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51. We thank our colleagues in the Lawrence Livermore National Laboratory ICF Program and elsewhere who led the projects reviewed in this article: L. Coleman started the experimental and diagnostic program on Novette; I. Stowers and W. Hatcher led the teams who produced the targets; K. Manes, R. Speck, and G. Suski led the Novette effort; P. Drake, R. Turner, Y. Kauffman, D. Bach, K. Estabrook, and B. Lasinski led the plasma physics experiments; F. Ze and S. Lane led the implosion experiments; and D. Matthews, M. Rosen, P. Hagelstein, N. Ceglio, and M. Eckhart led the x-ray laser effort. Supported by Lawrence Livermore National Laboratory (contract W-7405-ENG-48) under the auspices of the Department of Energy.

Defining Lipid Transport Pathways in Animal Cells

Richard E. Pagano and Richard G. Sleight

Summary. A new technique for studying the metabolism and intracellular transport of lipid molecules in living cells based on the use of fluorescent lipid analogs is described. The cellular processing of various intermediates (phosphatidic acid and ceramide) and end products (phosphatidylcholine and phosphatidylethanolamine) in lipid biosynthesis is reviewed and a working model for compartmentalization during lipid biosynthesis is presented.

Most of the enzymes responsible for lipid biosynthesis in animal cells reside on the rough and smooth endoplasmic reticulum (*1*); yet lipids are found in all the membrane systems of the cell, often with different intracellular organelles having different lipid compositions (*2*). Furthermore, some membranes exhibit an asymmetric distribution of lipids across the bilayer (*3*). Thus, a major problem in the cell biology of lipids is understanding how newly synthesized lipids are sorted into various intracellular compartments, and how these molecules are translocated or targeted to various destinations inside (or outside) the cell. This general problem is directly analogous to the study of cellular proteins whose assembly into membranes, turnover, and secretion is one of the most actively studied areas in cell biology (*4*).

Our laboratory has developed an approach for studying lipid transport by means of fluorescent lipid derivatives that appear to behave as analogs of their natural counterparts. With this methodology, it is now possible to examine the movements of fluorescent lipid molecules in the living cell by high-resolution fluorescence microscopy and correlate these data with the results of classical biochemical investigations. In this article we summarize recent findings obtained with this new technology and highlight possible future applications in cell biology.

Incubation of Fluorescent Lipids with Cells

Figure 1 shows the molecular structures of the three classes of fluorescent lipids we have used in our studies. The acyl chain–labeled lipids have high rates of spontaneous transfer in vitro (Table 1). For liposomes, this transfer occurs by dissociation of lipid monomers from one membrane, convection through the aqueous phase, and association with another membrane (*5*, *6*). This property permits us to readily integrate the fluorescent lipids into cellular membranes from exogenous sources.

Our strategy for examining the intracellular metabolism and translocation of fluorescent lipid analogs is to first incubate cells at 2°C with liposomes containing both an acyl chain–labeled C_6-NBD-lipid (*7*) and a nonexchangeable lipid [such as rhodamine-labeled phosphatidylethanolamine (*N*-Rh-PE) (*8*, *9*)]. During this incubation, large amounts of the acyl chain–labeled analog but only small amounts of the nonexchangeable marker become associated with cells, suggesting that most (typically 90 to 99 percent) of the NBD-lipid is transferred to the cells by a spontaneous diffusion process. The cells are then washed and examined by fluorescence microscopy, or the lipids are extracted and analyzed by conventional analytical procedures. The cells can also be washed after the liposome incubation and then warmed to various temperatures prior to analysis.

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Transport of Phosphatidylcholine

When cells are incubated with liposomes containing C_6-NBD-phosphatidylcholine (C_6-NBD-PC) at 2°C, their plasma membranes become fluorescently labeled (Fig. 2A). The fluorescent lipid is mobile in the plane of the plasma membrane (diffusion coefficient = 2×10^{-9} cm^2/sec), suggesting that the lipid is properly integrated in the membrane bilayer, and not simply adsorbed to the cell surface (*8*).

