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Crystal Structure Determination of β-Lactoglobulin From Electron Micrographs

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AN ABSTRACT OF THE THESIS OF Richard Foster for the Master of Science in Physics presented July 29, 1971.

Title: Crystal Structure Determination of β-Lactoglobulin from Electron Micrographs

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

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Often electron micrographs exhibit a repeating structure. Sometimes this repeating structure satisfies the definition of a crystal in that it has a three dimensional repeating structure. If the unit cell structure of this repeating structure can be determined, it can be used to help categorize different sections of a particular sample. In some cases, the use of optical diffraction analysis of electron micrographs with repeating structure is a method of determining the unit cell structure.

Samples of β-Lactoglobulin were prepared for viewing in the electron microscope using both the crystalline material and carbon

replicas of the crystal surface. Because the crystalline material was very unstable in the electron beam, images adequate for use as diffraction gratings could not be obtained. Electron images from the replicas were used to generate the optical diffraction patterns in this paper.

The structure of β-Lactoglobulin has been determined previously by X-ray diffraction analysis. This information was used to assist in the interpretation of the optical diffraction patterns.

Electron micrographs and optical diffraction patterns were recorded which were found to be consistent with the structure of β-Lactoglobulin as determined by X-ray diffraction analysis. The unit cell dimensions were determined to be a = 58±4Å, b = 59±3Å and c = 102±12Å.

CRYSTAL STRUCTURE DETERMINATION OF β-LACTOGLOBULIN FROM ELECTRON MICROGRAPHS

by

RICHARD ROETER

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
PHYSICS

Portland State University
1971

TO THE OFFICE OF GRADUATE STUDIES:

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CHAPTER I

INTRODUCTION

Inclusions which are distinguishable from their surrounding matrix are often found in electron microscope images. Sometimes the arrangement of these inclusions satisfies the definition of a crystal in that they are three dimensional periodic arrays. This repeating structure probably is important, in most cases, either structurally or functionally, in determining the properties of the systems being analysed. For example, in metallurgy, there might be a relationship between the inclusion structure and the chemical and physical properties of the material, and in biology, there might be a relationship between the inclusion structure and the cell structure or organization.

When the inclusion material can be adequately separated from its surrounding matrix, information about its three dimensional structure can often be determined using X-ray diffraction and/or optical crystallography techniques. If the inclusion material cannot be separated from its surrounding matrix it is often difficult to characterize more than the periodicities and the angular relationships between the arrays.

The inclusion material and its surrounding matrix can often be preserved or only slightly modified when prepared for viewing in the electron microscope. Provided the distortion is not too great, the

electron micrograph image provides a good representation of a section of the repeating structure. The electron micrograph image represents a plane section of the three dimensional periodic structure. Since, in general, different plane sections of a specimen will reveal different periodicities, the electron micrograph images may appear dissimilar when in fact they are different views of the same structure. This effect can lead to a great deal of confusion, especially if these electron micrograph images are to be compared with others found in the same or in different specimens. If the unit cell structure could be determined much of the difficulty could be eliminated.

The periodicity of the inclusion material can be measured directly from the print of a high magnification electron micrograph. Recently, Klug and DeRosier (1) have proposed optical diffraction techniques as a method of determining the three dimensional linear and angular parameters from electron micrographs which have a repeating structure. This was proposed to be accomplished by using the electron micrograph directly as a diffraction grating and analysing the resulting optical diffraction pattern. The use of optical diffraction for these measurements provides a few important advantages over the direct measuring method. First, the optical diffraction method provides an objective and accurate method of measurement. Second, there are often subtle periodicities which are not readily apparent to the naked eye but are easily seen in the optical diffraction pattern. Third, the optical diffraction patterns of different plane sections of the three dimensional material can be recombined in reciprocal space

to provide information about the unit cell structure. Fourth, the determination of the three dimensional unit cell structure can be used to provide a close approximation of the molecular weight of the material or at least place it within a certain range (2).

The purpose of this research project is to investigate the optical method of structure determination by attempting to determine the unit cell structure of a crystalline material whose unit cell dimensions have been determined by X-ray diffraction.

CHAPTER II

REVIEW OF LITERATURE

Generally, repeating structure in electron micrograph images is fairly easy to catagorize because a numerical value can be assigned to the various dimensions. Often the interpretation of this data is a far more complex problem. To extract the maximum amount of information from electron micrograph images with repeating structure, some innovative techniques have been developed. One such technique has been the use of optical methods to both enhance and analyse the repeating structure.

Markham, Frey and Hills (3) have developed a method of investigating rotational periodicities by superimposing photographs of an electron micrograph rotated repeatedly through a given angle. When the proper angle is chosen, the rotational structure is enhanced. Markham (4) has developed a stroboscopic technique which also serves to superimpose images in various angular arrays in a manner similar to the one above. Linear objects were treated in a similar manner by Markham, Hitchborn, Hills and Frey (5). The method consists of superimposing photographs of an electron micrograph which has a repeating structure translated repeatedly through a proper chosen distance to enhance the repeating structure.

