and Their Extracted Lipids at T = 35 °C

	a_0^{eff} (G)	S^{eff}
cells		
5-G_{M1}II	15.4	0.73
5-G_{M3}II	15.6	0.71
9-G_{M1}II	15.3	0.51
9-G_{M2}II	15.2	0.54
9-G_{M3}II	15.1	0.54
13-G_{D1a}II	14.3	0.36
13-G_{M1}II	14.3	0.36
13-G_{M2}II	14.3	0.36
13-G_{M3}II	14.3	0.36
13-G_{D3}II	14.3	0.35
lipids		
5-G_{M1}II	15.1	0.74
9-G_{M2}II	15.1	0.53

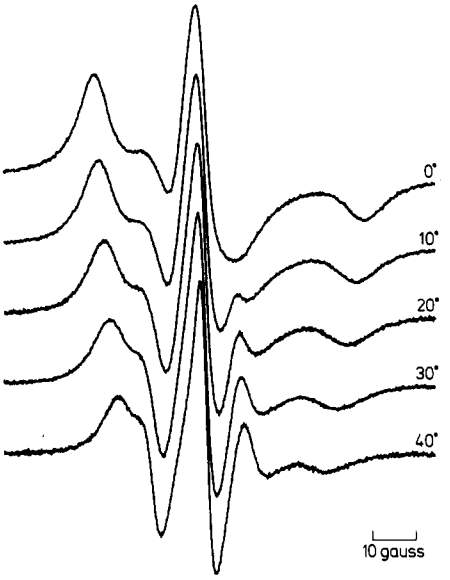


FIGURE 5: Temperature dependence of the ESR spectra of the 9-G_{M2}II ganglioside spin-label analogue in Clone-1D cells.

The outer and inner hyperfine splittings, of the spectra in Figure 4, can be used to calculate the order parameters and isotropic hyperfine splitting constants of the different labeled ganglioside analogues [see, e.g., Marsh (1982)]. Although there is a clear definition of the partial motional averaging, the line widths are rather broad. Thus, it cannot be excluded that the spectra, even at 35 °C, are approaching the slow motional regime in which not only the amplitude of motion but also the rate of motion contribute somewhat to the observed line splittings. For this reason the measured order parameters and isotropic hyperfine splitting factors must be considered as effective values. The effective order parameters and isotropic hyperfine splitting constants of the different spin-labeled ganglioside analogues, deduced from the spectra of Figure 4, are given in Table I. The order parameters give quantitative expression to the flexibility gradient of the fluid membrane. The isotropic hyperfine constants, which are sensitive to the environmental polarity, indicate that the spin-label groups positioned closer to the methyl end of the chain are situated deeper into the hydrophobic interior of the membrane, further demonstrating that the ganglioside analogues are incorporated into the membrane in a similar manner to the endogenous lipids.

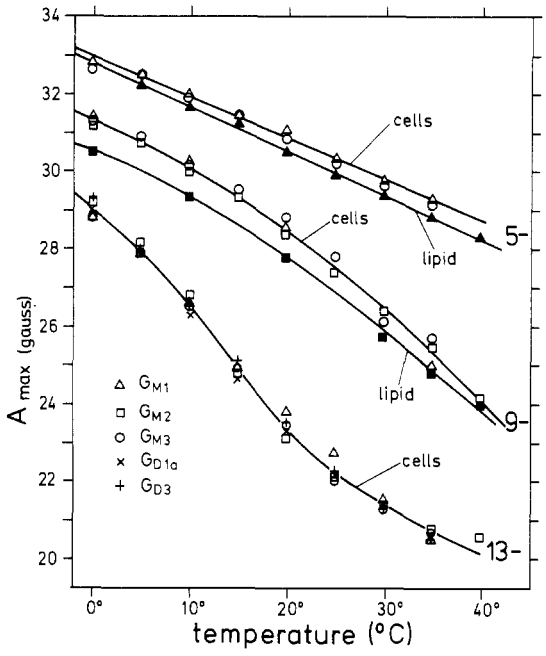


FIGURE 6: Temperature dependence of the maximum outer hyperfine splitting, $2A_{max}$, of spin-labeled ganglioside analogues in Clone-1D cells and in aqueous dispersions of the extracted membrane lipids. (Upper curves) 5-positional isomers; (middle curves) 9-positional isomers; (lower curves) 13-positional isomers. (△) *n*-G_{M1}II; (□) *n*-G_{M2}II; (○) *n*-G_{M3}II; (×) 13-G_{D1a}II; (+) 13-G_{D3}II. (Open symbols) Cells; (filled symbols) lipid dispersions.

