The Enzymes of Biological Membranes

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Physical and Chemical Techniques

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Preface

The romantic phase of membrane biochemistry characterized by conceptual developments and an essentially unlimited freedom of choice is gradually coming to a close. Attention is turning from the general, qualitative description of membrane structure toward the specific properties of membrane-linked enzymes and metabolic systems.

The purpose of this series is to serve this development by collecting and evaluating the mass of interesting information that is already available widely scattered in the literature. The emphasis will be upon a comprehensive treatment of membrane-linked enzymes from the viewpoint of modern enzymology. The general properties of membranes will be mentioned only to the extent that they are relevant to the discussion of the enzymes in question.

The first of the four volumes will deal with the physical and chemical techniques (X-ray crystallography, nuclear magnetic and electron spin resonance, fluorescence spectroscopy, immunology, etc.) used in the characterization of membrane enzymes. Chapters are also included on artificial bilayer membranes, chemical modification of membrane enzymes, and on the nature of lipid–protein interaction in membranes. In the next three volumes the enzyme systems participating in the biosynthesis of cell components, active transport, oxydative phosphorylation, and photosynthesis will be analyzed. A brief discussion of hormone receptors is also included. Subsequent volumes may fill in the few but significant gaps in the coverage that for various reasons could not be avoided.

The widely different levels of sophistication achieved in various areas of membrane research dictates different treatment in almost every chapter. While the structures of cytochrome c and cytochrome b_5 are known in atomic detail, the majority of membrane-linked enzymes have not even been isolated. Hopefully the heterogeneity in form will be justified by the relevance of the content. Some areas were entirely omitted from these volumes either because they were extensively reviewed recently or because there is not sufficient information to warrant a review at this time.

The field is very young and one is frequently reminded of a little poem by Robert Frost:

> We dance round in a ring and suppose
> But the Secret sits in the middle and knows.

We hope these volumes will serve as catalysts in the conversion of today's suppositions

into tomorrow's firm knowledge. Criticisms and suggestions which may help to achieve this aim are gratefully appreciated.

My warmest thanks to all who contributed to this work.

St. Louis, Missouri
January, 1976

ANTHONY N. MARTONOSI

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X-Ray Studies on Membranes

C. R. WORTHINGTON

I. Introduction

X-ray diffraction studies on biological tissues have a long history in that some of the early X-ray patterns were obtained soon after the discovery of X-ray diffraction by crystals. Biological tissues are invariably noncrystalline, and hence it was not surprising that these early X-ray patterns showed only a few reflections which were somewhat diffuse in comparison to the very sharp reflections obtained from crystals. Moreover, these X-ray patterns of noncrystalline cell components could not be interpreted correctly because valid procedures for structure analysis of these patterns did not exist at this time. Consequently, X-ray studies of cell components and, in particular, the study of the structure of biological membranes did not attract much attention during the period 1920–1960. However, since 1960 significant improvements in experimental technique and in methods of structure analysis have been accomplished. In recent years, these advances in the X-ray method have led to a revival of interest in X-ray studies on membranes.

II. X-Ray Diffraction Theory

A typical X-ray diffraction experiment is shown in Figure 1. The collimated X-ray beam is incident parallel to the surface of the membrane in order to record lamellar diffraction. The diffraction pattern is recorded on film. If the X-ray specimen contains a multilayered array of membranes, then discrete reflections are observed according to Bragg's law:

$$2d \sin \theta_h = h\lambda \tag{1}$$

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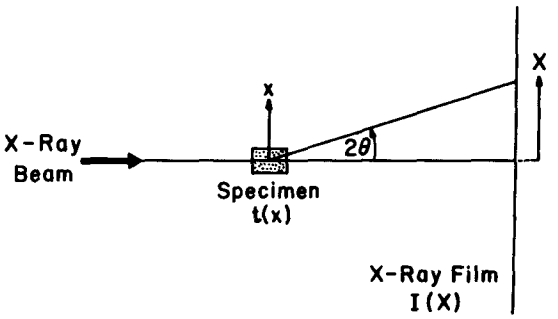