These early methods of using optical means of enhancing periodic structure have led researchers to seek other ways in which optical

methods could be used to interpret the images with periodic structure in electron micrographs. Klug and Berger (6) have suggested using the electron micrograph of an object exhibiting a repeating structure as a diffraction grating and analysing the repeating structure by means of optical diffraction. Using this method to analyse electron micrographs, they were able to analyse the repeat structure, state some conclusions about the negative staining process and distinguish between contrast arising from the "near" and "far" surfaces in the electron micrographs of TMV helix.

Klug and Berger performed their experiments using an optical diffractometer, an instrument which enables the operator to view the Fourier transform of an image and also to reconstruct images from their Fourier transforms. The primary use of this instrument has been to assist in the solution of X-ray diffraction problems (7). Taylor and Lipson (7), who have developed this instrument; have made valuable contributions in areas related to optical transforms. Berger (8) has introduced the gas laser as a light source for the optical diffractometer. Because of the high intensity of the laser light, exposure times for recording diffraction patterns are greatly reduced.

The three dimensional density map of the bacteriophage T4 has been reconstructed by DeRosier and Klug (1) using information contained in the electron micrograph, its optical diffraction pattern, and computer techniques. They further suggested that the projection of various optical views, i.e., electron micrograph images, of a material which has a three dimensional repeating structure could be recombined

in reciprocal space to determine the unit cell structure. Much of the theory related to the technique was developed earlier by Bragg (9). The use of optical diffraction techniques to determine unit cell structure was further developed by the theoretical considerations of Berger (2). Sternlieb and Berger (10) colaborated in the use of recombination techniques to determine the unit cell structure of mitochondrial crystals by optical diffraction analysis of electron micrographs of human liver biopsy specimens.

CHAPTER III

THEORY

To determine the crystal structure means to determine the distribution of scattering matter in the unit cell. For visualization it is convenient to consider the unit cell as made up of discrete scattering elements; however, the true representation is that of a continuous distribution of scattering matter with concentrations of scattering matter in the region of the unit cell elements.

One method of analysing this distribution of scattering matter is to express the density function of the crystalline material in terms of a suitable Fourier series. The spectrum of the radiation scattered from the crystalline material can be used to help determine the terms in the series, since each intensity peak in the diffraction pattern represents one of the terms of the series. When each term and its coefficient is determined, the unit cell structure is determined.

Because of the close relationship between the unit cell structure and its diffraction pattern, as expressed in terms of a Fourier series, a general expression of the series is helpful in visualizing the relationship of the unit cell structure to the optical diffraction method described in this paper. The Fourier series can be written in the following form when expressed in terms of the reciprocal lattice (9):

$$\text{Eq. (1)} \qquad \rho(r) = \frac{1}{V}\sum_{m} F(m) e^{-2\pi i(\bar{r}\cdot\bar{r}_m^*)}$$

where

$$\text{Eq. (2)} \qquad F(m) = \int \rho(r) e^{2\pi i(\bar{r}\cdot\bar{r}_m^*)} dv$$

$\rho(r)$ is the density function for a point (x,y,z) in the crystal, V is the volume of the unit cell, m is a single symbol used to represent the triple numbers h, k and l, $\bar{r}$ is the vector from the origin of the crystal lattice to the point (x,y,z) of the crystal lattice, $\bar{r}_m^*$ is the vector from the origin of the reciprocal lattice to the point (h,k,l) of the reciprocal lattice, F(m) is the structure factor and dv is the volume of the elementary parallelpiped whose sides are dx, dy and dz. The vector $\bar{r}_m^*(hkl)$ to the point (h,k,l) of the reciprocal lattice is perpendicular to the planes (hkl) of the crystal lattice and its magnitude is the reciprocal of the spacing of these planes.

To every spectrum that can be given by a crystal there corresponds one term in the Fourier series. Each such term represents a distribution of scattering matter whose density is given by a plane simple harmonic waveform. There is no spectrum for the zero order, and higher orders from a given set of planes give submultiples of the true lattice spacings of the planes.

The reciprocal lattice and the diffraction pattern will provide a representation of the spectra given by a crystal lattice. Each term in the Fourier series contains an amplitude component and a phase component. The amplitude component is the structure factor F(m). The value of the phase angle is given by (9):

Eq. (3) $$\phi_m = 2\pi(\bar{r}\cdot\bar{r}_m^*) = 2\pi(\bar{r}\cdot\bar{n}^*)/d_m$$

where $\bar{n}^*$ is the unit vector in the direction of the reciprocal lattice vector $\bar{r}_m^*$ and d_m is the spacing between the planes (hkl).

Experimentally, the intensity $|F(m)|^2$, is determined and the phase factor remains undetermined. For this reason and because of the finite number of terms of the Fourier series used during analysis, the Fourier series gives information about the unit cell structure but not the complete structure. Generally other experimental data and theoretical information are combined with the Fourier treatment to give a good representation of the complete structure.