When cells containing C_6-NBD-PC at their plasma membrane are warmed to 37°C, internalization of some of the lipid occurs. This internalization is dependent on endocytosis and is blocked at temperatures below about 8°C and by agents that lower internal adenosine triphosphate concentrations (*10*). The internalization of C_6-NBD-PC at 37°C for 60 minutes (in four different cell lines) results in one of two different patterns of intracellular fluorescence. In two of the cell types (V79 and CHO), the internalized C_6-NBD-PC accumulates both in a centrally located perinuclear region and as a number of small, punctate, intracellular fluorescent vesicles (Fig. 2B) (*10, 11*). By colocalizing the internalized fluorescent phosphatidylcholine with organelle-specific stains, we determined that the perinuclear fluorescence corresponds to the region of the Golgi apparatus (*10*). Since the location of punctate intracellular vesicles (Fig. 2B) does not correspond to that of known lysosomal markers (*12*), we tentatively refer to them as endocytic vesicles. In the other two cell types (BHK and CG-1), relatively small amounts of lipid are internalized after the 1-hour incubation, and accumulation of fluorescent lipid in the Golgi apparatus is not readily apparent (*11*). During 37°C incubations, some degradation of the fluorescent lipid occurs and results in the release of C_6-NBD–free fatty acid into the incubation medium. The C_6-NBD–free fatty acid is not reutilized for lipid synthesis and does not accumulate in V79 cells (*13, 14*).

When the internalization of C_6-NBD-PC is allowed to occur at 16°C, a large number of fluorescently labeled vesicles accumulate intracellularly (Fig. 2B, inset). This pattern of fluorescence is stable unless the temperature is raised above 18°C, at which time the vesicles disappear and the Golgi region becomes fluorescent (*10*). The accumulation of C_6-NBD-PC in endocytic vesicles may occur at 16°C because either delivery to or fusion of these vesicles with the Golgi apparatus is blocked at temperatures below 18°C.

When the plasma membranes of cells are labeled with fluorescent C_6-NBD-PC as well as a rhodamine-labeled lectin to mark glycoproteins, both fluorescent molecules are internalized simultaneously (*10*). However, while most of the lipid is delivered to the Golgi region, the majority of the lectin appears to be associated with small intracellular vesicles. Determination of the mechanism responsible for this separation may provide insights into the regulation of both protein and lipid transport.

Table 1. Characteristics of lipid movement in liposomes.

Lipid	Half-time for interbilayer transfer at 20°C* (min)	Transbilayer movement in liposomes†
C_6-NBD-sphingomyelin	0.04	–
C_6-NBD-cerebroside	0.11	–
C_6-NBD-ceramide	0.42	+
C_6-NBD-PA	0.55 (1.4)‡	–
C_6-NBD-PC	0.73	–
C_6-NBD-PE	1.54	–
C_6-NBD-DG	34.0 (168)‡	+
Dioleoylthioglycerol§	ND‖	+
Phosphatidylcholine¶	2.9×10^3	ND
Sphingomyelin**	$1–1.4 \times 10^5$	ND

* Half-time for equilibration of NBD-lipid between dioleoylphosphatidylcholine vesicles (*6, 11, 24*). † Procedures were as described (*9, 24*). ‡ Numbers in parentheses refer to measurements made at 5°C (*24*). § The derivative and its transbilayer movement have been described (*49*). ‖ ND, not determined. ¶ 1-Palmitoyl,2-oleoyl phosphatidylcholine; measurement made at 22°C (*31*). ** Palmitoylsphingomyelin; measurement made at 22°C (*32*).

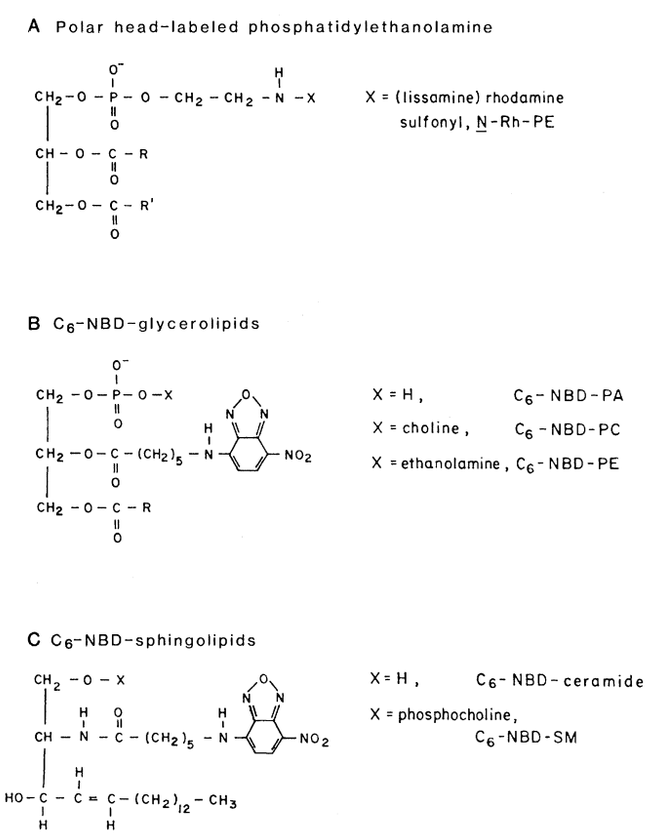

Fig. 1. Structures of fluorescent lipid derivatives. The polar head group–labeled rhodamine derivative is formed by reacting the free amino group of phosphatidylethanolamine with (lissamine) rhodamine B sulfonyl chloride (*43*). The acyl chain–labeled glycerolipids and sphingolipids have one of the naturally occurring fatty acids replaced with *N*-(4-nitrobenzo-2-oxa-1,3-diazole) aminocaproic acid (C_6-NBD-fatty acid) (*48*). R and R′ represent fatty acyl residues.

