ANNALS OF
THE NEW YORK ACADEMY
OF SCIENCES

VOLUME 414

BIOMEMBRANES AND CELL FUNCTION

Editors
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PUBLISHED BY THE NEW YORK ACADEMY OF SCIENCES
ANYAA9 414 1–195 (1983)

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The New York Academy of Sciences
New York, New York
1983

Library of Congress Cataloging in Publication Data

Main entry under title:

Biomembranes and cell function.

(Annals of the New York Academy of Sciences; v. 414)

Articles from a workshop entitled, The Role of biomembranes in the integrity and function of cells, held Aug. 3–6, 1982, New York City. Sponsored by the National Science Foundation and the Romanian Council for Science and Technology.

Includes bibliographical references and index.

1. Cell membranes—Congresses. 2. Cell physiology—Congresses. I. Kummerow, Fred A. II. Benga, Gheorghe. III. Holmes, Ross P. IV. National Science Foundation (U.S.) V. Consiliul Național pentru Știință și Tehnologie. VI. Series.
Q11.N5 vol. 414 [QH601] 500s [574.87'5] 83-26879
ISBN 0-89766-222-9
ISBN 0-89766-223-7 (pbk.)

Subject index prepared by Justine Cullinan

Cover: Negative stains of membranes formed by acyltransferase activity. Courtesy of David W. Deamer.

CCP
Printed in the United States of America
ISBN 0-89766-222-9 (cloth)
ISBN 0-89766-223-7 (paper)

INTRODUCTION

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The articles in this volume stem from a workshop held in New York City in August, 1982 to facilitate scientific exchange between the United States and Romania and between individual scientists. It was sponsored by the National Science Foundation and the Romanian Council for Science and Technology. The participants all have central research interests in the area of membranes and use a variety of experimental approaches to study their structural and functional properties. The aim of the workshop was to expose participants to these different techniques and approaches, to facilitate an understanding of their application, and ultimately to lead to a cross-fertilization of ideas and the development of new approaches. To this extent, the workshop was highly successful and a rewarding experience for all the participants. It is our belief that readers of the articles in this volume will obtain similar benefits from the application of the specialized techniques presented, and furthermore, will find valuable new information on a range of membrane properties.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 414

December 30, 1983

BIOMEMBRANES AND CELL FUNCTION*

Editors and Conference Chairmen
F. A. KUMMEROW, GHEORGHE BENGA, AND ROSS P. HOLMES

CONTENTS

* This volume is the result of a workshop entitled The Role of Biomembranes in the Integrity and Function of Cells, held on August 3–6, 1982 in New York City.

METABOLISM AND INTRACELLULAR DISTRIBUTION OF A FLUORESCENT ANALOGUE OF PHOSPHATIDIC ACID IN CULTURED FIBROBLASTS*

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Introduction

Lipid molecules are essential building blocks for virtually every membrane of the living cell. Despite their importance, little is known about the mechanism(s) by which different lipids are sorted, transported, and assembled into various intracellular membranes. In order to study these problems we have developed an approach employing fluorescent phospholipids,[1-6] which, in some cases, can be used as true analogues of their natural counterparts. This approach has many advantages over conventional methods of metabolic study, including the abilities to directly observe fluorescent lipid metabolites within the living cell by fluorescence microscopy, and to detect and analyze minute amounts of fluorescent material. This report highlights some of our recent studies[7] with 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidic acid (C_6-NBD-PA, Figure 1), a fluorescent analogue of phosphatidic acid, which is a key intermediate in glycerolipid biosynthesis.

Incubation of C_6-NBD-PA with Cultured Fibroblasts

We have used small unilamellar vesicles as a vector for introducing C_6-NBD-PA into cultured Chinese hamster fibroblasts. In a typical experiment, vesicles are first prepared from dioleoyl phosphatidylcholine, C_6-NBD-PA, and the non-exchangeable fluorescent lipid[2,3] *N*-(lissamine) rhodamine B sulfonyl dioleoyl phosphatidylethanolamine (*N*-Rh-PE) (77/20/3, mol %), and then incubated with cells either in monolayer cultures or in suspension for 60 min at 2°C. All incubations are carried out in a protein-free HEPES-buffered balanced salt solution at a total vesicle lipid concentration of 0.2 μmol/ml. Following this incubation, the cells are washed, and either examined by fluorescence microscopy, or the lipids are extracted and analyzed using conventional analytical procedures.