In 1929 Bragg (9) introduced a method for using Fourier series to analyse crystalline structure. Instead of determining the volume density, one attempts to determine the surface density of all the unit cell scattering matter projected onto a plane. The projection is made along any zone axis and if such a projection can be made on more than one plane, the information obtained is often sufficient to determine the unit cell structure. The Fourier method is particularly instructive since it provides a mathematical method of visualizing the distribution of scattering matter in the unit cell structure. James (9) has shown, using Fourier methods, that the two dimensional projection of the scattering matter of a crystalline structure along a zone axis onto the reciprocal net corresponds exactly with the distribution of the transmitted amplitude from a certain two dimensional grating having the same translations. This can be

restated to show the relationship to the optical diffraction method discussed in this paper. If the three dimensional crystalline structure can be projected along a zone axis and then transformed into reciprocal space, i.e., by optical diffraction, the reciprocal net represents the Fourier transform of a plane section of the crystalline structure. This Fourier transform passes through the origin of the reciprocal lattice and lies parallel to the plane of the projection.

The electron microscope can be used to project a three dimensional structure onto a plane. Due to the large depth of field of the electron microscope, the whole specimen is brought into focus in the image plane. Different levels of the specimen cannot be brought into focus excluding other parts of the specimen. Using a goniometer or tilting stage, it is possible to project different views of the three dimensional unit cell structure (if it is resolvable) along a zone axis onto a plane, i.e., the image plane.

As was stated earlier the diffraction pattern alone cannot be used to determine the complete unit cell structure in terms of a Fourier series because of the lack of information about the phase. However, one can analyse the spatial distribution of the diffraction maxima to determine a representation of the two dimensional projection of the crystalline structure.

To determine the three dimensional structure, one can generate optical diffraction patterns from electron micrographs which represent two dimensional projections of the crystalline structure. One then combines two dimensional diffraction patterns which contain one line

in common, that is, with an identical periodicity. These two diffraction planes intersect each other along their common line. Once the angle between the two planes is determined, the relative orientation of the two planes is uniquely determined. A practical method of solving this problem is to find a third two dimensional plane which intersects the first two. The constructed lattice now represents a section of all the unit cell structure in reciprocal space. Once a hypothetical unit cell structure in reciprocal space has been constructed, other diffraction patterns of different sections can be used to test for consistency. The unit cell structure in reciprocal space can then be converted mathematically into real space.

1. CAMERA CONSTANT METHOD

A ray diagram representing the Fraunhofer diffraction pattern generated from a double slit is shown in Figure 1. An intensity peak occurs in the diffraction pattern when the diffracted waves interfere constructively. If ϕ is sufficiently small, i.e., less than 6 degrees, the Bragg equation for the first order maximum can be written as

$$\text{Eq. (4)} \qquad \lambda = d\phi$$

where d is the spacing of the double slit and λ is the wavelength of the incident radiation. When ϕ is small, ϕ is approximately equal to θ and

$$\text{Eq. (5)} \qquad \phi = x/L$$

where L is the camera length and x is the distance from the center of the diffraction pattern to the spot to be indexed. Substituting

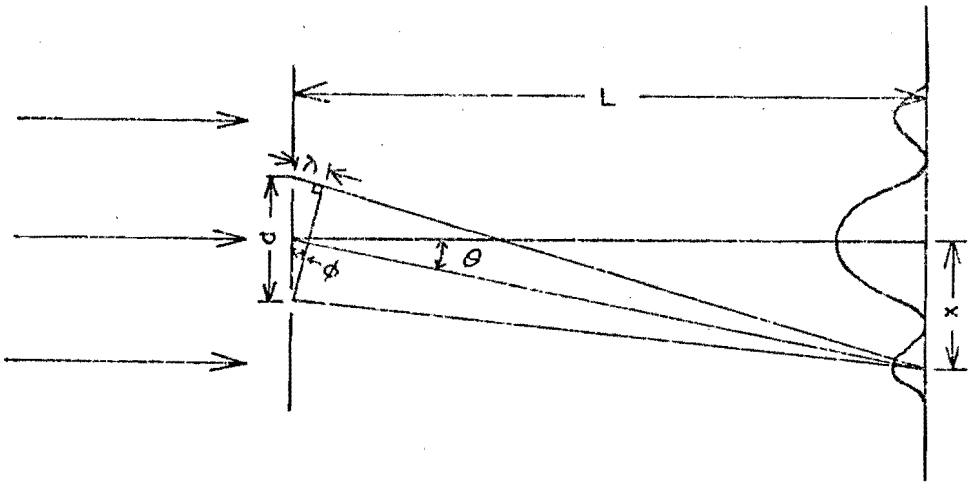

Figure 1. Schematic diagram showing the relation between the camera length L and the first diffraction maximum on the diffraction pattern.

Equation 4 into Equation 5, we get the equation

$$L\lambda = dx \qquad \text{Eq. (6)}$$

The term $L\lambda$ is known as the camera constant and is determined from measurements of x on a diffraction pattern of a substance of accurately known spacing. The procedure to index the diffraction pattern using the camera constant method is to measure the x value for each spot and calculate its d spacing from Equation 6.

II. ELONGATION OF THE RECIPROCAL LATTICE POINTS

A ray diagram showing the elongation of a reciprocal lattice spot generated from a diffraction grating is shown in Figure 2. Jenkins and White (11) list the angular half width of any principal maximum for a diffraction grating as

$$\text{Eq. (7)} \qquad \Delta\theta = \frac{\lambda}{Nd \cos \theta}$$

where $\Delta\theta$ is the angular half width of the diffraction maximum generated from the diffraction grating, θ is the angle at which a ray leaves the grating in Figure 2, λ is the wavelength of the incident radiation, d is the distance separating the centers of the scattering slits and N is the number of scattering slits.