Transport of Phosphatidylethanolamine

Like C_6-NBD-PC, the fluorescent phosphatidylethanolamine analog C_6-NBD-PE transfers from liposomes to the plasma membrane of cells at 2°C (Fig. 2C) and freely diffuses in the membrane bilayer (*8*). Furthermore, the lipid is localized exclusively in the outer leaflet of the plasma membrane bilayer (*8, 15*). The degradation rates of both C_6-NBD-PC and C_6-NBD-PE and the extent of release of C_6-NBD–free fatty acid from these lipids into the surrounding medium are identical (*10, 15*).

When cells that have been incubated with C_6-NBD-PE at 2°C are washed and

warmed to 37°C for 1 hour, the mitochondria, nuclear envelope, and Golgi apparatus become fluorescent (Fig. 2D). Although it is likely that the Golgi apparatus becomes labeled with the fluorescent phosphatidylethanolamine by the same pathway identified for the internalization of C_6-NBD-PC (*10, 15*), transport of C_6-NBD-PE to the nuclear envelope and mitochondria is independent of endocytosis and appears to result from transmembrane movement at the plasma membrane followed by translocation to internal organelles.

Transmembrane movement of C_6-NBD-PE. This process can be studied in experiments in which C_6-NBD-lipid present on the outer leaflet of the plasma membrane bilayer is removed during incubation with nonfluorescent liposomes (back exchange) (*8, 10, 15, 16*). Complete removal of C_6-NBD-PE from the plasma membrane by back exchange at 2°C is dependent on the temperature at which the labeled cells were previously incubated. As long as cells containing C_6-NBD-PE at their plasma membranes are never warmed above 6°C, no internalization is observed and complete removal of the fluorescent lipid by back exchange at 2°C occurs. If, however, the cells are warmed above 6°C, only a fraction of the lipid at the plasma membrane can be removed by back exchange at 2°C (*15*). This suggests that when the cells are warmed above 6°C some of the fluorescent lipid at the plasma membrane becomes inaccessible to back exchange because it resides in the inner leaflet of the membrane. It remains to be determined whether transmembrane movement of C_6-NBD-PE is a physical process or a protein-mediated event.

Translocation of C_6-NBD-PE from the plasma membrane to intracellular membranes. The endocytosis-independent pathway of C_6-NBD-PE internalization appears to exclude delivery via intracellular lipid vesicles. Since C_6-NBD-PE can move spontaneously between liposomes (Table 1) or between liposomes and cells, it is likely that the same process occurs for translocation from the plasma membrane to intracellular membranes. It is also possible that lipid transfer proteins (*17*), which increase the rate of C_6-NBD-PE intermembrane transfer in vitro (*18*), may play a role in the intracellular transport of this lipid. In addition, a permanent or transient interconnection between the inner leaflet of the plasma membrane and other intracellular organelles (*19*) may exist, providing a pathway for internalization of C_6-NBD-PE via lateral diffusion. Immediately after brief incubations at elevated