Table 1 presents some typical results on the uptake of C_6-NBD-PA and *N*-Rh-PE by cells under these conditions. As seen from these data, significant amounts of the NBD-lipid are transferred to the cells with relatively little uptake of *N*-Rh-PE. This results in a ratio of NBD to rhodamine fluorescence in the washed, vesicle-treated cells that is much greater than in the applied vesicle suspension. This ratio demonstrates that the uptake of C_6-NBD-PA is due to preferential exchange or lipid transfer,[2,6,7] and is not due to the association of intact vesicles with cells.[6,8,9] Uptake of intact vesicles would cause the ratio of NBD to rhodamine fluorescence in the cell extracts to be identical to that found in the starting vesicles. Similar results to those

* Supported in part by a grant from the Whitehall Foundation and U.S. Public Health Service Grant GM22942.

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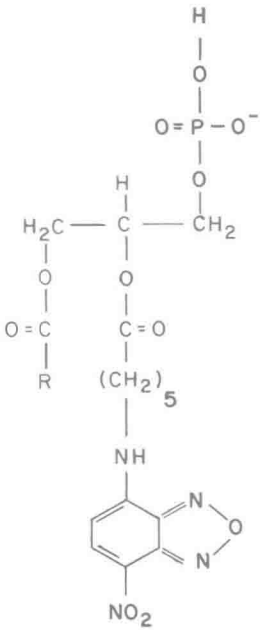

FIGURE 1. Structure of a fluorescent analogue of phosphatidic acid, 1-acyl-2-(*N*-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidic acid. R, fatty acyl residue.

presented in TABLE 1 were obtained using monolayer cultures in place of cells in suspension (unpublished observations).

Extraction and analysis of the fluorescent lipid associated with the C_6-NBD-PA–treated cells revealed that 80–90% of the C_6-NBD-PA is hydrolyzed to 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminoacyl diglyceride (NBD-DG), even though the cells are maintained at 2°C throughout the experiment. Most of the remaining fluorescent lipid is in the form of C_6-NBD-PA, although small amounts of NBD-labeled phosphatidylcholine (NBD-PC) are sometimes also detected. If vesicle-cell incubations are carried out at 37°C, significantly more NBD-PC is formed, as well as 1,3-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminoacyltriglyceride (NBD-TG). Thus, C_6-NBD-PA is metabolized to the fluorescent products expected from the estab-

TABLE 1

CELLULAR UPTAKE OF C_6-NBD-PA BY CHINESE HAMSTER FIBROBLASTS AT 2°C*

	Cells	Applied Vesicles
pmol NBD-lipid/10^7 cells	3383	–
pmol *N*-Rh-PE/10^7 cells	16	–
(pmol NBD-lipid/pmol *N*-Rh-PE)	211	12.5

* Cells were incubated with small unilamellar vesicles composed of dioleoyl phosphatidylcholine/*N*-Rh-PE/C_6-NBD-PA (75.7/1.8/22.5, mol %) for 60 min at 2°C. The cells were then washed, the lipids extracted, and the amount of cell-associated NBD-lipid and *N*-Rh-PE quantified. Identical extractions and analyses were also made using an aliquot of the starting vesicle suspension.

lished lipid biosynthetic pathways (reviewed in References 10 and 11) in mammalian cells. In contrast, when NBD-PC is used in place of C_6-NBD-PA in vesicle-cell incubations, substantial amounts of fluorescent lipid become cell-associated, but no metabolism of this fluorescent lipid can be detected.[1,7]

Intracellular Distribution of NBD-Fluorescence in C_6-NBD-PA-treated Cells[7]

When vesicles containing C_6-NBD-PC are incubated with Chinese hamster fibroblasts in suspension for 60 min at 2°C, the fluorescent lipid is incorporated almost exclusively into the plasma membrane.[1] This results in peripheral ring fluorescence as seen in Figure 2a, with little, if any, fluorescent lipid being observed inside the cell. By contrast, when identical incubations are carried out with C_6-NBD-PA, no labeling of the plasma membrane is seen. Rather, the cell-associated NBD-lipids appear to be totally intracellular (Figure 2b), with labeling of the nuclear membrane being particularly prominent.