FIGURE 1. Diagram of a typical X-ray diffraction experiment. The membrane specimen has electron density $t(x)$, and the intensity recorded on the X-ray film is $I(X)$ where r, R are real and reciprocal space coordinates. The diffraction angle is 2θ and refers to the angle between the incident X-ray beam and the diffracted X-ray beam.

where d is the repeat period, $2\theta_h$ is the diffraction angle, h is the order of diffraction, and λ is the wavelength of the X radiation.

Only an elementary treatment of diffraction theory is presented; a more detailed account can be found elsewhere (Worthington, 1969*a*, 1971*a*; Worthington, *et al.*, 1973). Let $t(x)$ refer to the one-dimensional electron-density distribution of the unit cell of width d and let $T(X)$ be the corresponding Fourier transform where r, R are real and reciprocal space coordinates. In the case of a multilayered array of membranes, discrete values of $T(X)$, where $X = h/d$, are obtained. The relation between the Fourier transform magnitude $|T(h)|$ and the observed intensity $I(h)$ is

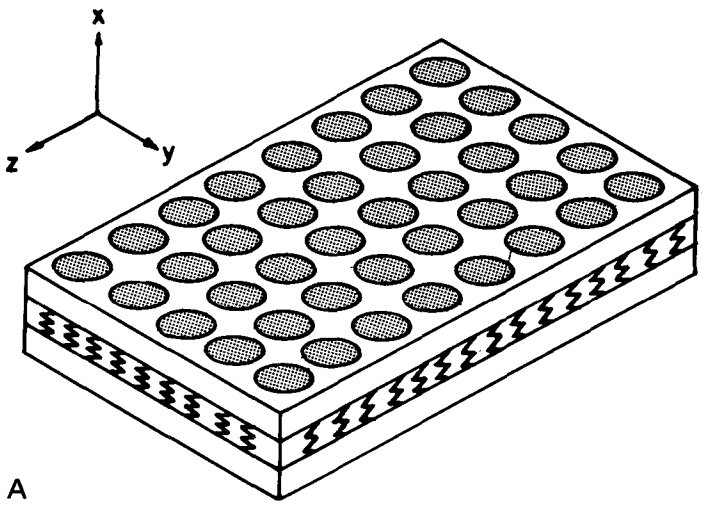
$$|T(h)|^2 = KI(h)C(h) \tag{2}$$

where $C(h)$ is the correction factor and K is the normalization constant. It is convenient to assume $K = 1$ but when an absolute scale is considered, a precise value is assigned to K.

A membrane is a three-dimensional structure, and a drawing of a single membrane is shown in Figure 2A. Let $t(r)$ represent the electron density distribution of this membrane and we may write $t(r) = t(x)t(y, z)$, where $t(x)$ is the lamellar structure and $t(y, z)$ is the subunit structure. The lamellar structure refers to the one-dimensional electron-density distribution in a direction at right angles to the membrane surface, whereas the subunit structure refers to structure within the plane of the membrane. The diffraction arising from $t(x)$ and $t(y, z)$ can be distinguished; this is shown in Figure 2B. An X-ray beam parallel to the surface of the membrane gives rise to lamellar diffraction whereas an X-ray beam at right angles to the surface of the membrane gives rise to the subunit diffraction.