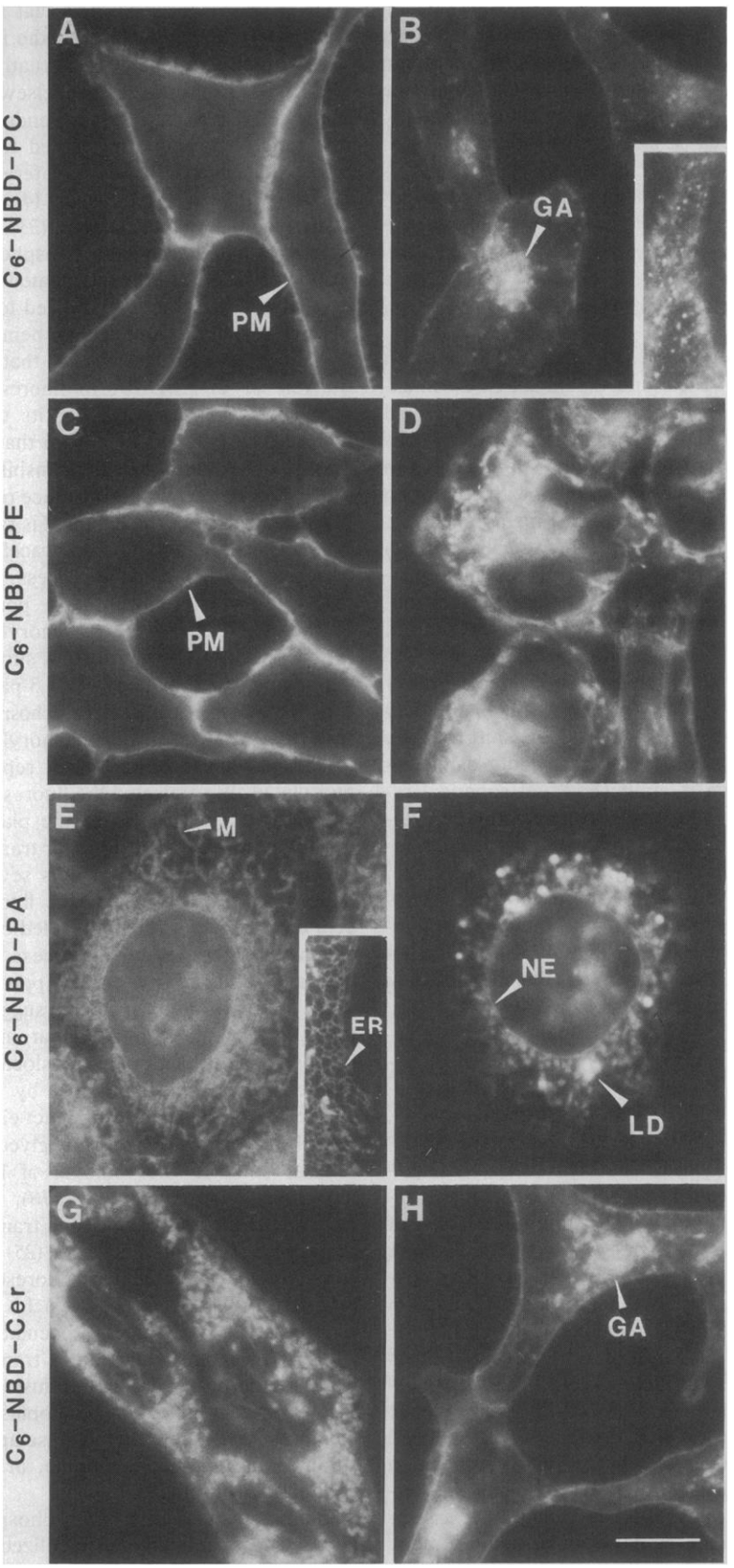

Fig. 2. Fluorescence micrographs of cells treated with C_6-NBD-PC, C_6-NBD-PE, C_6-NBD-PA, or C_6-NBD-ceramide. Chinese hamster fibroblasts were incubated with liposomes containing the indicated fluorescent lipid at 2°C for 30 to 60 minutes and then washed (left column). Some of the cells were then further incubated at either 37°C (B, D, F, and H) or 16°C (panel B inset) for 30 to 60 minutes (right column). In (B) and (D) the amount of fluorescence at the plasma membrane was reduced by incubating the cells with dioleoylphosphatidylcholine vesicles at 2°C (*10, 15*). Inset in (E) shows the endoplasmic reticulum (ER) at cell periphery. GA, Golgi apparatus; LD, lipid droplet; M, mitochondria; NE, nuclear envelope; PM, plasma membrane. Bar is 10 μm.

temperature, no intracellular labeling with C_6-NBD-PE can be seen, even though the amount of this lipid that can be removed from the plasma membrane by back exchange at 2°C is substantially reduced (*15*). Therefore, we suggest that transbilayer movement of this lipid is much faster than its intracellular translocation.

Transport of Phosphatidic Acid

In contrast to the results obtained with C_6-NBD-PC and C_6-NBD-PE, essentially all of the C_6-NBD-phosphatidic acid (C_6-NBD-PA) that is transferred to cells during a 2°C incubation is internalized to cytoplasmic membranes, with little or no labeling of the plasma membrane (Fig. 2E). The internal membranes that become labeled are the endoplasmic reticulum, mitochondrial membrane, and nuclear envelope (*20*). A different pattern of intracellular fluorescence is observed if cells that have been treated with C_6-NBD-PA at 2°C are washed and then warmed to 37°C. After the shift to 37°C, the fluorescence of the endoplasmic reticulum is diminished and spherical regions of fluorescence appear in the cytoplasm, corresponding to intracellular lipid storage droplets (Fig. 2F). Although these droplets are present at all times during the experiment, they are not fluorescent after incubation with C_6-NBD-PA at 2°C but become labeled only when the temperature is shifted to 37°C.