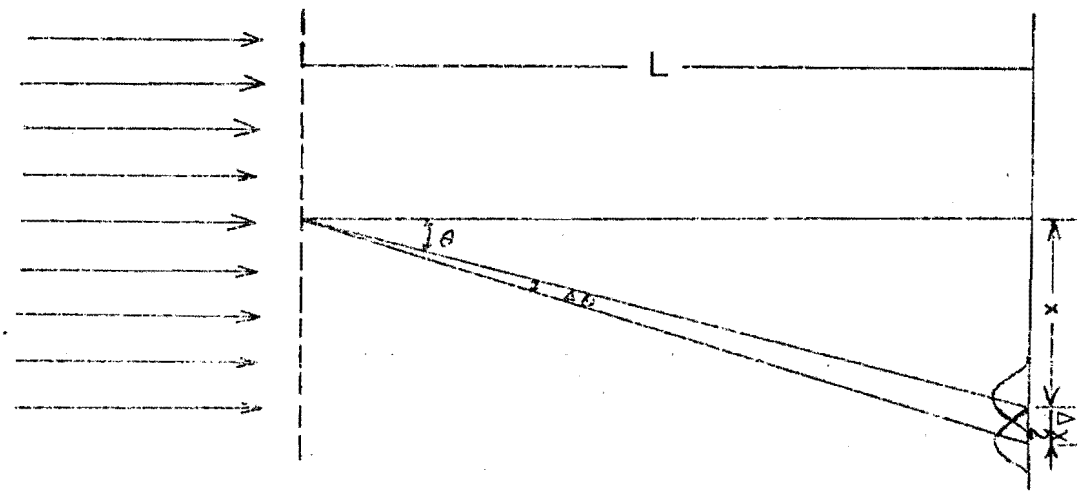

Figure 2. Schematic diagram showing the relation between the elongation of the reciprocal lattice spots and the number of scattering slits.

In the optical diffraction arrangement θ is small, i.e., less than 6 degrees, and $\theta \approx \tan \theta = x/L$, so that the angular half width of any principal maxima can be expressed as

$$\text{Eq. (8)} \qquad \Delta\theta = \frac{\Delta x}{2L}$$

where Δx is the elongation of the reciprocal lattice point. Combining Equations 6, 7 and 8, the elongation of the reciprocal lattice point

can be expressed as

$$\text{Eq. (9)} \qquad \Delta x = \frac{2x}{N}$$

where x is the magnitude of the reciprocal lattice vector from the origin of the diffraction pattern to the diffraction maximum of interest and can be measured directly. The value of N can also be determined experimentally. This can be accomplished by photographing both the back focal plane and the image plane of the magnifying lens of the optical diffractometer. The image plane will contain the image of all the scattering objects which scatter the radiation from the laser beam of finite diameter. Another method is to prepare a mask for the region from which a diffraction pattern has been obtained in an electron micrograph. The number of scattering objects intercepted by the laser beam will depend on the dimensions of the mask in the particular direction related to a diffraction spot. One can photograph the mask area, i.e., using an enlarger, and count the number of scattering objects along the direction related to a particular diffraction spot. In general, the value of N will depend upon the magnification of the electron micrograph and the area in the electron micrograph exposed to the incident radiation.

III. SOURCES OF ERROR

Because of inherent difficulties in the diffraction method of analysis and the numerous problems associated with the specimen, the constructed lattice will be subject to some error. Sternlieb and

Berger (10) listed some of the inaccuracies associated with the specimen. Compression of the material during sectioning can make lattice determination difficult since the analysis is based upon the combination of reciprocal lattices with identical periodicities. Some inaccuracies may be due to swelling or shrinking of the specimen during fixation and dehydration. Berger (2) states that is some cases there may be more than one type of crystal lattice in a single specimen. However, if enough sections are analysed some discrepencies should be apparent which prevents construction of the unit cell structure.

A serious source of error can be related to the type of symmetry of the crystalline material. If the crystalline material has a glide or screw axis, the lattice parameters may have the wrong dimensions. However, the constructed unit cell is useful information in these cases because it is related by some constant to the real repeating pattern. Other methods can be used to determine the value of this constant.

The diffraction maxima in the reciprocal lattice are generally not lattice points. Because of this there is a certain amount of error introduced in combining reciprocal lattices with identical periodicities. When the elongated reciprocal lattices are combined there is a range of orientations possible. The elongation of the reciprocal lattice point can be mathematically expressed by Equation 9. This equation shows that the reciprocal lattice maximum is a point when the number of scattering objects is infinite. Ideally, the experimental apparatus should provide the largest possible number of scattering points without producing unwanted other effects. The

laser beam diameter can be chosen or else expanded so that the maximum number of scattering objects will be intercepted without introducing abberation or distortion of the optical diffraction pattern due to the dimensions of the lens.

For a thin lens the focal point for the rays coming from the outer part of the lens is closer to the lens than the rays from the inner part of the lens. This effect cannot be totally eliminated but it can be reduced by selecting only the rays near the optical axis.

