STUDIES OF MEMBRANE STRUCTURE BY FREEZE-ETCHING
Progress Report

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I. ABSTRACT

The structure and organization of biological membranes has been studied using a variety of physical and biochemical techniques together with electron microscopy and freeze-etching. These studies have provided knowledge relating the architecture of cellular membranes to their role in regulating the transport of molecules into and out of cells. A number of model systems, including Acholeplasma laidlawii and human erythrocyte membranes have been probed. The role of lipids and phase transitions in membrane structure and permeability has been analyzed by lipid supplementation and temperature shift experiments. The role of lipid-lipid and protein-protein interactions in controlling the mobility of cell surface macromolecules have been studied using freeze-racture, protein extraction and SDS-polyacrylamide gel techniques.

II. INTRODUCTION

A. Introduction

This is a proposal to continue investigations of membrane structure which are ultimately intended to provide a better understanding of the parameters which regulate the movement of materials from the outside environment to the inside of living cells.

My laboratory has been concerned with the relationship between the structure of membranes seen in the electron microscope and the details of their physical, chemical and functional organization. Two short reviews (Branton, 1971, Phil. Trans. Royal Soc. London B, 261:133-138 and Pinto da Silva and Branton, 1971, Chem. Phys. Lipids 7:9-14) as well as a more complete analysis (Branton and Deamer, 1972, Protoplasmatologia, Vol. II E1) have been published. These reviews explain the rationale for our efforts and may be consulted for a more detailed introduction and background for this report. These reviews show how the approaches and methods we have developed provide clear evidence for the existence of discrete, structurally differentiated sites within the plane of most biological membranes. Our recent work shows that the distribution of these sites is dependent on chemical gradients in both the hydrophylic and the hydrophobic portions of the membrane. Although physical and averaging techniques such as X-ray diffraction, circular dichroism, and paramagnetic resonance have proven to be powerful tools for studying certain aspects of the overall membrane matrix, they have not indicated much about the structure or distribution of these discrete sites. In view of mounting evidence that these sites correspond to functionally important centers and because many of the functions of biological membranes may involve only a small fraction of the total membrane, averaging techniques must be supplemented by and combined with methods capable of exposing structure within individual areas of a single membrane. It is in this context that we have applied freeze-etch methods of electron microscopy. These methods have unique advantages as they provide extensive high resolution views of structure within the plane of single membranes and are readily combined with a variety of physiological, chemical and physical manipulations. This combination of ultrastructural and experimental approaches is the basis for current work in my laboratory.

III. PROGRESS REPORT

A complete progress report covering this project agreement was submitted at the termination of the last review period for this contract in fall, 1972. Thus, the report here covers only the last 18 month period. During this period, I have closed down my laboratory at Berkeley and I have transferred activities to my new laboratory facilities at Harvard University.

Planning and accomplishing the transfer has been demanding and our research productivity during this period has been somewhat reduced.

A detailed account of some of our AEC sponsored work during the past year is contained in our publications. The publications are listed below together with their summaries. Reprints or preprints are enclosed and should be consulted for a more complete presentation.

James, Robert and Daniel Branton. 1973. Lipid and temperature-dependent structural changes in Acholeplasma laidlawii cell membranes. Biochim. Biophys. Acta, 323:378-390.

The lipids in cell membranes of Acholeplasma laidlawii were enriched with different fatty acids selected to produce membranes showing molecular motion discontinuities at temperatures between 10°C and 35°C. Molecular motion in these membranes was probed by electron spin resonance after labelling with 12-nitroxide stearate, and structure in these membranes was examined by electron microscopy after freeze-etching.

Freeze-etching and electron microscopy showed that under certain conditions the particles in the A. laidlawii membranes aggregated, resulting in particle rich and particle depleted regions in the membrane, this aggregation could begin at temperatures well above the ESR-determined discontinuity. Aggregation increased with decreasing temperature but was completed at or near the discontinuity. However cell membranes grown and maintained well below their ESR-determined discontinuity did not show maximum particle aggregation until after they had been exposed to temperatures at or above the discontinuity.