Specific events in lipid metabolism accompany both the initial uptake of C_6-NBD-PA and its subsequent redistribution at 37°C. During incubation at 2°C, approximately 80 to 90 percent of the C_6-NBD-PA is converted to C_6-NBD-diacylglycerol (C_6-NBD-DG), with the remaining lipid consisting principally of intact C_6-NBD-PA. When cells are warmed to 37°C, the C_6-NBD-DG is converted largely to C_6-NBD-triacylglycerol (C_6-NBD-TG) and C_6-NBD-PC, while small amounts of other C_6-NBD-lipids are also formed (*13, 21*). Thus, the redistribution of intracellular fluorescence during the 37°C incubation is associated with a marked change in the composition of cell-associated NBD-lipids. Furthermore, only C_6-NBD-TG becomes associated with the intracellular lipid droplets, while other NBD-lipids remain in other intracellular membranes (*13*). Hence the cell recognizes the different classes of fluorescent glycerolipids as they are formed, and can transport or "sort" them to different cytoplasmic locations. Although the molecular mechanism (or mechanisms) underlying this process is not yet known, it is possible that some C_6-NBD-TG is synthesized at the intracellular lipid droplet sites. Alternatively, C_6-NBD-TG may be formed elsewhere in the cell (for example, at the endoplasmic reticulum) and translocated to the intracellular lipid droplets by lateral diffusion (*22*), vesicular transport (*4*), lipid transfer (*17*), or emulsification (*23*).

When a nonhydrolyzable phosphonate analog of C_6-NBD-PA is incubated with cells at 2°C, it becomes localized to the plasma membrane, rather than being internalized (*24*). This suggests that hydrolysis of C_6-NBD-PA to fluorescent diacylglycerol is required for its entry into cells. We have also shown that the C_6-NBD-DG undergoes transbilayer movement to the cytoplasmic face of the plasma membrane from which it is rapidly translocated to other intracellular membranes by a facilitated process. During or after internalization, a portion of the C_6-NBD-DG is phosphorylated (at 2°C) back to C_6-NBD-PA. A surprising finding was obtained with 1,3-palmitoyl, NBD–aminocaproyl glycerophosphate (β-NBD-PA), which is dephosphorylated to 1,3-diacylglycerol but is not rephosphorylated. We expected the fluorescent diacylglycerol to remain at the plasma membrane since its half-time for transfer between lipid vesicles at 5°C is several hours (Table 1). However, the fluorescent diacylglycerol rapidly labeled cytoplasmic membranes by a process that was not inhibited by energy poisons. This internalization strongly suggests that the labeling of intracellular membranes is due to facilitated translocation of the diacylglycerol, possibly by specialized cytosolic proteins that act either directly as carriers (*17*) of diacylglycerol, or that enhance the removal of lipid monomers from membranes (*18*). Our finding that the nonspecific lipid transfer protein isolated from beef liver (*25*) can stimulate the movement of fluorescent diacylglycerol between lipid vesicles (*18*) is consistent with such speculation. Alternatively, this facilitated transfer could be the result of lateral diffusion of the fluorescent lipid along cytoplasmic membranes that are either in close apposition to the plasma membrane, or are continuous with it (*19*).

In animal cells, endogenous phosphatidic acid is normally metabolized to diacylglycerol, triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine, and by the CDP-diacylglycerol route to the anionic lipids phosphatidylinositol, phosphatidylglycerol, and cardiolipin. We have never found expression of the anionic lipid pathway in cells treated with C_6-NBD-PA. Initial studies have shown that C_6-NBD-PA can be metabolized to fluorescent CDP-diacylglycerol and phosphatidylinositol in cell homogenates (*26*). This suggests that, in the intact cell, the substrate may not reach the appropriate intracellular compartment for synthesis to occur. Alternatively, fluorescent anionic lipids may be formed but undergo rapid remodeling of their acyl chains, thereby releasing C_6-NBD–free fatty acid into the medium.

Transport of Ceramide

When cells are incubated at 2°C with liposomes containing C_6-NBD-ceramide, the initial pattern of intracellular fluorescence is similar to that seen with C_6-NBD-PA; namely, the mitochrondria, nuclear envelope, and endoplasmic reticulum become fluorescently labeled (Fig. 2G) (*27*). On the basis of its in vitro properties (Table 1), we speculate that during incubation at 2°C, the fluorescent ceramide is inserted into the outer leaflet of the plasma membrane bilayer, but readily undergoes transmembrane movement to the cytoplasmic face of the membrane. From there, it could be rapidly translocated to intracellular membranes by a spontaneous or a facilitated diffusion process (or both).