The distribution of intracellular fluorescence observed following treatment with C_6-NBD-PA is better resolved using monolayer cultures as seen in Figure 3. Two prominent intracellular features are seen. First, a portion of the fluorescence is localized in a reticular network in the cytoplasm; and second, bright "dots" of fluorescence are distributed throughout the cytoplasm. These two regions of the cell have been identified as the endoplasmic reticulum and mitochondria, respectively. Identification of the reticular network was accomplished as follows. Cells were treated with C_6-NBD-PA for 60 min at 2°C, washed, and photographed. The cells were then fixed, permeabilized, and treated with a rhodamine-conjugated lectin, *Lens culinaris* agglutinin, which has been shown to stain the endoplasmic reticulum.[12] Subsequent photography of the same cell using optics appropriate for rhodamine fluorescence demonstrated that the reticular network stained during C_6-NBD-PA treatment and the endoplasmic reticulum stained by the lectin were the same cytoplasmic structure. Identification of the bright "dots" of fluorescence was accomplished in an analogous manner using rhodamine 3B, a cationic fluorescent probe that is specifically accumulated by the mitochondria of living cells.[13]

Thus, when cells are incubated with C_6-NBD-PA at 2°C, the mitochondria, endoplasmic reticulum, and nuclear membrane (an extension of the endoplasmic reticulum) are the principal intracellular sites of fluorescence localization.

Metabolism and Redistribution of Intracellular Fluorescence at 37°C

In preliminary experiments we have found that if cells are treated with C_6-NBD-PA for 60 min at 2°C, washed, and then warmed to 37°C in a simple, balanced salt solution, labeling of the endoplasmic reticulum was decreased, while new cytoplasmic regions became labeled. Particularly prominent were fluorescent structures about 0.5–2 μm in diameter (Figure 4), which we have tentatively identified as intracellular lipid droplets. Specific events in NBD-lipid metabolism accompanied this redistribution of intracellular fluorescence. Namely, the cell-associated NBD-DG was metabolized to substantial amounts of NBD-labeled phosphatidylcholine and triglyceride. In the future we hope to determine which of these lipids is present in the lipid droplets, and whether, after long-term incubations at 37°C, some of the intracellular fluorescent lipids are transported to the cell surface.