Another method of increasing the number of scattering points is by selecting an optimum magnification for the electron micrograph image. There will be a range of magnification possible for the analysis of electron micrograph images using optical diffraction. This range will be limited at one extreme by the resolution of the electron microscope and/or sample preparation and at the other extreme by the number of scattering objects intercepted by the beam of radiation of a finite diameter incident on the electron micrograph.

One attempts to establish the optimum conditions for the determination of the reciprocal lattice. This will generally mean generating a diffraction pattern which is an optimum between the angle of the diffracted beam being small and the number of scattering objects being large.

Knowing the number of scattering objects and the magnitude of a particular reciprocal lattice vector, the elongation of that point can be determined. This elongation can be used to indicate the range of possible orientations when two diffraction patterns are combined.

CHAPTER IV

METHODS AND TECHNIQUES

I. SAMPLE AND SAMPLE PREPARATION

The three dimensional structure of many crystals which have a repeating structure which is resolvable with the electron microscope has been determined. β-Lactoglobulin is one such crystal.

Air dried β-Lactoglobulin crystals were obtained fron the Mann Research Laboratory. Senti and Warner (12) have reported some of the properties of β-Lactoglobulin crystals they have grown. They reported an average value of 9.8% water content in air dried β-Lactoglobulin crystals with slight variations from day to day. Senti and Warner also found that two types of β-Lactoglobulin crystals formed, both orthorhombic, but different in habit. One batch had thick rectangular tablets somewhat elongated along the [100] and showing (001) dominance. The other batch contained prisms which were considerably elongated along the [010] and usually showed (100) dominant, although (001) was sometimes as developed as much as (100). These differences were also observed by Riley (13). The optical properties of the β-Lactoglobulin crystals used in the work reported in this paper were not examined. This information is probably directly related to the sections observed in the electron microscope because of the preferential fracturing along certain planes of the crystal during sample preparation. In the light

microscope, the crystals appeared to be translucent flakes. Figure 3 shows typical β-Lactoglobulin crystals as observed with the light microscope.

There have been several reports of the three dimensional unit cell structure of β-Lactoglobulin. There is a certain amount of difference in the values for the lattice parameters. Much of this discrepency is due to the uncertainty in the water content. For air dried crystals Crowfoot (14) reported unit cell dimensions of a = 60Å, b = 63Å, c = 110Å, Fankuchen (15) reported a = 60Å, b = 62Å, and c = 111Å, and the values of a = 60.7Å, b = 63.0Å and c = 112.4Å were found by Senti and Warner (12). The error in the Senti and Warner lattice parameters is estimated to be no greater than 0.5% (12). To give an idea of the variation possible in the lattice parameters due to the water content, Senti and Warner reported the following values for the lattice parameters with crystals having an average water content of 46.2%: a = 69.29Å, b = 70.42Å and c = 156.5Å.

The lattice parameters of β-Lactoglobulin are well within the resolution limit of the electron microscope. Dawson (16) has recorded electron images which show planes of unit cell dimensions for β-Lactoglobulin. He reported a view of the (100) face of β-Lactoglobulin crystal with a periodicity of c = 105±5Å. In addition, from a measurement of shadow length he determined a = 50±10Å, with b being undetermined. Considering that the water content was probably reduced in the evaporator during sample preparation, the results are in good agreement with the X-ray diffraction results for the

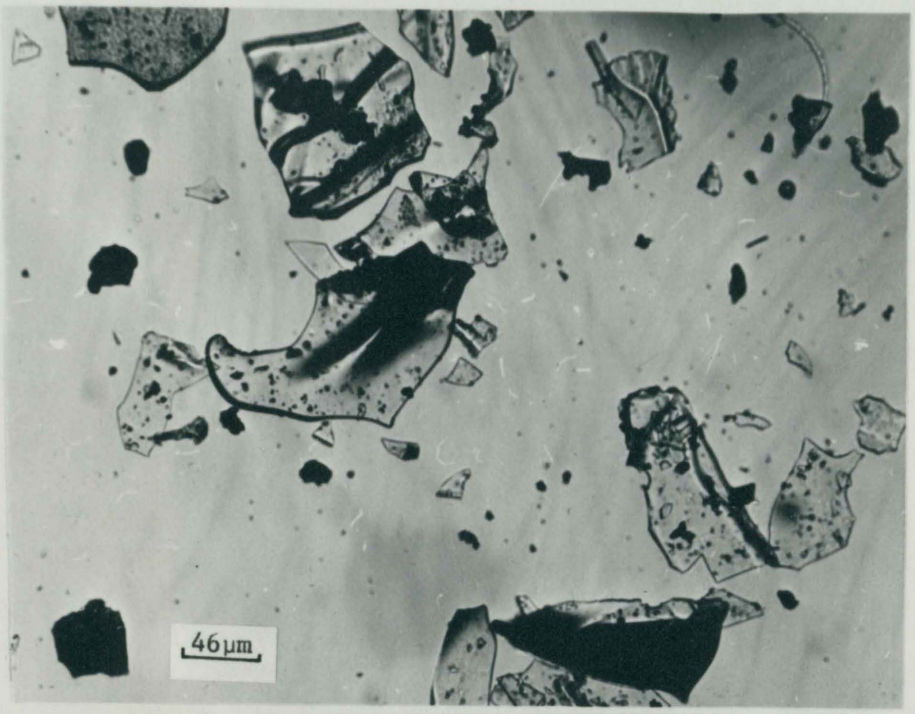

Figure 3. Photograph of a typical collection of β-Lactoglobulin crystals as seen in the light microscope. Magnification 240X.

determination of the unit cell dimensions.

