MASTER

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Annual Technical Progress Report
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Title of Project: Studies of Membrane Structure by
Freeze-Etching
Annual Report: January 1, 1980 - December 31, 1980

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PROGRESS REPORT

A. Background

We are continuing investigations of membrane structure that are ultimately intended to provide a better understanding of the parameters which modulate cell behavior in response to environmental perturbants and which regulate the movement of materials from the outside environment to the inside of living cells.

A major gap in our ability to interpret freeze-etch images persists. After freeze-etching, the fracture faces of most membranes appear as smooth sheets interrupted by bumps commonly termed intramembrane particles. It is now usually accepted that these intramembrane particles represent transmembrane proteins (but see Verkleij, 1979). But a more detailed understanding of these particles is wanting. Even in the case of the erythrocyte membrane, whose molecular architecture has been extensively documented (Juliano, 1973; Steck, 1974) and where the intramembrane particles have been equated with one or both of the known major transmembrane proteins (Pinto da Silva *et al.*, 1971; Pinto da Silva and Nicolson, 1974), one cannot ascribe a defined polypeptide composition to a single particle. For example, in the human erythrocyte membrane, the major transmembrane proteins (glycophorin and band 3) have been identified and partially characterized (Tomita and Marchesi, 1975; Yu and Steck, 1975), but it is not known whether each intramembrane particle represents an association between band 3 and glycophorin; or whether one population of particles represents a band 3 oligomer and another population represents a glycophorin oligomer; or whether all of the particles represent band 3 oligomers.

In this contract we proposed to develop the labelling procedures required to answer such detailed, interpretational questions. Before developing the actual labelling procedures, several related problems had to be solved so that we could evaluate the labelling procedures as they were developed:

1. To what extent and under what conditions does fracturing a frozen membrane break covalent bonds in transmembrane proteins?

2. How can membrane fracturing be quantified? (Measurement of covalent bond breakage must be scaled in terms of the unit area of membrane that is fractured.)

3. To what extent can ultra low-temperature freeze-fracturing and rotary replication reduce plastic distortion and improve our ability to discern small labels attached to specific transmembrane proteins?

During the past year we have focussed much of our energies on questions 2 and 3.

B. Results

We have investigated the effect of integral membrane proteins upon the fracturing of frozen lipid bilayers. We have developed an experimental protocol utilizing lectin affinity columns which should detect small amounts of covalent bond breakage during the fracture of liposomes containing purified (Marchesi, V.T. and E.P. Andrews, Science 174, 1247 (1971)) glycophorin (a transmembrane glycoprotein of human erythrocyte membranes). To fracture liposomes in bulk, frozen liposomes are ground repeatedly under liquid nitrogen. Failure to detect any significant covalent bond breakage (contrary to Edwards, H.H., Mueller, T.J. and M. Morrison, Science 203, 1343 (1979)) led us to question the effectiveness of our grinding procedure in fracturing and splitting lipid bilayers.

Fluorescence resonance energy transfer methods were used to measure the fracture of frozen lipid bilayers (Kirchanski, S. and D. Branton, J. Cell Biol. 75(2), 216 (1977)).. Because a frozen membrane is expected to split down its hydrophobic center (an area of weak, non-covalent interactions) thereby separating the two lipid monolayers, it should be possible to monitor this process by covalently attaching an acceptor fluorochrome to one monolayer and a donor fluorochrome to the other monolayer. If the two fluorochromes have the proper overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor, and if the molecules are properly oriented and sufficiently close (maximum distance is approximately 100 Å), light energy absorbed by the donor will be transferred to and reemitted by the acceptor molecule. Because this energy transfer is strongly distance dependent (proportional to the sixth power of the donor to acceptor separation), it should be an ideal way of assessing the fracture of frozen membranes.

The major difficulty in testing the technique is in producing lipid vesicles which are correctly labeled with one fluorochrome on the inner monolayer and a different fluorochrome on the outer monolayer. Many dye pairs with nearly ideal spectral overlaps had to be discarded because we were unable to develop suitable asymmetric labeling techniques. The simplest approach to asymmetric labeling is first to produce vesicles uniformly labeled with one dye either by using prelabeled lipids or by using a penetrating labeling reagent. The dye molecules on the outer monolayer are removed or altered using a suitable non-penetrating reagent. This produces a vesicle which is labeled with the desired fluorochrome only on the inner monolayer. The final step is to attach the second fluorochrome to the outer monolayer, again using a non-penetrating reagent.

Our most successful attempts at removing or destroying a fluorochrome on the outer monolayer of a vesicle have been attained using the amino reactive reagent fluorescamine (maximum emission 474 nm). We initially observed that this label was somewhat unstable at acid pH and hypothesized that this instability might be caused by the acid activation and subsequent reaction of the fluorescamine carboxyl group (see Figure 1). After considerable experimentation we found that this process of fluorescamine self-destruction could be greatly

accelerated by the use of a water soluble carbodiimide at pH 5-6. Optimal destruction occurs only in the presence of pyridine-HCl buffer.

This technique can readily be applied to fluorescamine destruction in lipid vesicles which contain labeled phosphatidylethanolamine. In this case the vesicles are suspended in pyridine-HCl buffer (pH 5.5) and are reacted with a water soluble carbodiimide, EDC (1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide hydrochloride). The fluorescence at 474 nm begins to rapidly decrease, and within about 30 min stabilizes at a value equal to about 50% of the initial value (Figure 2). This represents destruction of the fluorochrome on the outer monolayer. If more carbodiimide is added, further dye destruction does not ensue unless the vesicles are disrupted by freeze-thawing (Figure 3).