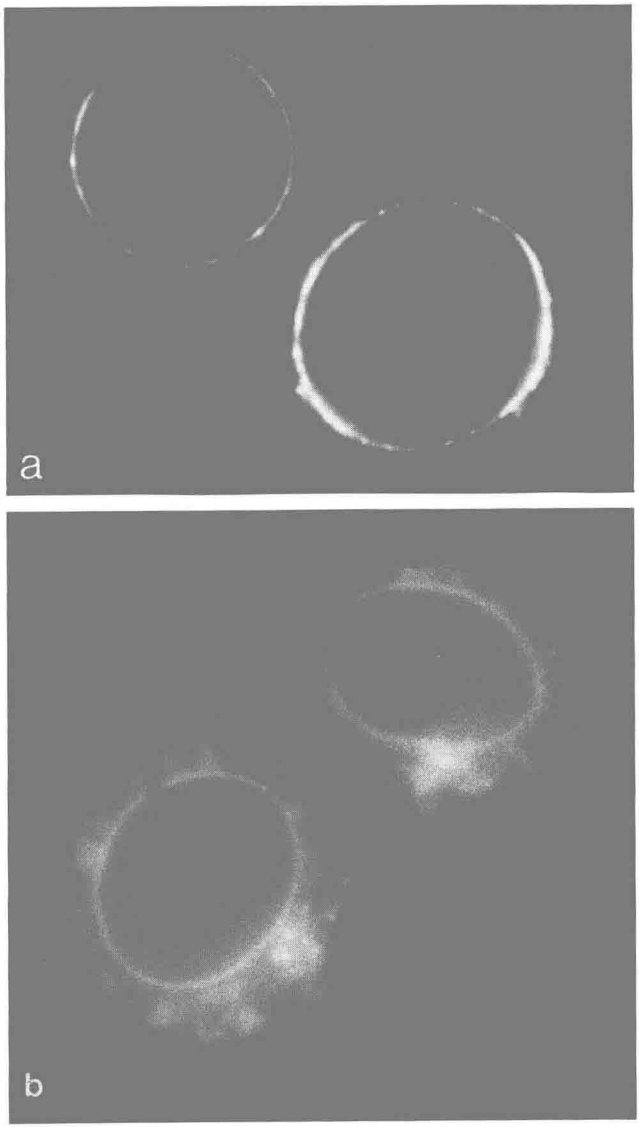

FIGURE 2. Fluorescence micrographs of Chinese hamster fibroblasts in suspension after incubation with C_6-NBD-PC- or C_6-NBD-PA-containing vesicles at 2°C. Incubations were performed at 2°C for 1 hr with dioleoyl phosphatidylcholine vesicles containing 5 mol % (a) C_6-NBD-PC or (b) C_6-NBD-PA.

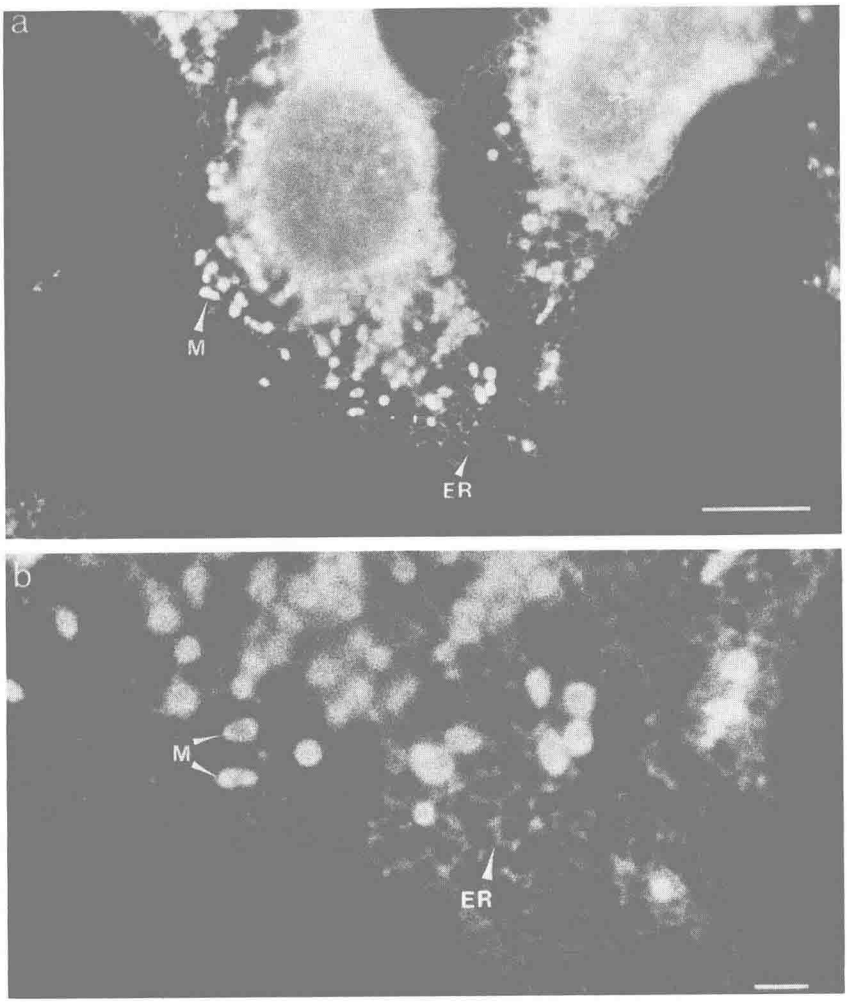

FIGURE 3. Fluorescence micrographs of Chinese hamster fibroblast monolayer cultures incubated with C_6-NBD-PA–containing vesicles for 60 min at 2°C. (a) Bar, 10 μm. (b) The same cell at higher magnification. Bar, 2 μm. ER and M designate areas of fluorescently stained endoplasmic reticulum and mitochondria, respectively. (From Pagano *et al.*[7] By copyright permission of The Rockefeller University Press.)

