SUBMICROSCOPIC CYTOCHEMISTRY

II

MEMBRANES, MITOCHONDRIA, AND CONNECTIVE TISSUES

Edited by

ISIDORE GERSH

Submicroscopic Cytochemistry

Volume II

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To Dr. Louis B. Flexner
for his inspiration and support over a period of forty years

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Preface

In this age of specialization and fragmentation the opportunity for an individual to carry out a comprehensive laboratory study is rare indeed. Through a series of accidental happenings, I found myself in a position which impelled me to continue a long-term study of a general type. It is difficult to express briefly the fascination and absorbing interest in developing subtle interrelationships of methods and ideas and in molding complex materials into a coherent view of some aspects of cellular activity. The first eight years of this study were devoted largely to developing and testing cytochemical methods to the point of satisfaction, and the next five years chiefly to applying them to biological studies. The analytic processes continued up to and even after the time of writing of the last chapter.

Three major topics are considered in this two-volume work: (1) the pattern of distribution of nucleic acids at the molecular level in various cells and states of activity and in relation to protein synthesis; (2) the molecular and macromolecular organization of cellular membranes; and (3) the origin and distribution of the major macromolecular aggregates of connective tissue. The first major topic comprises Volume I; the remaining ones, Volume II. All three are integrated in the last chapter of this volume.

There is a high premium in this work on morphology, that is, on the distribution of cell components and their organization in cells at the macromolecular level. Such studies required that new methods be developed to preserve these molecules and molecular aggregates very nearly in the position they occupied in the living state and to identify them more or less selectively. The crucial tests were chiefly for proteins, nucleic acids, lipids, and acid mucopolysaccharides. A glance at the Contents will indicate the wide range of biological problems which could be approached with the new methods. The last chapter is the most general one, and serves to bind together all the others. Here morphology, through cytochemistry, is integrated with parts of genetics, biochemistry, cytophysiology, developmental biology, and pathology.

The main emphasis in this work is on the presentation of laboratory findings and theoretical aspects involved or derived from them. These are related, whenever possible, to the main body of thought on the various topics discussed by spe-

cific or general references. The latter are so varied, competent, and numerous that it was felt they would suffice in most instances to relate the original work to contributions from the literature available to most workers. Since there was no need for encyclopedic reviews of the various fields, the references cited are minimal, except in certain specific instances where the reviews were incomplete or otherwise unsatisfactory. An effort was made to update the literature citations up to the time the manuscript went to the publisher.

A few words should be written about prints. Nearly all negatives were printed directly at a magnification of two times. Where the contrast was marked, as with apatite crystals (Chapter 9, Volume II), some prints were magnified five times. Only a few negatives were printed with some dodging or burning. A small number of negatives were double printed to give enhanced, selective contrast to some specific component. These were printed from the original negative, usually on a soft paper. This was then photographed on a second EKTAPAN (Kodak) film, which was printed again usually on a soft paper. The final magnification was two to four times the original electron micrograph. Such prints are labeled "G2 print" in the figure legend. In taking some of the electron micrographs for such prints, an appreciable astigmatism was introduced in order to improve resolution and contrast. This method was suggested by F. S. Sjöstrand ("Electron Microscopy of Cells and Tissues," Volume I, p. 127, Academic Press, New York).

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I am indebted to the following for their essential and loyal technical work: Faustina Manelis and Elizabeth Vilkas, of Chicago; Vivien Catlin, Eugénie Ford, Patsy Jo Terrell, and Eliana Muñoz, formerly of Philadelphia; and George J. Grigonis, Jr., also of Philadelphia. It was a great comfort when Diane Weinstock took over the typing. Diagrams were developed by Mary Jo Larsen, who was of great aid, in addition, in connection with other illustrations. I wish to thank Janice Heald for facilitating work in the library and for checking the references.

I am pleased to recall the perceptive advice of Dr. Herbert S. Anker, Department of Biochemistry, University of Chicago, and of numerous other chemists. For advice on matters pertaining to Chapter 2, Volume II, I am indebted to Dr. Donald A. Abt, of the Department of Medicine, and to Dr. James A. Dvorak, when he was in the Department of Pathobiology in the School of Veterinary Medicine, University of Pennsylvania.

In addition to working jointly on the material described in five chapters, my wife has helped me significantly to interpret observations in other chapters from a genetic point of view. She also helped me clarify the analyses of the various problems studied and to express the thoughts involved. I enjoyed the collaboration of several colleagues, past and present, who are coauthors of several chapters: Dr. Peter L. Amenta, Department of Anatomy, Hahnemann Medical College, Philadelphia; Dr. Peter J. Hand, Department of Animal Biology, University of Pennsylvania, Philadelphia; Dr. Zelma Molnar, Department of Pathology, University of Chicago, Chicago; and Dr. Giovanni L. Rossi, Institut für Tierpathologie, Universität Bern, Bern.

This work was started when I was Professor in the Department of Anatomy at the University of Chicago, and was continued with very little interruption at the University of Pennsylvania.