In the study of lamellar diffraction, the X-ray specimen often has a multilayered array of membranes, and in this usual case the Fourier series representation for $t(x)$ is given by

$$(2/d) \sum_{1}^{h} |T(h)| \cos (2\pi hx/d - \alpha_h) \tag{3}$$



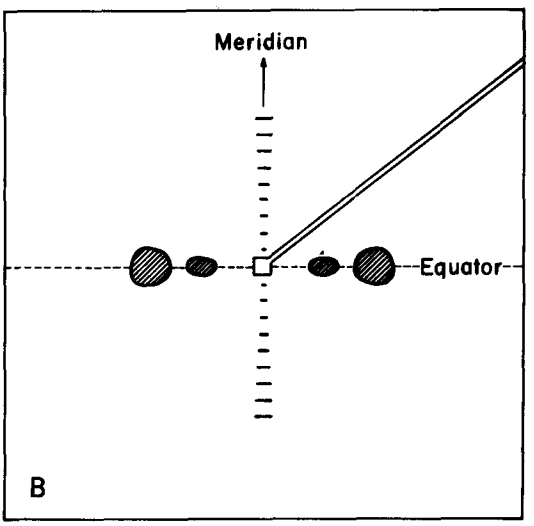


FIGURE 2. (A) A three-dimensional drawing of a triple-layered membrane. The one-dimensional lamellar structure refers to the electron-density distribution along *x*. Possible subunit structure is shown in the top layer of the planar membrane. (From Worthington, 1973*b*.) (B) A drawing of the X-ray diffraction pattern from a multilayered arrangement of planar membranes. The membranes are similar to the one shown in Figure 2A, and the lamellar repeat of the assembly is along *x*. The pinhole collimated X-ray beam is at right angles to *x*, the lamellar repeat direction. The layer line reflections along the meridian are orders of the lamellar repeat distance. The subunit diffraction is on the equator of the X-ray film. A liquid-like planar array of subunits is assumed so that diffuse reflections are obtained. A beam stop is positioned in the center of the X-ray film to prevent the incident beam reaching the X-ray film.

where α_h is the phase angle associated with the amplitude $T(h)$ and $T(h) = |T(h)|e^{i\alpha_h}$. The phases vary from 0 to 2π. However, Eq. (3) has not been used in membrane research because there is no way to derive the correct set of phases. On the other hand, if the unit cell has a center of symmetry, then there is a simplification for the phases are either 0 or π, that is, $T(h) = \{\pm\}|T(h)|$. The resulting set of signs $\{\pm\}$ are referred to as the phases. The Fourier series representation for $t(x)$ with a center of symmetry is given by

$$(2/d)\sum_{1}^{h}\{\pm\}|T(h)|\cos 2\pi hx/d \tag{4}$$

Eq. (4) has been used to derive the electron-density profiles of nerve myelin, retinal photoreceptors, and sarcoplasmic reticulum membranes. The resolution of the Fourier profile is given by $d(2h)^{-1}$, where h is the number of diffraction orders used to compute the Fourier synthesis.

III. Progress in the X-Ray Method

The X-ray method is conveniently divided into four parts: experimental, data processing, structure analysis, and molecular distribution. These are discussed in turn.

A. Experimental

The experimental problem of recording X-ray diffraction from membranes was a serious one prior to 1960 when X-ray cameras consisted of pinholes or slits and when water-cooled stationary anode X-ray sources were mainly used. Exposure times were very long, on the order of weeks. In 1959, an optically focusing X-ray camera was developed for biological research (Elliott and Worthington, 1963). The combination of this camera and a microfocus X-ray source shortened the X-ray exposure times to days. In recent years, the use of a rotating anode microfocus X-ray source provides even shorter exposure times. Thus, the technical problem of recording X-ray patterns from membranes has been largely overcome.

A suitable X-ray specimen should be about 1 mm thick, if copper $K\alpha$ radiation is used, as is usual. Most X-ray studies on biological membranes have been carried out on naturally occurring multilayered structures which have a well-defined lamellar repeating distance. For instance, studies have been made on nerve myelin (Schmitt *et al.*, 1941; Blaurock and Worthington, 1969) and retinal photoreceptors (Finean *et al.*, 1953; Gras and Worthington, 1969; Blaurock and Wilkins, 1969). Also, it is now possible to artificially prepare a variety of cell membranes in a multilayered form by sedimenting in the centrifuge. Thus, X-ray studies have been made on cell membranes which do not occur naturally in a multilayered form. For instance, studies have been made on bacterial cell walls (Burge and Draper, 1967), red-blood-cell membranes (Finean *et al.*, 1966), and sarcoplasmic reticulum membranes (Coleman *et al.*, 1969; Worthington and Liu, 1973; Dupont *et al.*, 1973).