As an acceptor dye for use with the fluorescamine we chose fluorescein (excitation maximum about 496 nm in the vesicles) because it is easy to use and has a high quantum yield. Dyes with a better spectral overlap with fluorescamine should be investigated. We have used DTAF (dichlorotriazinyl amino fluorescein) as the labeling reagent, but initially found some problems in obtaining uniform, non-penetrating labeling. This problem was solved by complexing the DTAF with cyclodextrin (Nakaya, K., Yabuta, M., Iinuma, F., Kinoshita, T. and Y. Nakamura, BBRC 67, 760 (1975)). The complex is more readily water soluble and appears to be active for a longer period of time than is DTAF alone.

In a typical experiment liposomes are produced by dialysis from a cholate solution of DPPC (dipalmitoyl phosphatidylcholine) and DPPE (dipalmitoyl phosphatidylethanolamine) (10 mole percent) which also contains 1 mole percent of fluorescamine labeled DPPE and 0-10% (by weight) purified glycophorin. The fluorescamine on the exterior monolayer is destroyed by reaction with 25 mM EDC in 0.1 M pyridine-HCl, pH 5.5. After fluorimetric verification of dye destruction the vesicles are labeled with cyclodextrin-DTAF (1 mg/ml) in 0.1 M sodium borate, pH 9.0. Finally the vesicles are rapidly frozen in 0.1 M sodium phosphate, pH 7.0 by injection into vigorously stirred freezing-nitrogen slush (prepared by pulling a partial vacuum on stirred liquid nitrogen).

Control vesicles are allowed to thaw directly after freezing while ground vesicles are pulverized repeatedly while remaining under liquid nitrogen. After all samples have thawed, they are diluted with 0.1 M sodium phosphate, pH 7.0 and analyzed with a photon counting spectrofluorimeter. The extent of energy transfer is assessed using the acceptor excitation method (described in Stryer, L., Ann. Rev. Biochem. 47, 819 (1978)). The ratio of fluorescein emission when the vesicles are excited at 390 nm and 330 nm is used as an indication of the extent of energy transfer. The percent change in this ratio between ground and non-ground samples is taken as the apparent measure of membrane splitting. The actual ratios vary somewhat from experiment to experiment probably because of variation in labeling and

Figure 1. Fluorescent form of fluorescamine. N is from amino of DPPE.

Figure 2. Fluorescence destruction with EDC. Top trace is zero time. Successive traces at 10 min intervals.

Figure 3. Fluorescence destruction with EDC. At arrow fresh EDC is added. Dotted line represents vesicles that are made leaky by freezing at the same time fresh EDC is added.

because of a considerable background energy transfer caused by imperfect asymmetric labeling. However, the percentage changes in transfer are repeatable and consistently show a decrease of transfer upon grinding.

As can be seen from Table I grinding of vesicles containing no protein results in the largest apparent membrane splitting. If one batch of vesicles is divided into several lots some of which are ground more extensively than others, the extensively ground membranes show more apparent membrane splitting. If glycophorin is incorporated into the membranes, a considerably smaller apparent membrane splitting is seen. Trypsin treatment of the glycophorin vesicles generally results in reduced apparent membrane splitting. Since this treatment removes the outward facing oligosaccharides but leaves the hydrophobic membrane penetrating section of glycophorin intact, this result may indicate that the sugar groups are somehow affecting the DTAF label or are actually increasing the extent of membrane fracture.

The use of fluorescence resonance energy transfer appears to allow a bulk measure of membrane fracture. Exact quantitation of the splitting has not been achieved, but comparisons allow us to determine the relative efficiency of splitting. For example, incorporation of glycophorin into the bilayer decreases membrane splitting. We are currently attempting to extend the observations to other integral membrane proteins (both cis and trans membrane) and to further ensure that the measurements do not represent an artifact of dye modification or loss brought about by grinding under nitrogen.

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February 9, 1981

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We are transmitting copies of documents submitted in accordance with our requirements under the subject contract.

Gaile Higashi, Contract Specialist
Acquisition and Assistance Unit

AA:CH

Enclosures:

1. Progress Report No. DOE/EV/02423-1 (4 cys.)
2. Financial Statement (1 cy.)
3. Form DOE 536 (3 cys.)

cc: Brookhaven Office of Patent Counsel, Brookhaven Area Office - w/encl. 1
Technical Information Center, Oak Ridge, TN
(THRU BROOKHAVEN OFFICE OF PATENT COUNSEL) - w/encl. 1 and 2, and Form
427 for enclosure 1

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1. DOE Report No. DOE/EV/02423-1		3. Title	
2. Contract No. DE-AC02-76EV02423.A007		Studies of membrane structure by freeze-etching	
4. Type of Document ("X" one)			
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8. Submitted by		Name and Position (Please print or type)	
Daniel Branton		Principal Investigator, Professor of Biology	
Organization Harvard University			
Signature		Date 30 Jan 81	

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9. Patent Clearance ("X" one)

- ☐ a. DOE patent clearance has been granted by responsible DOE patent group.
- ☐ b. Report has been sent to responsible DOE patent group for clearance.
- ☒ c. Patent clearance not required.

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