STUDIES OF MEMBRANE STRUCTURE BY FREEZE-ETCHING
Progress Report

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I. ABSTRACT: Technical Scope

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 are providing 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 proved to determine the distribution and asymmetry of component lipids and proteins. For this purpose, a novel combination of freeze-fracture and electron microscope autoradiographic techniques has been developed and is being tested.

II. 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, Protoplasmalogia, 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. Publications

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

1. Elgsaeter, A. and D. Branton, 1974. Intramembrane particle aggregation in erythrocyte ghosts I. The effects of protein removal. Journal of Cell Biology, 63:1018-1030.

Freeze-etching and SDS-polyacrylamide gel electrophoresis was 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 aggregation in freshly prepared ghosts. This suggests that the spectrin molecules form a molecular meshwork which limits the translational mobility of the erythrocyte membrane particles. In other cell types, 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. (reprints attached)

2. Fisher, K. and D. Branton, 1974. Application of the freeze-fracture technique to natural membranes. Methods in Enzymology, 32: 35-44.

A brief description of the methods and techniques to be used in freeze-fracturing membranes for electron microscopic examination. (Academic Press, the publisher of this series, has not made reprints available).

3. Southworth, D., K. Fisher and D. Branton, 1975. Principles of freeze-fracturing and etching. Techniques of Biochemical and Biophysical Morphology, 2:247-282.

The various operations and the underlying theory of freeze-etching are explained to show how each step influences the final image and its interpretation. (John Wiley & Sons, Inc., the publisher of this series has not made reprints available).

In addition to the above studies, we have continued our evaluation of our newly developed freeze-fracture autoradiographic technique (FARG). Before applying FARG to study the distribution and localization of molecules in membranes, we felt it essential to develop a rapid, controllable assay to establish the qualitative and quantitative merits of our procedure. For this purpose, we required a simple biological membrane system that could be labelled to extremely high specific activity in a known but asymmetric

manner. High specific activity was sought so that during the early stages of our research, any given autoradiograph would be ready for examination within one or two days. To assure a known but asymmetric distribution of radioactivity we developed a labelling method using cationized ferritin molecules whose position on one surface of a membrane could be verified by direct inspection.

Ferritin is cationized by carbodiimide (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide - EDC) promoted amide formation between a ferritin carboxyl group and a diamine such as methyl lysine

After cationization, the ferritin binds to the outside surface of an erythrocyte and cannot be washed off even in 150 mM NaCl.

For our purposes, the ferritin has to be made radioactive. Because we wish to evaluate FARG with both tritium and I^{125} , both of these radiolabels were attached to the ferritin. High specific activity cationized ferritin was easily obtained by iodination in the presence of chloramine-T (Table). High specific activity tritium labelled cationized ferritin was produced by either of three methods. The first two introduced the tritium label via the diamine used to cationize the ferritin:

Method 1: Cationization using methyl-lysine. Commercially available high specific activity lysine was methylated in the presence of HCl,

and this methyl ester of lysine used as the tritiated diamine in the cationization reaction (equation 1).

Method 2: Cationization using sodium borohydride reduced keto-diamines. Commercially available diaminobutanone is reduced by tritiated sodium borohydride.

and the resultant diaminobutanol used as the tritiated diamine in the cationization reaction (equation 1). Alternatively, the ferritin can be cationized with the diaminobutanone first and the cationized ferritin and protein subsequently treated with tritiated sodium borohydride.

Method 3: Acetylation of cationized ferritin with tritiated acetic anhydride

As shown by the table below, the ^3H -methyl cationized ferritin gave the highest specific activity and has been selected as the method we are using to tritiate and cationize our ferritin. In separate experiments, we have found that approximately 1 mg cationized ferritin binds to $10^{11} \mu^2$ of red cell surface. Using ferritin with a specific activity of about $10^4 \text{ mCi}/\mu\text{mole}$ this results in approximately 400 disintegrations per day per square micron of membrane surface. In other words, we have achieved the high specific activity, selective labelling desired to run test FARG's in one or two days.

TABLE. RADIOACTIVE LABELLING OF FERRITIN

Source of radioactivity	Isotope specific activity, mC/ μmole	No. of bound isotopes per ferritin molecule	Ferritin specific activity, mC/ μmole
Tritiated Lysine methyl ester	30	150-560	$1 \times 7 - 4 \times 5 \times 10^3$
Tritiated NaBH_4	10	200	2×10^3
Tritiated Acetic anhydride	2	300	6×10^2
^{125}I	2000	13	$2 \times 2 \times 10^5$