SUMMARY

We have shown that a fluorescent compound, C_6-NBD-PA, behaves as an analogue for phosphatidic acid, an important intermediate in glycerolipid biosynthesis. This derivative is preferentially transferred from phospholipid vesicles to cultured Chinese hamster fibroblasts at 2°C, while the C_6-NBD-PA–derived fluorescence is localized at the nuclear membrane, endoplasmic reticulum, and mitochondria. Ex-

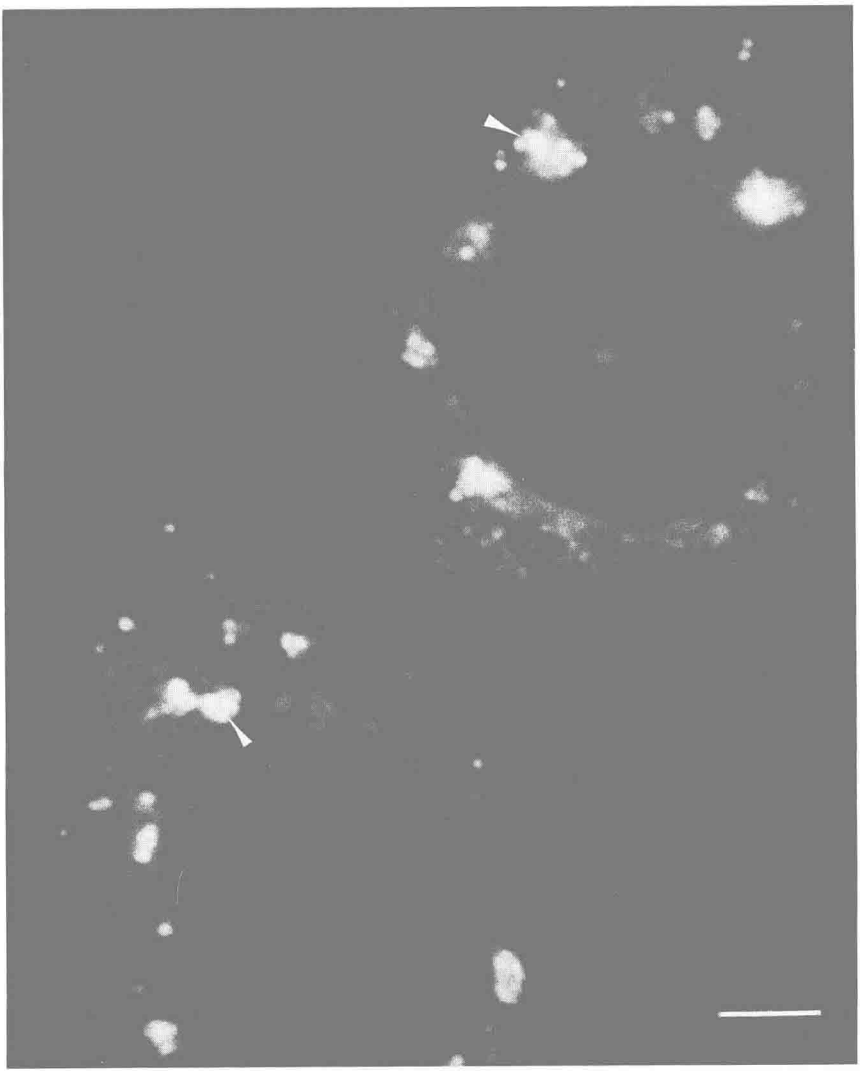

FIGURE 4. Fluorescence micrograph of Chinese hamster fibroblast monolayer culture incubated with C_6-NBD-PA for 60 min at 2°C, washed, and subsequently warmed to 37°C for 30 min. Arrows indicate fluorescent intracellular lipid droplets. Bar is 10 μm.