Contents of Volume I

Proteins and Nucleic Acids

Contents

1

Cytochemical Studies on Lipid and Protein Components of Membranes in Pancreatic Acinar and Hepatic Cells of the Mouse

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Research on cell membranes has been very lively. Some of the activity has revolved on differences between various kinds of membranes in their position in cells, in their geometry, thickness, chemical composition, enzymic activity, immunological and electrical properties, and in their relations to studies on transport and permeability. These aspects are reviewed in various books and symposia *(9, 13, 14, 66, 67)*. One thread which runs through a seemingly chaotic jumble of data is the unit membrane hypothesis *(58)*. According to this hypothesis, virtually all cellular membranes of all cells comprise a lipid bimolecular leaflet with protein or polysaccharide on the external surfaces of the lipid layer in association with the hydrophilic ends of the oriented lipid molecules. Proof for the existence of such organized lipid layers in myelin and retinal rods, and in artifical layers seems quite overwhelming, but is, in general, lacking for almost all other cells *(30, 31)*. Recently, the proposed unique structure of the *in vitro* membranes was challenged by studies which showed that lipids may be organized in several regular manners, which may be in equilibrium with the bimolecular leaflet *(20, 40, 41)*. The possibility arose that unit membranes (except for myelin sheath and retinal rods) may be an artifactual rearrangement of lipid molecules, which takes place during fixation of cells, to its most stable form, the bimolecular leaflet *(40)*. An additional uncertainty is that lipid molecules may be oriented at least in some membranes, artificial and cellular, in the form of a lipid globular pattern with associated intercalated protein *(19, 21, 24, 38, 39, 66, 67, 72, 76, 77)*. Numerous modifications of the unit membrane hypothesis have been suggested and criticized in reviews of this topic *(16, 32, 42, 62, 63, 72, 75, 76, 79)*. Some studies of mo-

lecular distortions, which take place during the fixation of membranes, have also been reported *(21, 44, 50, 51)*. Danielli *(11)* showed that lipid layers must exist as bimolecular leaflets, but the thermodynamic analysis may not help in ascertaining whether such leaflets occur in living cells. As far as cellular membranes are concerned, a basic cause for the ambiguities enumerated above is that none of the morphological methods used in electron microscopy is sufficiently specific chemically to be regarded as a positive identification of phospholipid *(30, 31)*.

Numerous recent reports emphasize structural proteins of membranes. These are regarded by some as noncatalytic structural proteins which are self-assembled to form membranes in conjunction with phospholipids. Others regard the structural proteins of membranes as capable of enzymic activity. In both cases, the membranes are regarded as largely hydrophobic in nature, because of a preponderance in the inner parts of polypetide chains of nonpolar amino acids, such as glycine, alanine, leucine, valine, and proline. Phospholipids are considered important in stabilizing the folded configuration of many proteins through weak interactions involving chiefly short-range forces, especially electrostatic and hydrophobic bonds *(59)*. Some of the forces involved have been discussed and numerous models incorporating these newer data have been published *(3, 4, 8, 26, 33, 34, 45–47, 73, 74, 78)*.

Certainly, recent work on isolated membranes includes analyses of both lipid and protein components, with special emphasis on enzymes *(4, 26, 33, 45–47, 75)*.

Isolated membrane proteins were found to be a complex array of varying molecular weight. They were rapidly labeled, through with varying rates of turnover, suggesting that the membrane proteins were heterogeneous *(27, 28)*. In general, such membranes are composed of protein molecules, whose interior is largely hydrophobic and whose surface is charged and hydrophilic. The surface of the protein molecules is such that, in the presence of phospholipids, they aggregate as mono- or bilayered sheets. The phospholipid molecules are arranged in this sheet with their charged head on or near the outer surface of the protein sheet, and their aliphatic chains extending internally between the hydrophobic polypeptide coils of the protein molecules. Some of the lipids may also form small bilaminar mosaic patches between the predominantly protein molecules. This is the general view of some biochemists, who have separated various kinds of mitochondrial particles whose enzymic activity has been studied before and after reconstitution *(2, 10, 20, 22, 23, 54–56, 60)*. A generally cautious note on the technical limitations in the study of membrane proteins and on the possible physiological roles of such membranes was expressed in a very recent review *(25)*. While these fractionations have been controlled by electron microscopy, the difficulty has been to ascertain whether the particulates preexist as such in living cells, and whether the reconstituted "membranes" are, indeed, similar to the original membranes in living cells.