When cells that have been treated with C_6-NBD-ceramide are washed and warmed to 37°C, the Golgi apparatus and later the plasma membrane become intensely fluorescent (Fig. 2H) (*27*). During this redistribution of intracellular fluorescence, the C_6-NBD-ceramide is metabolized to fluorescent sphingomyelin and cerebroside (*27*). In agreement with the increasing fluorescence at the plasma membrane over time, increasing amounts of each fluorescent metabolite can be removed from the cell surface by back exchange to nonfluorescent acceptor liposomes (*16*). Monensin inhibits the delivery of the fluorescent metabolites to the cell surface, but does not inhibit their synthesis. There is a concomitant increase in fluorescence at the Golgi apparatus, strongly suggesting that both the fluorescent sphingomyelin and glucocerebroside analogs are first synthesized intracellularly from C_6-NBD-ceramide and are then translocated through the Golgi apparatus to the cell surface (*16*). The finding that monensin inhibits the appearance of C_6-NBD-glucocerebroside at the plasma membrane was not unexpected since most evidence indicates that glycosylating enzymes are localized to microsomal or Golgi fractions (*28*). However, inhibition of C_6-NBD-sphingomyelin transport to the cell sur-

face by monensin may be particularly significant since the exact site of sphingomyelin biosynthesis is in question, and has recently been suggested to be the plasma membrane (*29*).

Comparison of in Vitro and in Vivo Properties of C_6-NBD-Lipids

In cells, the various NBD-lipids behave differently from one another, not only with respect to their metabolism but also with respect to their initial localization at 2°C and subsequent redistribution at 37°C (Table 2). Each NBD-lipid, once delivered to the cell, is processed differently. How can this be, given the high rates of spontaneous transfer in vitro of the NBD-lipids compared to native lipids (Table 1) (*30–32*)? Indeed, from these data, we might predict that each of the NBD-lipids and their metabolites should be randomly scrambled among all intracellular membranes once inserted into a given cellular membrane. We suggest that this does not occur because the various fluorescent lipids are sometimes compartmentalized within the cell (*33*). That is, they may be restricted to the luminal surface of an organelle or intracellular vesicle, and therefore cannot transfer between intracellular membranes because of their inability to undergo transbilayer movement. Thus they can only move from one place to another by a process such as vesicle movement.

The compound C_6-NBD-PC can be inserted into the plasma membrane of cells at 2°C and is internalized, upon warming to 37°C, to discrete structures such as endocytic vesicles and the Golgi apparatus (*10*). From the topology of the internalization process, we suggest that the fluorescent lipids initially present in the external leaflet of the plasma membrane become localized to the inner leaflet (or lumen) of endocytic vesicles. If, as for liposomes, the NBD-lipids do not readily flip-flop in vivo, they could not reach the opposite leaflet of these endocytic vesicles and spontaneously transfer into other intracellular membranes. What about lipids such as C_6-NBD-DG and -ceramide, which can readily undergo transbilayer movement (Table 1) (*34*)? The ability of these lipids to rapidly move between intracellular membranes as well as across them by flip-flop may result in their continuous supply to intracellular sites where further metabolism may occur. Once converted to end products that are no longer able to undergo transbilayer movement, they would be trapped on the side of the membrane on which synthesis occurred, generating an asymmetry. Such an asymmetry could be disrupted by proteins specialized for the specific translocation of certain lipids (*35*) across the lipid bilayer.

One way of testing the compartmentalization of the NBD-lipids is by microinjection experiments. If the ideas presented above are correct, then, when an NBD-lipid is injected into a cell, it should be able to transfer by diffusion through the cytosol and label all intracellular membranes. Figure 3 shows the distribution of intracellular fluorescence shortly after microinjection of C_6-NBD-PC. Although the most intense fluorescence labeling is of the mitochondria and nuclear envelope, labeling of the endoplasmic reticulum, plasma membrane, and possibly other organelles is also seen. Similar results have been obtained after microinjection of C_6-NBD-PA and C_6-NBD-PE (*11*). Thus, the intracellular distribution of C_6-NBD-PC after its internalization from the plasma membrane (Fig. 2B) is different from the distribution after microinjection (Fig. 3); this finding supports the compartmentalization model.

We conclude that (i) some cellular

Table 2. C_6-NBD-lipid processing by cells.