The temperature dependence of the ESR spectra of the 9-G_{M2}II ganglioside analogue incorporated into cells is given in Figure 5. The extent of spectral anisotropy decreases with increasing temperature, corresponding to an increasing amplitude of motion, characteristic of the temperature dependence of chain mobility in lipid membranes. The temperature dependence of the outer hyperfine splitting, A_{max}, is given for the different positional isomers of the different ganglioside analogues in Figure 6. In all cases the outer splitting decreases with increasing temperature, for the cells, in a very similar manner to that for aqueous dispersions of the extracted lipids. The temperature dependence is steeper for the positional isomers closer to the terminal methyl end of the chain, which is again characteristic of the behavior in lipid membranes.

In Table II the total amount of double-labeled ganglioside analogue bound to the cells is compared with the amount which cannot be removed from the cells by trypsin. At any given incubation time the trypsin-resistant fraction is more than half of the total, irrespective of the nature of the oligosaccharide moiety. The amount of ganglioside analogue actually incorporated into Cl-1D cell membranes is approximately 15 nmol/mg of total cellular protein after 18 h of incubation. For comparison, the corresponding value for the ganglioside G_{M3} is about 19 nmol/mg of cellular protein (see Figure 1), similar to the amount of incorporated ganglioside analogue. On the other hand comparison of Table II and Figure 1 indicates that at incubation times shorter than 11 h the uptake kinetics of ganglioside G_{M3} differ from those of the ganglioside analogues.

Table II: Total and Trypsin-Resistant Uptake of Double-Labeled Ganglioside Analogues by Cl-1D Cells[a]

ganglioside analogue	incubation time (h)	total uptake (nmol/mg of protein)	trypsin-resistant (nmol/mg of protein)
13-G_{M3}II	4	15.0	8.6
	11	24.7	15.0
	18	23.0	14.4
13-G_{D3}II	4	13.0	7.6
	11	22.7	13.8
	18	23.0	15.0
13-G_{M2}II	4	17.4	9.7
	11	28.1	16.5
	18	27.6	17.0
13-G_{M1}II	4	16.7	8.6
	11	25.8	15.9
	18	24.4	16.1
13-G_{D1a}II	4	11.7	7.1
	11	19.2	11.7
	18	17.1	10.8

[a] Cl-1D monolayer cells (ca. 1 mg of total cellular protein) were incubated at 37 °C with 5×10^{-5} M double-labeled ganglioside analogue in 2.1 mL of medium. After a given incubation time cells were washed extensively with PBS and detached by trypsin treatment as described under Materials and Methods. The amount of the trypsin-resistant incorporated ganglioside analogue was calculated from the radioactivity remaining with the cells. The amount of total cell-bound ganglioside analogue was calculated from the sum of the radioactivities remaining with the cells and the trypsin-supernatant.

Discussion

The experiments with the ^{3}H-labeled and double-labeled ganglioside analogues clearly indicate that they are taken up by the cultured cells, with two different modes of association, one of which is resistant to trypsin treatment of the cells. The ESR experiments with the nitroxide-labeled analogues indicate that the ganglioside analogue which is removed by trypsin treatment is still present as ganglioside micelles, which presumably had been adhering to the cell surface. The trypsin-resistant portion of the cell-associated ganglioside analogue is almost wholely incorporated and dispersed within the cell membrane. The characteristic anisotropy and temperature dependence of the spectra, the comparison of the different positional isomers, and the comparison with dispersions of the extracted lipids all indicate that the trypsin-resistant ganglioside analogues are located in the cell membrane in a similar manner to the endogenous lipids.

The exact cellular membrane location of the inserted ganglioside analogues is not definitely known. However, the high polarity of the ganglioside sugar groups suggests that these lipids would be unlikely to flip-flop, and thus, in the absence of appreciable endocytosis, they would most likely be incorporated into the outer leaflet of the plasma membrane. The high effective order parameters observed for the different analogues (Table I) would also suggest a location in the plasma membrane which will have a high cholesterol content, rather than in the intracellular membranes which generally have low cholesterol content [see, e.g., Marsh (1975)]. The effective order parameters of the ganglioside analogues in fluid dimyristoylphosphatidylcholine bilayer membranes at 35 °C are in contrast approximately 0.63, 0.41, and 0.16 for the 5-, 9-, and 13-isomers, respectively.