Initially, an attempt was made to view the lattice planes of β-Lactoglobulin directly and correlate the planes of the crystal with the selected area electron diffraction pattern. β-Lactoglobulin crystals were crushed into a very fine powder. This powder was dusted onto a copper mesh grid which was coated with a thin carbon film substrate. The grid was then shaken to remove any crystals which did not adhere to the carbon film. The crystals were then viewed directly with 50kv and 125kv Hitachi electron microscopes. These crystals were very unstable even under very low beam currents, eg. 5 μamps. Any repeating structure in the crystal was not readily apparent. The electron image changed from dark to light when viewed with bright field with very little contrast except when gross changes were occurring. No repeating structure was apparent using dark field imaging. The initial electron diffraction pattern was generally very strong and faded in a short length of time (generally in a few minutes). These effects made direct viewing of the crystal lattice planes impossible.

Figure 4 shows a print of a bright field image of a single β-Lactoglobulin crystal. Figure 5 shows a print of a bright field image of the same β-Lactoglobulin crystal after exposure to the electron beam for about 4 minutes. During this time there were numerous dynamical changes in contrast conditions. Not shown in the print is a halo of material which has presumably resulted from a reaction of the electron beam with the β-Lactoglobulin crystal. Dark field images for β-Lactoglobulin crystals were recorded but not used

Figure 4. Photograph shows a bright field image of a single β-Lactoglobulin crystal as recorded using a Hitachi HS-7S electron microscope. Magnification 52,500X.

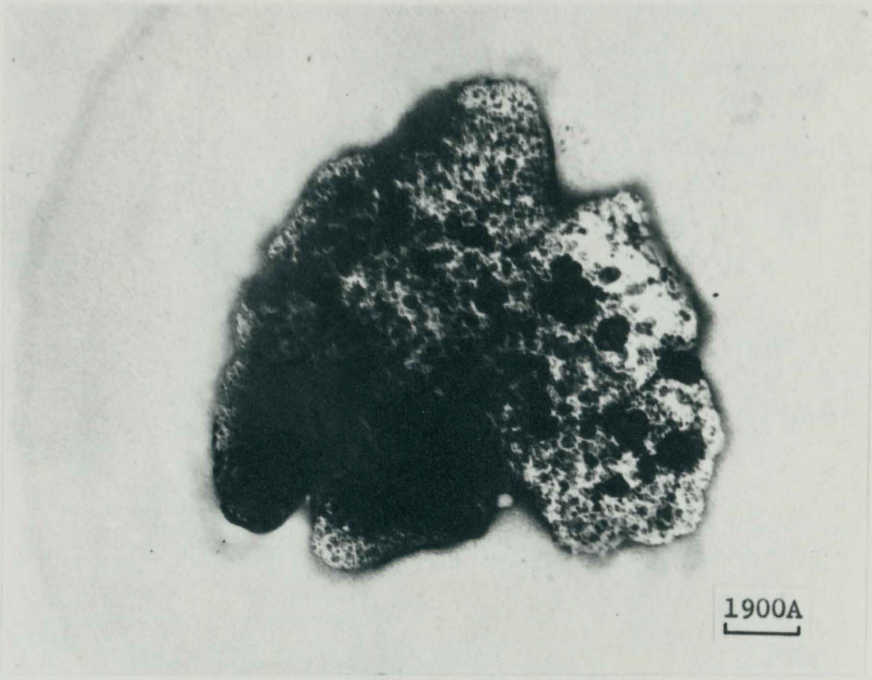

Figure 5. Photograph shows the same crystal as seen in Figure 4 after about 4 minutes exposure to the electron beam. Magnification 53,500X.

in this paper. Although the electron diffraction pattern had intense scattered beams, the image from these scattered beams did not reveal periodicities. Figure 6 shows a typical electron diffraction pattern obtained from β-Lactoglobulin crystal.

Preparations for indirect viewing of the crystal planes in the electron microscope were made using a psuedo-replica technique similar to that used by Wyckoff (17). β-Lactoglobulin crystals were crushed between two glass slides and dusted onto a parlodion film. An alloy containing 80% platinum 20% palladium was shadow cast in a vacuum evaporator onto the crystals from two directions at right angles to each other. One direction was elevated 20^{o} above the horizontal and the other direction 45^{o} above the horizontal. Each shadowcasting deposited approximately 40A of the alloy on the crystals. Carbon was then deposited from vertically above the crystals in order to form a continuous replica film. The copper mesh grid was then placed sample side up in a dish containing a fine sponge filled with acetone to dissolve the parlodion film. After ten minutes, the copper mesh grid was transferred from the acetone bath to a second dish containing a fine sponge filled with a 0.1 M solution of sodium chloride in distilled water to dissolve the β-Lactoglobulin. After thirty minutes, the copper mesh grid was transferred to a third dish containing a fine sponge filled with distilled water to rinse the sample. The sample was then removed from the water and air dried in preparation for viewing in the electron microscope. If small pieces of the original materials adhere to the carbon support, they may be removed with