B. Data Processing

The problem here is to find the correction factor $C(h)$ for the membrane system under study. If the X-ray specimen was a crystalline powder, then the correction factor (or Lorentz factor) is h^2 for low-angle X-ray data. Since X-ray intensities are measured by integrating over the whole arc, in the present notation $C(h) = h$ for a powder. A correction factor analysis based upon diffraction theory has been presented (Worthington, 1973*a*). It has also been pointed out that an experimental study of the membrane diffraction using fine pinhole collimation is helpful in establishing the correct form of $C(h)$ (Worthington, 1973*a*).

C. Structure Analysis

The first important consideration is whether the membrane assembly within the unit cell has a center of symmetry. Three distinct experimental situations are illustrated in Figures 3A, B, and C. Consider a dispersion of vesicles consisting of single membranes, with each membrane asymmetric about its center. The cross section of one of these membranes is shown in Figure 3A. The X-ray diffraction pattern $I(X)$ is continuous and, because there is no way to derive the phases, the dispersion data from membranes have not yet been interpreted. Figure 3B shows a regular packing of single membranes; each membrane is asymmetric about its center. This situation might arise as a result of the formation of a concentric layered vesicle or some condensation phenomena. The X-ray diffraction pattern $I(h)$ is discrete and, because there is no way to derive the phases, this kind of diffraction pattern cannot be interpreted. In Figure 3C the unit cell contains two membranes, each membrane is asymmetric about its center, but there is a plane of symmetry at the center of the unit cell. This arrangement occurs naturally in nerve myelin and in retinal photoreceptors and is also obtained as a result of an orderly packing of flattened vesicles. The X-ray diffraction pattern $I(h)$ is discrete, and $t(x)$ is obtained using Eq. (4) provided that the $\{\pm\}$ phases can be found.

The use of direct methods of structure analysis has enabled the phases to be uniquely assigned in the centrosymmetric case. These methods are based upon obtaining additional data points on the Fourier transform by swelling. However, the normal and swollen X-ray data have to fit on the same transform. The direct methods refer to deconvolution analysis or reconstruction analysis using the sampling theorem and Fourier series expressions (Worthington *et al.*, 1973).

The direct methods of structure analysis are ambiguous for two structures are obtained; the positive structure, $+t(x)$, and the negative structure, $-t(x)$. A choice between these two structures can be made by carrying out a further swelling experiment but using a different fluid of electron density F_2 in place of the original fluid F_1. One set of phases will show electron density levels F_1 and F_2 in the Fourier profile with $F_2 > F_1$, whereas the other set of phases will give the reverse result, $F_1 > F_2$. Thus, from knowledge of F_2 and F_1, a choice is made between the two possible structures.