The mode of insertion of the ganglioside analogues into the membrane is demonstrated by the progressive decrease both of the effective order parameters and of the effective isotropic splitting constants, as the spin-label position is stepped down the fatty acid chain toward the terminal methyl end. The isotropic hyperfine constants reflect the decreasing polarity toward the center of the membrane. For comparison the corresponding values of a_0 for spin-labeled phosphatidylcholines in lipid membranes of dipalmitoylphosphatidylcholine plus 50 mol % cholesterol are approximately 15.1, 14.7, and 14.3 G for the 5-, 9-, and 13-isomers, respectively (Marsh & Watts, 1981). The values of a_0^{eff} for the ganglioside analogues in cells are somewhat higher than those in the bilayer model mem-

brane. This may in part be an artifactual effect of the slow-motion nature of the spectra mentioned previously but will also reflect both the different structural and compositional features of the two systems. The measurements of a_0^{eff} and S^{eff} thus indicate that the ganglioside analogues are incorporated in a fluid lipid environment similar to that found in lipid bilayer model membranes. Although the spectra cannot definitively distinguish between various possible liquid-crystalline structures, it seems very likely that the exogenous ganglioside analogues are incorporated into lipid bilayer regions of the cell plasma membrane in a similar manner to the endogenous lipids. An interesting feature of the temperature dependence in Figure 6 is the steep decrease in A_{max} observed for the 13-positional isomers between 10 and 20 °C. This is not accompanied by a similar discontinuity in the temperature dependence of the other isomers and thus should not be attributed to a lipid phase transition as such. A bulk phase transition would not be expected for a plasma membrane with a high cholesterol content. The steep temperature dependence probably arises from the fact that the 13-isomer is positioned below the steroid nucleus in the membrane and hence is less motionally restricted by cholesterol than are the other isomers. It should also be noted that at 0–10 °C the spectra are in the slow-motion regime of ESR spectroscopy and hence may be expected to have a reduced motional sensitivity.

The spectral characteristics of the 13-positional isomers of the different ganglioside analogues are all very similar (Table I and Figure 6), indicating that there is little difference in the mode of incorporation or structural perturbation at this depth into the membrane. The relatively small differences observed with the 5- and 9-isomers also lie within the experimental variation between different preparations. In addition, the results of Table II indicate that the uptake kinetics and the extent of incorporation of the different analogues are similar. Thus, the ganglioside analogues are similarly anchored in the hydrophobic phase of the membrane, but specificity between the different types can be expected in interactions at the cell surface.

Previous studies on the uptake of spin-labeled gangliosides (Kanda et al., 1982a,b) have concentrated mainly on single-tailed lipids, which are detergent-like in nature, and have been restricted to red blood cells. The present study has used solely double-tailed ganglioside analogues. The experiments using radioactive ganglioside analogues have shown that the extent of incorporation of these ganglioside analogues is similar to that of the true ganglioside G_{M3}, at least in the C1-1D cell system. In addition, the incorporation has been studied with living mammallian cells. Viability after uptake of the spin-labeled ganglioside analogues was demonstrated by the further growth capability of the cells after washing the cells and restoring the serum-containing medium. This work thus paves the way to a study of the functional role of gangliosides at the cell surface. Of particular importance is the combination of radioactive tracer techniques with ESR spectroscopy by using double-labeled analogues to combine the quantitative sensitivity of the former method with the structural and dynamic sensitivity of the latter.