Exogenous lipid	At 2°C		After warming to 37°C		Proposed mechanism for observed distribution
	Subcellular location	Major metabolic products	Subcellular location*	Major metabolic products	
C_6-NBD-PC	PM	C_6-NBD-PC			Insertion of monomers into outer leaflet of PM
			PM	C_6-NBD-PC	Incomplete internalization or recycling (or both)
			Golgi	C_6-NBD-PC	Endocytosis, vesicular transport
C_6-NBD-PE	PM	C_6-NBD-PE			Insertion of monomers into outer leaflet of PM
			PM	C_6-NBD-PE	Incomplete internalization or recycling (or both)
			Golgi	C_6-NBD-PE	Endocytosis, vesicular transport
			Mito, NE	C_6-NBD-PE	Transmembrane movement followed by diffusion†
C_6-NBD-PA	ER, Mito, NE	C_6-NBD-DG, C_6-NBD-PA			Hydrolysis to C_6-NBD-DG at PM, transmembrane movement followed by diffusion† and some rephosphorylation
			ER, Mito, NE	C_6-NBD-PA, -PC, and -TG	Further local metabolism of C_6-NBD-PA and C_6-NBD-DG
			Fat droplets	C_6-NBD-TG	Local synthesis or selective transport after synthesis (or both)
C_6-NBD-Cer	ER, Mito, NE	C_6-NBD-Cer			Insertion of monomers into PM, transbilayer movement followed by diffusion†
			ER, Mito, NE, and Golgi	C_6-NBD-SM and -cerebroside	Local metabolism of C_6-NBD-Cer or vesicular transport (or both)
			PM	C_6-NBD-SM and -cerebroside	Vesicular transport from the Golgi apparatus

* PM, plasma membrane; Golgi, Golgi apparatus; Mito, mitochondria; NE, nuclear envelope; ER, endoplasmic reticulum. †May include spontaneous transport, protein-facilitated transport, lateral diffusion, or a combination of these processes.

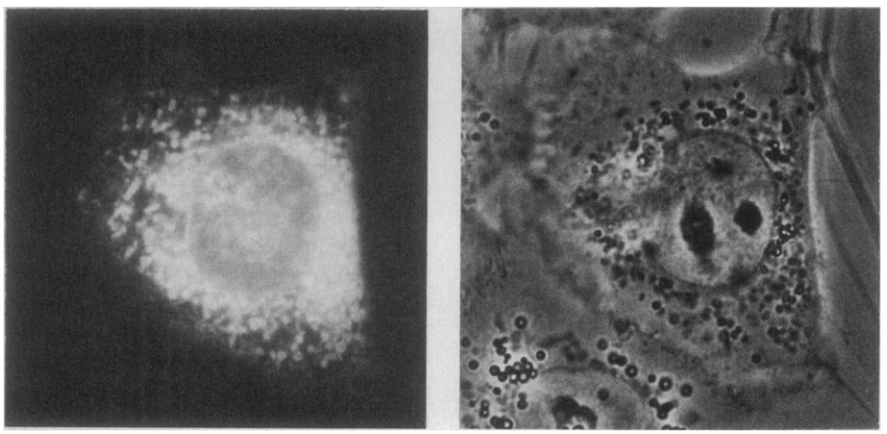

Fig. 3. Microinjection of an NBD-lipid. Fluorescence and phase micrographs of a Chinese hamster fibroblast taken immediately after microinjection of liposomes containing self-quenching (*46*) amounts of C_6-NBD-PC.

lipid metabolism occurs on the noncytoplasmic face of the endoplasmic reticulum, and (ii) flip-flop of some of the fluorescent lipids from the lumen to the cytoplasmic face of this organelle is negligible. These conclusions are in disagreement with current ideas about lipid metabolism and transmembrane movement in the endoplasmic reticulum (*36*). However, it must be emphasized that our current understanding of these processes is derived from studies of isolated microsomes. Because the endoplasmic reticulum might be different in the intact, living cell, a rigorous study of the sidedness of lipid biosynthesis could best be achieved with an in situ approach, perhaps by means of the fluorescent lipid analogs described here. Molecules that cannot cross membranes but are potent quenchers of NBD-fluorescence [for example, anti-NBD-antibodies (*37*)] may be useful for studying the sidedness of NBD-lipids within the various membrane systems of the living cell.

Do C_6-NBD-Lipids Accurately Reflect the Behavior of Endogenous Lipids?

At present we do not know how precisely the metabolism and intracellular translocation of the C_6-NBD-lipids mimic that of their endogenous counterparts. However, the following points suggest that these molecules are good analogs:

1) We have found that when another fluorescent moiety (dansyl) is used in place of NBD, the fluorescent phosphatidic acid and phosphatidylcholine derivatives behave the same as the corresponding NBD-analogs (*38*). This suggests that the chemistry of other parts of the molecule, rather than the fluorescent moiety, plays the dominant role in determining the intracellular fate of the lipids.

2) Both C_6-NBD-PA and C_6-NBD-ceramide are metabolized in various cell types to many of the fluorescent end products predicted from classical metabolic pathways. Furthermore, when radioactively labeled and fluorescent ceramides are added to subcellular fractions, they are metabolized without preference to the corresponding sphingomyelins and glucocerebrosides (*16*). This suggests that the NBD-moiety does not bias these processes.