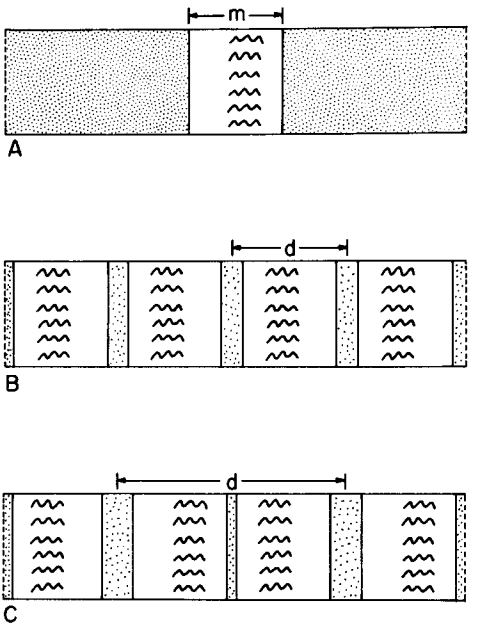


FIGURE 3. (A) A drawing of a cross-sectional view of a single membrane which is asymmetrical about its center. The membrane has width m. This situation is obtained in the study of a dispersion of membrane vesicles. (B) A drawing of a membrane assembly of single membranes with fluid layers of equal width. The unit cell has width d and contains one asymmetric membrane and one fluid layer. (C) A drawing of a membrane assembly where the inner and outer surfaces are separated by fluid layers. The fluid layers in the drawing are unequal in width. The unit cell has width d and contains two membranes and two fluid layers. The unit cell is centrosymmetrical about its center.

D. Molecular Distribution

The Fourier synthesis for $t(x)$ describes the electron-density distribution within the unit cell but at a finite resolution given by $d(2h)^{-1}$. This derived electron-density profile is not the true density profile for it has a ripple contour superimposed on the true structure as a result of finite resolution. This means that bumps or ripples in the profile cannot be directly assigned to molecular components unless extensive molecular labeling experiments are carried out and interpreted.

The Fourier synthesis for $t(x)$ is on a relative scale but, in order to interpret the Fourier profile in molecular terms, knowledge of an absolute electron-density scale in electrons/Å^3 is required. This absolute scale is obtained from knowledge of either the average electron density of the membranes and the electron density of the swelling fluid or from swelling experiments using two different fluids which have different electron densities. It is evident that any proposed molecular model should have an electron-density contour in harmony with the derived Fourier synthesis. Thus, possible models are found while many other models are rejected.

The knowledge of the electron-density profile of a membrane on an absolute

scale allows a general description of the molecular distribution to be given. That is, the hydrocarbon chains which have low densities are assigned to the low-density regions of the membrane, while the ionic head groups of lipids and the protein molecules which have comparatively high densities are assigned to the high-density regions.

It is often convenient to describe the electron-density distribution in terms of an electron-density strip model. The bilayer-type model chosen has three different layers with high–low–high densities. The bilayer has width m and has a uniform low-density region of width l. The model is asymmetric when the high-density layers have different densities and widths.

IV. Lamellar Structure of Membranes

X-ray diffraction studies on membranes have been mainly carried out on the naturally occurring multilayered structures such as nerve myelin, retinal rod photoreceptors, and chloroplasts. Drawings of typical low-angle X-ray diffraction patterns arising from the lamellar structure of nerve myelin and retinal photoreceptors are shown in Figure 4. The structure analysis of these lamellar patterns has advanced considerably in recent years. A recent development is that X-ray diffraction studies on single membranes are now possible provided that these membranes, which do not occur naturally in linear arrays, can be organized into multilayers by sedimenting in the ultracentrifuge. A drawing of a typical low-angle X-ray diffraction pattern from sarcoplasmic reticulum (SR) membranes is also shown in Figure 4.

A. Nerve Myelin

The molecular organization of nerve myelin has been extensively studied by diffraction and microscopy. It is known from electron microscopy that the myelin sheath of peripheral nerve is derived from a spiral wrapping of the Schwann cell membranes around the axon of the nerve fiber. Moreover, the concentric myelin layers have a precise multilayered assembly which is eminently suited for analysis by X-ray diffraction. Thus, it is appropriate to pursue the study of nerve fibers as far as the diffraction method will allow.

It is realized that the nerve myelin membrane is a very special membrane containing more lipid than protein and containing a high proportion of cholesterol. Perhaps this relates to the passive role of the myelin sheath during nerve excitation, for the myelin sheath is presumed to act solely as an insulator. Thus, it could be argued that the cell membranes which possess various dynamic biological functions may not necessarily follow the design principles of the nerve myelin membrane. This remains to be demonstrated, however.

Myelinated nerve specimens when examined by low-angle X-ray diffraction