traction and analysis of the fluorescent lipids associated with the cells after treatment with vesicles at 2°C or 37°C revealed that a large fraction of the fluorescent phosphatidic acid is converted to fluorescent diglyceride, phosphatidylcholine, and triglyceride. Although we do not yet know how accurately the metabolism and intracellular distribution of C_6-NBD-PA and its metabolites reflect those of endogenous phosphatidic acid, it is encouraging that this fluorescent analogue is apparently me-

tabolized through the diglyceride pathway to give fluorescent analogues of diglyceride, triglyceride, and phosphatidylcholine. This metabolism suggests that the presence of the fluorescent group on the acyl chain of the phosphatidic acid analogue does not inhibit the enzymes involved in phosphatidic acid metabolism.

We conclude that fluorescent lipid analogues such as C_6-NBD-PA may be useful in correlating biochemical studies of lipid metabolism with studies of the intracellular localization of lipid metabolites by fluorescence microscopy.

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HETEROGENEITY IN THE PLASMA MEMBRANE LIPIDS OF EUKARYOTIC CELLS

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The so-called fluid mosaic model[1] of cell membrane organization has served well as the intellectual basis for much work on membrane organization and function. This model emphasized the hydrophobic interaction of membrane integral proteins with the lipid bilayer, and, though other alternatives were considered, the independence of membrane components and the tendency for these components, both lipids and proteins, to randomize by diffusion in the plane of the membrane. The idea of a fluid membrane whose components were free to diffuse in the plane of the membrane developed from work on the movement of membrane proteins in the plasma membranes of isolated mammalian cells, lymphocytes,[2] or cultured fibroblasts.[3] The impact of these experimental demonstrations of capping and diffusion of membrane proteins obliterated the observations on the restrictions to lateral diffusion that were evident in tissue cells, for example in the localization of membrane enzymes to one face only of an epithelial cell (discussed by Edidin[4]) or in the isolation of membrane fractions of plasma membrane with greatly differing lipid and protein compositions.[5,6]

Renewed interest in the heterogeneity of membrane organization was awakened by a series of experiments and speculations on the organization of membrane lipids. A series of papers on synthetic lipid vesicles by McConnell and co-workers developed phase diagrams for mixtures of lipids and showed clearly that several lipid phases could co-exist in a single vesicle.[7,8] Jain and White[9] offered a model in which membrane lipids are segregated by species into a series of immiscible domains.

The existence of lipid domains was also postulated in order to explain discrepancies in the behavior of lectin receptors or enzymes in cells with modified fatty acid composition. Thus, Horowitz and co-workers[10] reported that the lectin-mediated agglutination of 3T3 or SV3T3 mouse fibroblasts was a function of temperature and membrane fatty acyl composition, but that the critical temperature for agglutination was different for the lectins concanavalin A and wheat germ agglutinin. They argued that the receptors for the two lectins were sited in different lipid environments. This argument was supported by later work[11] in which a spin label reported two apparent transitions in the membrane lipid phases whose temperatures correspond to the critical temperatures for agglutination by the lectins. Similar arguments have been applied to differential effects of temperature or lipid substitution enzyme activity (reviewed by Jain and Wagner[12]). In other cases, we infer that physical probes of membrane lipids, for example spin labels, are in different environments than membrane enzymes or receptors whose activity is being followed biochemically.[13] All of these experiments suggest, if only indirectly, that membrane lipids are heterogeneously distributed. They do not address the extent of domains around particular proteins, though recent work on boundary lipids[14] and on the functional requirements for reconstitution of lipid-requiring membrane enzymes (review by Jain and Wagner[12]) suggest that boundary domains are short lived and rather disordered and that there is usually little lipid specificity shown in reconstituted enzyme preparations.

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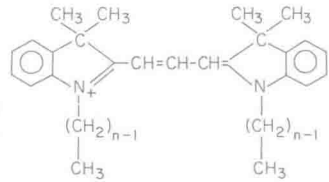

3,3'-diacylindocarbocyanine Iodide

FIGURE 1. 'diI' a carbocyanine dye family with alkyl chains varying from 10 to 22 carbons in length.