3) The intracellular sorting of fluorescent triacylglycerol supports the idea that the C_6-NBD-lipids do not randomly disperse throughout the cytoplasm, but rather assume a unique intracellular distribution during their metabolism.

4) The time required for the translocation of newly synthesized fluorescent sphingomyelin and cerebroside to the plasma membrane (*27*) is consistent with the time required for an isotopically labeled neuronal ganglioside (*39*) to appear at the plasma membrane.

5) Monensin inhibits the appearance of both isotopically labeled glycosphingolipids (*40*) and fluorescent glucocerebroside (*16*) at the cell surface.

6) Both C_6-NBD-PE (*15*) and de novo–synthesized radioactively labeled phosphatidylethanolamine (*41*) appear to undergo transbilayer movement at the plasma membrane.

Conclusions and Perspectives

The use of fluorescent lipids represents a novel approach to research in the cell biology of lipids. While it is impossible to predict which uses of this methodology will be the most rewarding, we expect considerable progress to be made in the following areas:

1) It should now be possible to examine the recycling of lipids (*42*) between the plasma membrane and cell interior.

2) NBD- and rhodamine-labeled lipids make excellent donor-acceptor pairs for resonance energy transfer studies (*43*). We and others have used fluorescent lipid analogs to study the extent of lipid intermixing as the result of membrane fusion (*43, 44*), lipid transfer (*8, 18*), and transmembrane movement (*9, 24*). Future experiments with resonance energy transfer may allow direct measurements to be made of the kinetics of translocation of a fluorescent lipid from one intracellular compartment to another.

3) Recent developments in image intensification and digital image processing (*45*) make fluorescence observations possible at extremely low light levels with excellent resolution. Thus, it should now be possible to examine fluorescent lipid translocation in single, living cells, over long periods of time.

4) It will be of interest to study the behavior of NBD-lipid analogs of sphingomyelin, phosphatidylserine, phosphatidylglycerol, cardiolipin, and CDP-diacylglycerol to learn whether any unique intracellular distributions and translocation pathways for these lipids exist. In addition, the use of NBD-lipids with unusual chemistry may give further insights into these processes. For example, self-quenched (*46*) fluorescent lipids bearing two NBD–fatty acyl chains and D-stereoisomers of lipids may allow us to distinguish certain metabolic pathways from one another and observe their effects on lipid translocation.

5) Finally, in light of the recent discovery that the polyphosphoinositides and their metabolites play a central role in signal transmission and cell regulation (*47*), it will be particularly exciting to examine the intracellular movements of fluorescent analogs of these molecules in a living, stimulated cell.

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The Generative Grammar of the Immune System

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Grammar is a science that is more than 2000 years old, whereas immunology has become a respectable part of biology only during the past hundred years. Though both sciences still face exasperating problems, this lecture attempts to establish an analogy between linguistics and immunology, between the descriptions of language and of the immune system. Let me first recall some of the essential elements of the immune system, with which I shall be concerned. In 1890, von Behring and Kitasato (*1*) were the first to discover antibody molecules in the blood serum of immunized animals and to demonstrate that these antibodies could neutralize diphtheria toxin and tetanus toxin. They also demonstrated the specificity (*2*) of antibodies: tetanus antitoxin cannot neutralize diphtheria toxin, and vice versa. During the first 30 years or more after these discoveries, most immunologists believed that all cells of our body are capable of producing antibodies, and it took until the 1950's before it became clear, and until 1960 before it was demonstrated (*3*), that only white blood cells, named lymphocytes, can produce antibodies. The total number of lymphocytes represents a little more than 1 percent of the body weight of an animal. Thus, it would not be wrong to say that our immune system is an organ consisting of about 10^{12} lymphocytes, and a mouse, which is 3000 times smaller than we are, has an immune system consisting of about 3×10^8 lymphocytes. This brief description of the immune system disregards the fact that lymphocytes interact with most other cells in the body, which in my definition do not belong to the immune system in a strict sense.

Let me draw attention to the fact that this number of lymphocytes in the immune system is at least one order of magnitude larger than the number of neurons in the nervous system. Also, we should note that lymphocytes travel among most other cells of our body, that they circulate in blood and lymph, and that they occur in large concentrations in spleen, lymph nodes, appendix, thymus, and bone marrow. Strangely enough, however, they seem to be excluded from the brain. The 1960's was a very fruitful decade of immunological discoveries, of which I shall name a few. In the beginning of the decade, the primary structure of antibody molecules was clarified (*4*); then followed the demonstration that the dictum of Burnet (*5*) was correct, namely that all antibody molecules synthesized by one given lymphocyte are identical;

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