Acknowledgments

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Registry No. 5-G_{M1}II, 82447-24-3; 5-G_{M3}II, 86854-98-0; 9-G_{M1}II, 86854-99-1; 9-G_{M2}II, 86862-72-8; 9-G_{M3}II, 86855-00-7; 13-G_{D1a}II, 86855-01-8; 13-G_{M1}II, 86855-02-9; 13-G_{M2}II, 86855-03-0; 13-G_{M3}II, 86855-04-1; 13-G_{D3}II, 86855-05-2; ganglioside G_{M1}, 37758-47-7; ganglioside G_{M2}, 19600-01-2; ganglioside G_{M3}, 54827-14-4; ganglioside G_{D3}, 62010-37-1; ganglioside G_{D1a}, 12707-58-3; II^{3}Neu5AcGgOse$_4$, 52659-37-7; II^{3}Neu5AcGgOse$_3$, 75652-60-7; II^{3}Neu5AcLac, 35890-38-1; II^3(Neu5Ac)$_2$Lac, 38598-36-6; IV^{3}Neu5Ac,-II^{3}Neu5AcGgOse$_4$, 71494-27-4; 1-amino-1-deoxymonosialogangliotetraitol, 86855-06-3; 1-amino-1-deoxymonosialogangliotriaitol, 86855-07-4; 1-amino-1-deoxymonosialolactitol, 52659-35-5; 1-amino-1-deoxydisialogangliotetraitol, 86855-08-5; 1-amino-1-deoxydisialolactitol, 86862-73-9; 1-[(2-azidooctadecanoyl)amino]-1-deoxymonosialogangliotetraitol, 73954-26-4; 1-[(2-azidooctadecanoyl)amino]-1-deoxymonosialogangliotriaitol, 86862-74-0; 1-[(2-azidooctadecanoyl)amino]-1-deoxymonosialolactitol, 86855-09-6; 1-[(2-azidooctadecanoyl)amino]-1-deoxydisialogangliotetraitol, 86855-10-9; 1-[(2-azidooctadecanoyl)amino]-1-deoxydisialolactitol, 86862-75-1; 1-[(2-aminooctadecanoyl)amino]-1-deoxymonosialogangliotetraitol, 73942-19-5; 1-[(2-aminooctadecanoyl)amino]-1-deoxymonosialogangliotriaitol, 86855-11-0; 1-[(2-aminooctadecanoyl)amino]-1-deoxymonosialolactitol, 86855-12-1; 1-[(2-aminooctadecanoyl)amino]-1-deoxydisialogangliotetraitol, 86855-13-2; 1-[(2-aminooctadecanoyl)amino]-1-deoxydisialolactitol, 86855-14-3; 2-bromooctadecanoic acid, 142-94-9; 2-azidooctadecanoic acid, 73942-18-4; *N*-succinimidyl 2-azidooctadecanoate, 86855-15-4; 4-(4′,4′-dimethyl-2′-tridecyl-3′-oxyoxazolidin-2′-yl)butanoic acid, 29545-48-0; 8-(4′,4′-dimethyl-2′-nonyl-3′-oxyoxazolidin-2′-yl)octanoic acid, 50613-97-3; 12-(4′,4′-dimethyl-2′-pentyl-3′-oxyoxazolidin-2′-yl)dodecanoic acid, 74920-77-7.

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Electron Paramagnetic Resonance and Optical Spectroscopic Evidence for Interaction between Siroheme and Tetranuclear Iron–Sulfur Center Prosthetic Groups in Spinach Ferredoxin–Nitrite Reductase†

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ABSTRACT: Spinach ferredoxin–nitrite reductase (NiR) is a monomeric enzyme containing one siroheme (high-spin Fe^{3+}) and one oxidized Fe_4S_4 cluster per active molecule. When NiR is photochemically reduced with ethylenediaminetetraacetate (EDTA)–deazaflavin, the free enzyme and its CN^- complex display two phases of reduction by visible absorption spectroscopy. The second phase in both cases shows changes in absorption bands associated with the heme even though the heme is already in the Fe^{2+} state. Similarly, the CO complex of NiR, which is formed only when the heme is in the Fe^{2+} state, shows changes in heme absorption bands upon reduction. In the CO and CN^- complexes, these spectral changes are associated with the appearance of electron paramagnetic resonance (EPR) signals of the classical "$g = 1.94$" type characteristic of reduced Fe_4S_4 clusters. The line shapes and exact g values of these EPR signals vary between the two complexes. The second phase of reduction of free NiR is associated with the appearance of three distinct EPR signals: a small $g = 1.94$ type signal (0.11 spin per heme); a signal of the novel type previously observed in the *Escherichia coli* and spinach sulfite reductases (SiRs, also siroheme–Fe_4S_4 enzymes) with $g_\perp = 2.40$ and $g_\parallel = 2.19$ (0.15 spin per heme); an "$S = 3/2$" type signal with $g = 5.07$, 2.91, and 2.09 (0.44 spin per heme), which is similar to the two $S = 3/2$ signals found in *E. coli* SiR. Fully reduced NiR in the presence of 20% dimethyl sulfoxide has greater spin concentration in its $g = 2.40$ ($S = 1/2$) type signal ($g_\perp = 2.35$, $g_\parallel = 2.12$; 0.32 spin per heme) than free NiR. In the presence of 5 mM KCl, the $S = 3/2$ and $S = 1/2$ signals are enhanced while the $g = 1.94$ type signal is diminished. After either treatment, the enzyme remains active. These data suggest the presence of a strong magnetic interaction between the siroheme and Fe_4S_4 centers in spinach NiR. It is suggested that the novel EPR signals of reduced free NiR arise from exchange interaction between $S = 1$ or 2 ferroheme and $S = 1/2$ reduced Fe_4S_4.