The results show that temperatures at or near a phase transition temperature can induce aggregation of the membrane particles. This suggests that temperature induced changes in the lipid phase of a biological membrane can induce phase separations which affect the topography of associated proteins.

Branton, Daniel, 1974. The fracture process of freeze-etching. in Freeze-etching techniques and applications, E. Benedetti and P. Favard, ed. Soc Francaise Microscopie Electronique, Paris.

A brief review showing how weak entropic interactions guide the fracture process during freeze-etching.

Elgsaeter, Arnljot and Daniel Branton, 1974. Erythrocyte intramembrane particle mobility 1. Effect of protein removal. Submitted to Journal of Cell Biology.

Freeze-etching and SDS-polyacrylamide gel electrophoresis were used to study the conditions under which the intramembrane particles of the human erythrocyte ghost may be aggregated. The fibrous membrane protein, spectrin, can be almost entirely removed from erythrocyte ghosts with little or no change in the distribution of the particles. However, after spectrin depletion, particle aggregation in the plane of the membrane may be induced by conditions which cause little or no particle redistribution in untreated ghosts. This suggests that the spectrin molecules form a molecular meshwork which limits the translational mobility of the erythrocyte membrane particles.

Our results showing that spectrin molecules limit the mobility of the erythrocyte ghost intramembrane particles may have important implications for the structural arrangement of other cell membranes. The association between spectrin and the particles, which are a means whereby proteins within a cell could affect the distribution of cell surface components. Although abundant quantities of spectrin have not been detected on other cell membranes, a similar association between intrinsic membrane components and an underlying network of fibrous or filament forming protein may be a common mechanism whereby the mobility of surface macromolecules is controlled by other cellular constituents.

In addition to the above studies, we have recently designed and constructed equipment for *in vacuo* transfer of autoradiographic emulsions to a completer freeze-fracture replica. This has made it possible to combine standard techniques of electron microscopic autoradiography with our freeze-etch methods. Freeze-fracture autoradiography (FARG) provides a way to measure asymetry in chemical composition of each half of a biological membrane. Because freeze-fracturing splits biological membranes to expose extended face views of those components in either one or the other half membrane, an autoradiograph of that half membrane can yield information about membrane asymmetry that would otherwise require unobtainably high resolution using thin section E.M. radiography. FARG is accomplished by keeping the replicated sample frozen after routine freezing, fracturing, and shadowing in a Balzer's device. The frozen, replicated sample is coated, *in vacuo* with a dry monolayer of autoradiographic emulsion. After exposure at -80°C the emulsion-replica-specimen sandwich is developed, fixed, and washed. The specimen is then removed from the emulsion-replica sandwich so that the location of silver grains over the replica can be viewed in the electron microscope. To assess the feasibility of the technique, several physical and biological test systems have been examined. Physical test systems have included non-diffusible "point sources" of radioactivity in the form of low energy iodine-125 bound to anion exchange resin beads or tritium as tritiated oleic acid partitioned into a suspension of oil droplets in distilled water. Biological test systems included erythrocyte ghosts containing I-125 bound to the outer portion of the membrane by lactoperoxidase iodination. (See Fig. 1&2). Although the technique of FARG has been designed to gain new information about membrane structure, it should also be useful in an examination of diffusible and extractable substances.

Figure 1.

Figure 2.

Figure 1. Freeze-etch autoradiography. The overall procedure employs the usual freeze-etch steps (a-f) following which the emulsion is applied to the tissue at -75°C (g). After exposure, the sandwich is brought up to room temperature and passed through standard photographic processing chemicals. Any remaining tissue is then cleaned from the replica and the replica and emulsion overlay is examined in the electron microscope (h).

Figure 2. Example of FARG showing portion of an iodinated (I^{125}) erythrocyte-membrane (from the thesis work of K. Fisher).