Recent work with lipid-soluble fluorescent probes has given somewhat more direct evidence for domains in the lipids of native membranes. The data show that a given probe or members of a series of related probes reside in different environments in a single membrane, though we still cannot determine either the size or the lifetime of these domains. The two groups of published experiments strongly suggesting the presence of lipid domains are by Klausner and co-workers[15,16] and by Wolf and co-workers.[17,18,19] Klausner *et al.*[15] examined the fluorescence polarization and fluorescence lifetime of diphenylhexatriene (DPH), a hydrophobic fluorescent lipid probe, in synthetic lipid vesicles and in native lymphocyte membranes. The work with synthetic vesicles showed that membrane perturbants that had a differential effect on DPH polarization in native membranes did not show any differential in single component vesicles. These compounds did have differential effect on DPH in mixed vesicles containing both fluid and gel phases. This argued that the differential effects (of *cis* and *trans* unsaturated fatty acids) reflected the presence of fluid and gel domains in native plasma membranes. This interpretation was reinforced by the analysis of fluorescence lifetime data. DPH fluorescence in native membranes and in mixed-phase vesicles could only be analyzed in terms of two or more lifetimes while DPH in single-component vesicles gave a single lifetime by phase fluorometry.

Wolf and co-workers used a similar logic to infer the presence of multiple domains in native membranes of sea urchin (*S. puperatus*)[17] and mouse (*M. musculus*)[18] eggs. They measured the lateral diffusion of a series of lipid probes, the diI's (3,3'-

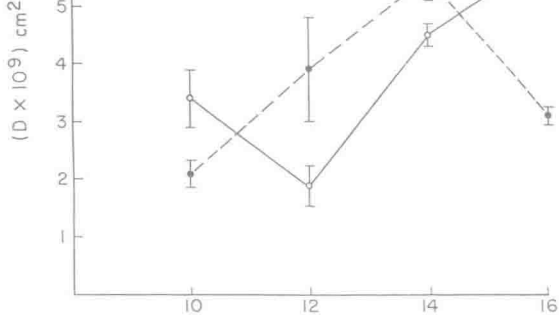

FIGURE 2. Lateral diffusion of diI's of various alkyl chain lengths in membranes of unfertilized and fertilized *S. purperatus* eggs.[17] ———, unfertilized. - - -, fertilized. Diffusion was measured by the method of fluorescence photobleaching and recovery (FPR).[26,27]

dialkylindocarbocyanines) (FIGURE 1) in single component and multicomponent lipid vesicles and in plasma membranes of intact cells. The results from native membranes, in which the diffusion of the diI's was a function of alkyl chain length, could be mimicked only in mixed-phase vesicles, not in single component vesicles[19] (FIGURES 2 and 3). The diI's, like the probes used by Klausner *et al.*, appear to partition preferentially into gel or fluid phases as shown by fluorimetric[19] and calorimetric[20] methods. It is not clear if the dyes probe gel and fluid domains in native membranes, or if they partition into immiscible fluid domains of different compositions. As noted above, the size of the putative domains cannot be estimated from these experiments. The diffusion behavior of the probe reports on domain viscosity, on partition between different domains and on the lifetime of these domains. If the partition coefficients can be established for the dyes, we should be able to determine the size of the domains, assuming that a given domain persists for a period on the order of a minute or more.

At this point, we have strong indications from work on lipid probes of the differentiation of plasma membranes into lipid domains, and we also have considerable evidence from studies of protein distribution and function that particular proteins may be associated with such domains. In some cases mentioned above, changes in lipid composition or organization are not readily associated with changes in protein