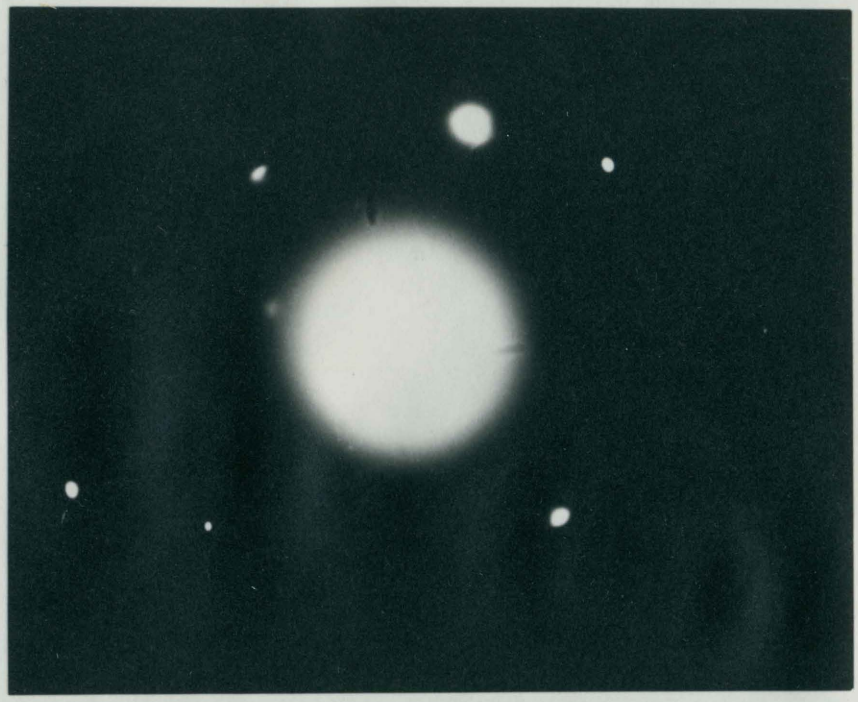

Figure 6. Photograph of the electron diffraction pattern obtained from the β-Lactoglobulin crystal in Figure 4.

solvents or, in many cases, left in place, since the structure observed in the electron microscope is mainly that of the metallic film.

II. APPARATUS

The specimens of β-Lactoglobulin, prepared as described in the previous section were viewed with a HS-7S electron microscope at an accelerating potential of 50kv and photographically recorded on electron image plates. Magnifications of 50,000 to 60,000 times enabled one to obtain electron micrographs with repeating structure. This structure was generally not apparent when viewed directly in the electron microscope. These electron micrographs were used to generate optical diffraction patterns in the apparatus described below. The optical diffraction patterns were recorded on Plus-X Pan Professional 4147 film with exposure times of about 1 second. In addition, a mask was prepared for each section in the electron micrograph from which an optical diffraction pattern was obtained. This was done to identify the section and record the orientation.

A diagram of the optical diffractometer used to obtain optical Fraunhofer diffraction patterns from electron micrographs is represented diagrammatically in Figure 8. Light coming from a Spectra-Physics Model 132 helium-neon gas laser, whose specifications are listed in Table 1, passes through a microscope objective lens, 10X, and a pinhole which form a spatial filter for expanding and cleaning up the beam, i.e., removing unwanted intensity variations. L_1 is a collimating lens that renders the divergent light parallel. Next in

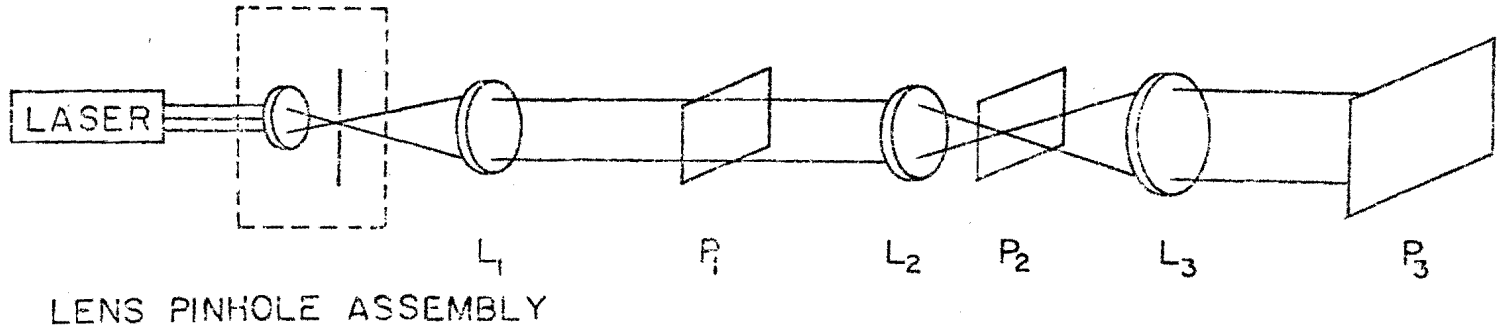

Figure 7. Schematic diagram of the optical diffractometer apparatus.