Spinach NiR[1] (EC 1.7.7.1) is a monomeric enzyme of 61 000 daltons [Vega & Kamin, 1977; but see Hirasawa-Soga & Tamura (1982) for evidence that the native molecular weight of NiR is 84 000] that catalyzes the six-electron reduction of NO_2^- to NH_3 and SO_3^{2-} to S^{2-} with either reduced ferredoxin or MV^+ as electron donor. It contains a catalytic unit consisting of one siroheme and one Fe_4S_4 cluster per polypeptide (Lancaster et al., 1979; Hirasawa & Tamura, 1980; Knaff et al., 1980). The *Escherichia coli* SiR hemoprotein[2] (Siegel et al., 1982) and spinach SiR (Krueger & Siegel, 1982a) also contain this set of prosthetic groups.

To study this catalytic center further, it is desirable to be able to reduce these enzymes at both iron centers. Unfortunately, the classical strong reductant dithionite is an unsuitable agent, since one of its oxidation products is SO_3^{2-}, a substrate for each of these enzymes (with a K_m much higher than that for NO_2^- in the case of NiR), thus effecting a turnover state that contains little reduced Fe_4S_4 (Siegel et al., 1973, 1982; Vega & Kamin, 1977). MV^+ in equilibrium with H_2 and hydrogenase produced a small $g = 1.94$ type signal, typical of reduced Fe_4S_4 clusters, in these enzymes (Siegel et al., 1982; Lancaster et al., 1979), but quantitative production of such a signal had only been achieved for enzyme complexed with CO (Lancaster et al., 1979; Siegel et al., 1982) until Janick & Siegel (1982) applied the photochemical reducing system described by Massey & Hemmerich (1978) to *E. coli* SiR hemoprotein. This system, of very negative reduction potential, utilizes photoactivated Dfl to extract an electron from EDTA, whereupon it is converted to the strongly reducing Dfl radical species. These radicals react with each other to form a stable dimer species when removed from the light.

Mössbauer studies on *E. coli* SiR hemoprotein have shown that the siroheme and Fe_4S_4 centers are exchange coupled, probably through a bridging ligand, both in oxidized and two-electron-reduced uncomplexed enzyme (Christner et al., 1981). Janick & Siegel (1982, 1983) have reported three novel EPR signals associated with fully reduced SiR: one with $g = 2.53$, 2.29, and 2.07 ($S = 1/2$ type signal) and two with presumed $S = 3/2$ ground state with features at $g = 5.23$ and

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[1] Abbreviations: Dfl, 5′-deazaflavin; Fe_4S_4, tetranuclear iron–sulfur center; Fe_2S_2, binuclear iron–sulfur center; MV^+, reduced methyl viologen; NiR, nitrite reductase; SiR, sulfite reductase; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; EPR, electron paramagnetic resonance.

[2] The hemoprotein is a subunit of the native enzyme that is multimeric as isolated, having an $\alpha_8\,\beta_4$ subunit structure (Siegel et al., 1973, 1982; Siegel & Davis, 1974). The flavoprotein (α_8) moiety accepts electrons from NADPH, the physiological donor. The isolated monomeric hemoprotein (β) subunit is fully catalytically competent in the presence of a suitable electron donor, such as MV^+ (Siegel et al., 1982).

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