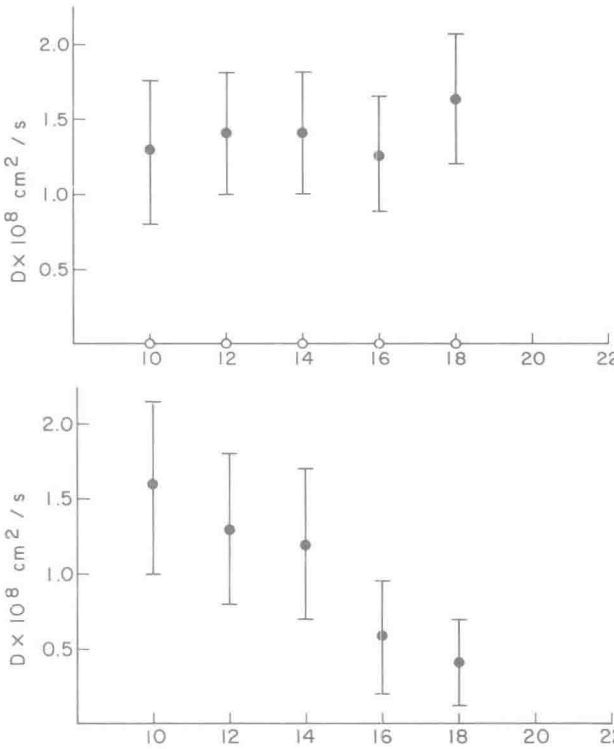

FIGURE 3. Lateral diffusion of diI's of various alkyl chain lengths in synthetic lipid vesicles.[19] (Top) Single component (●) fluid or (○) gel vesicles. (Bottom) Mixed gel and fluid vesicles.

function, while in other instances transitions in membrane lipids reported by fluorescence probes are reflected in the behavior of membrane enzymes.[21]

In the rest of this paper we will summarize some work on activity of membrane enzymes, membrane lipid composition, and membrane physical properties obtained in a single-cell system, cultured fish fibroblasts. The data reinforce the observations briefly summarized above in that they indicate that membrane enzymes are differentially affected by alterations in membrane acyl chain and cholesterol content. They also show that, even in a membrane in which these differences suggest organization into domains, both spin labels and probes of protein lateral diffusion fail to indicate the presence of such domains.

Fathead minnow (*Pimephales promelas*) fibroblasts (ATCC-42) were of interest to us since they are derived from a poikilothermic animal whose environment (freshwater ponds) undergoes considerable temperature changes during the course of a day and during the course of the seasons. Consonant with this temperature range in the native habitat, it has been found that the cells will grow in culture at temperatures ranging from under 10°C to 37°C. We were able to confirm this wide range of growth temperatures, but found that 15°C was about the lowest practical limit for

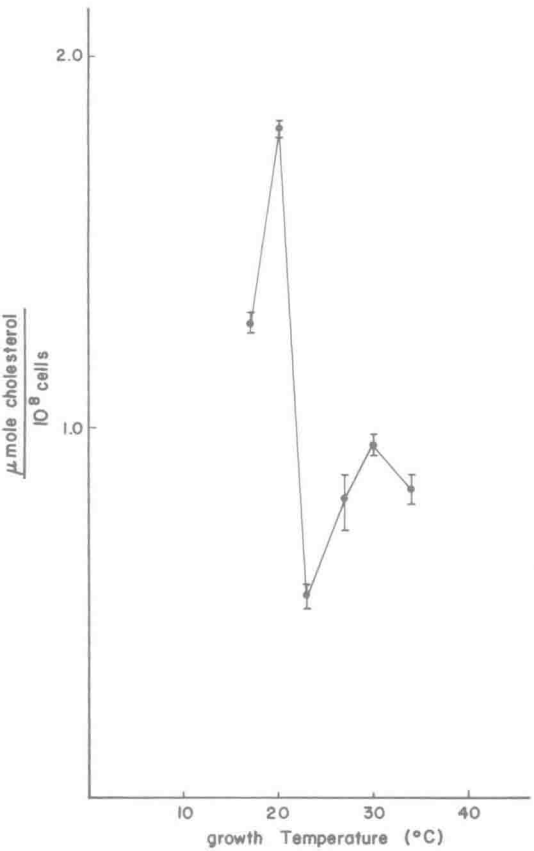

FIGURE 4. Cholesterol content of FHM cells cultured for 3 days at the temperatures indicated. Cholesterol was assayed by the cholesterol oxidase method of Allain and coworkers.[28] Bars indicate standard deviations of replicate assays.