On the other side, is the work of Sjöstrand and Barajas *(70)*, who have identified globular molecules in certain membranes, and have interpreted them as their basic elements. They appear largely as nonstainable (hydrophobic) regions enclosed by extremely fine stained (hydrophilic) walls, giving the impression that each one

is a globule. Their relations to each other and to lipids are interpreted more or less as in the preceding paragraph. The hope is projected that "complexes of molecules could be recognized and possibly identified due to the characteristic distribution of stain sites within the complex" *(68)* and that protein molecules could be identified in thin sections provided that (1) the distribution in space of sites that bind the stain were known, and (2) the conformation of protein molecules could be maintained sufficiently close to their native conformation to allow recognizing the characteristic of stain sites *(67)*. Again, "It is quite conceivable that in the future at least certain types of protein molecules with, for instance, a characteristic arrangement of identical subunits will be identified in tissue sections and their location in the cellular structures determined in this way" *(67)*. These are, in my opinion, extremely remote possibilities which will be difficult or impossible to realize because of the molecular distortions which are inevitable with the addition or removal of water and with cross-linking, whether too much or too little. Moreover, the enzymic properties of these globular molecules still remain to be demonstrated.

Thus, it seems that the biochemical and morphological emphasis on the important roles of proteins in membranes are convergent. However, it should be kept in mind that, on the one side, the reality of the biochemically characterized particulates of membranes in living cells is uncertain, and, on the other side, the morphological particles (globules) of membranes in cells are not biochemically characterized. Another discussion of membrane proteins is presented in this volume (Chapter 3) which deals with membranes in mitochondria.

The purposes of the work reported here are: (1) to test the question whether lipid membranes of cells as commonly conceived are real or artifactual; (2) to identify the lipid nature of such membranes as might exist by a chemical method which does not involve the use of osmium tetroxide or potassium permanganate; (3) to test the hypothesis of the unit membrane; (4) to ascertain whether there might be differences in the lipid components in different parts of the membrane as well as between different membranes; and (5) to compare the distribution of lipid and protein components of membranes.

The most reactive components of the fatty acid moiety of phospholipids are the carboxyl ester linkage and the unsaturated carbon bonds of the aliphatic chain *(12, 35, 37, 53, 57, 80)*. There are many reactions for the former, but the most promising seemed to be conversion to an amide by use of an amine of high molecular weight to increase contrast in electron micrographs. There are even more reactions for marking the unsaturated double bonds of the aliphatic chain, but in the interests of simplicity it was thought wise to use the same amine used to form amides. At the same time, efforts were made to define the conditions of the test so as to prevent or reduce molecular rearrangements or displacements of lipid molecules. I cannot claim complete success in overcoming these technical requirements, either in attaining an absolute specificity or in preventing all molecular displacements, but the results are sufficiently satisfactory to merit presentation.

A discussion of the general aspects of staining freeze-dried specimens for protein has been presented in Vol. I, Chapter 2.

The results will be presented in three parts. In the first (this chapter), the morphological distribution of lipid and protein components of membranes of hepatic and exocrine pancreatic cells will be described. In the second (Chapter 2), certain of these morphological patterns will be analyzed quantitatively based on densitometric tracings, in an effort to construct three-dimensional (but not molecular) models of lipid components of the rough endoplasmic reticulum (RER), the nuclear membrane, and Golgi structures. In the third part (Chapter 3), observations on lipids and proteins of mitochondria will be presented.

Methods

Lipids

The methods to be described were developed to overcome certain difficulties encountered in lipid cytochemistry. These include extraction of lipids (with probable displacement of the remaining lipids), the flowing and coalescence of lipids because of the low melting point of many lipids and their intersolubility, and the tendency of certain lipids to separate from their associated protein when water is removed as during freezing and drying, and thus to be displaced significantly *(14, 15, 35–37)*. There is also a strong tendency for lipids to seek the most stable form of molecular orientation *(40)*. Finally, the chemical reaction used to characterize lipids in the electron microscope should be clearly distinquishable as increased contrast over a low or near zero background.

The methods to be described overcome these difficulties to a very large degree. The specimens were frozen ultrarapidly without ice crystal formation. [The general acceptance of freeze-etching and of freeze-fracturing carries with it the implication that specimens can be frozen sufficiently rapidly to preclude ice crystal formation *(7, 29, 49)*.] The artifacts that were regarded as having been caused by ice crystals *(5, 61, 65, 69, 71)* should be attributed instead to the entrapment of noncondensable and insoluble gases during the infiltration of the specimen with fluids, after the specimens have been dried. In nearly all specimens, which were properly infiltrated with the plastic monomer mixture, submicroscopic holes (now attributed to gas entrapment) were altogether absent. Bullivant *(5)* also attributed the holes to improper infiltration with the embedding materials. Evidence is presented in Chapters 1 and 2 (Vol. I) that shows that no ice crystals are formed, during freezing and drying, of a size sufficiently large to be visible with the electron microscope. When suitably postfixed and stained, the spaces formerly claimed to be empty (i.e., occupied only by ice crystals before drying) were shown to be filled with protein.

During the time that water was subliming from the specimen at some temperature lower than $-30°C$, the reactive groups of the lipids (as they were exposed) reacted *in vacuo* with vapors, which raised markedly the melting point of the affected lipids and decreased their solubility. There is no evidence that ice crystals formed at this temperature, since no holes could be observed in electron micrographs of sections. The reactive groups were the carboxyl ester linkage and the