TABLE I

SPECIFICATIONS FOR SPECTRA-PHYSICS MODEL 132 LASER

Wavelength	6328A (visible spectrum, deep red)
Output Power	1.0 milliwatts
Spatial Mode	Tem_{oo}
Beam Diameter @ $1/e^2$ Points	0.8 millimeters
Beam Divergence	1.0 milliradians

in the plane P_1 is the input film, usually contained in a liquid gate assembly. A fluid whose index of refraction matches that of the film base and/or emulsion is used to eliminate phase noise - the random variation of optical phase across the film caused by variations in film thickness. Since in this apparatus no liquid gate assembly was used, variation in film thickness was a source for some error. Lens L_2 extracts the Fourier transform of the input film and presents it as a complex amplitude distribution in the plane P_2, the Fourier transform plane. The optical diffraction pattern can be recorded in the plane P_2. Generally, the diffraction pattern in the plane P_2 is difficult to resolve adequately. A convergent lens L_3 was used to magnify the diffraction pattern and position it in the final plane P_3. The plane P_2 is located a distance behind L_2 equal to the focal length of L_2.

Taylor (7) has listed the ideal conditions for the attainment of optical diffraction patterns in the optical diffractometer. In general, it is important to have monochromatic light, high quality

lenses and proper alignment of the system. Precautions were taken to approach these ideal conditions. Lenses were chosen which were free from bubbles and minute imperfections. In addition, care was taken to insure the lenses were clean and free from grease spots and condensation. The Spectra-Physics Model 132 Laser provided a sufficiently intense source of monochromatic, coherent light. Finally, the diffraction apparatus was aligned so that all the components of the system had a common optical axis. Focusing is generally a very critical operation in this type of apparatus.

CHAPTER V

DATA

A Cornell Interference and Diffraction Slitfilm Demonstrator was used as a reference pattern for the camera constant method of analysis. The particular spacing chosen consisted of 14 slits with a center to center spacing of 0.0176 cm. The diffraction pattern generated from this set of slits had principal maxima with separations of 1.0837 cm. Using Equation 6, it was determined that the camera constant for this particular position of camera and magnifying lens was 0.01907 cm^2. This camera constant was used in all calculations related to the optical diffraction pattern analysis, since the position of the camera and the magnifying lens was not changed in the apparatus.

A series of photographs was taken for each area of the electron micrographs from which an optical diffraction pattern was obtained. These photographs consisted of a general area from which the optical diffraction pattern was obtained, the area enclosed by the mask which contained the scattering objects for the optical diffraction patterns and the optical diffraction pattern itself.

Some general features of the diffraction patterns are worth commenting upon. The intense central region is contributed to not only by the zero order diffracted beam but also the halation ring arising from reflections of this beam at the back surface of the photographic film. This phenomena is commonly met with in photography

of strong sources of light. The two, approximately perpendicular spikes of high intensity which pass through the center of the diffraction pattern represent the aperture function of the subject, i.e., the diffraction pattern of the mask used to frame the region illuminated. Because the short end of the mask has not always been set strictly perpendicular to the long edge, these spikes are not necessarily mutually orthogonal. The very fine periodicities within the spikes are functions of the mask dimensions. The use of the mask enables one to rotate the optical diffraction pattern into the image so that the particular planes in the image can be identified.

A summary of the data resulting from the analysis of the optical diffraction patterns is listed in Tables II and III. Table II shows a comparison of the possible plane spacings for β-Lactoglobulin crystalline material using the lattice parameters of Senti and Warner (12) and those plane spacings which were determined experimentally. The theoretical and experimental values for the angles between the possible planes are listed in Table III. The data was determined to be consistent if plane spacings and angular relations were both reasonably close to the X-ray results.

Figure 8 is a print obtained from an electron micrograph of β-Lactoglobulin crystal surface. The magnification of the image is 168,000X and the magnification of the insert is 336,000X. Figure 8 shows the (110) and (012) planes for β-Lactoglobulin. Although the image appears to be quite structured, the periodicity is not readily apparent. Figure 9 shows a photograph of the optical diffraction

TABLE II

COMPARISON OF POSSIBLE PLANES RESOLVABLE IN β-LACTOGLOBULIN CRYSTAL AND EXPERIMENTAL RESULTS

hkl	d (Å) (12)	d_{exp} (Å)	Scattering Objects
001	112.4	102±12	17
010	61.0	59±3	20
100	60.7	58±4	10
002	56.2	----	--
011	53.6	----	--
101	53.4	53±3	20
110	43.0	44±2	20
012	41.3	43±2	20
102	41.2	----	--
111	40.2	----	--
112	32.6	----	--
020	30.5	----	--
200	30.4	----	--

TABLE III

THEORETICAL AND OBSERVED ANGLES BETWEEN DIFFRACTION PLANES

PLANES	ANGLE	$ANGLE_{measured}$
(010), (101)	90^{o}	89^{o}
(001), (100)	90^{o}	89^{o}
(110), (012)	61.5^{o}	61.5^{o}

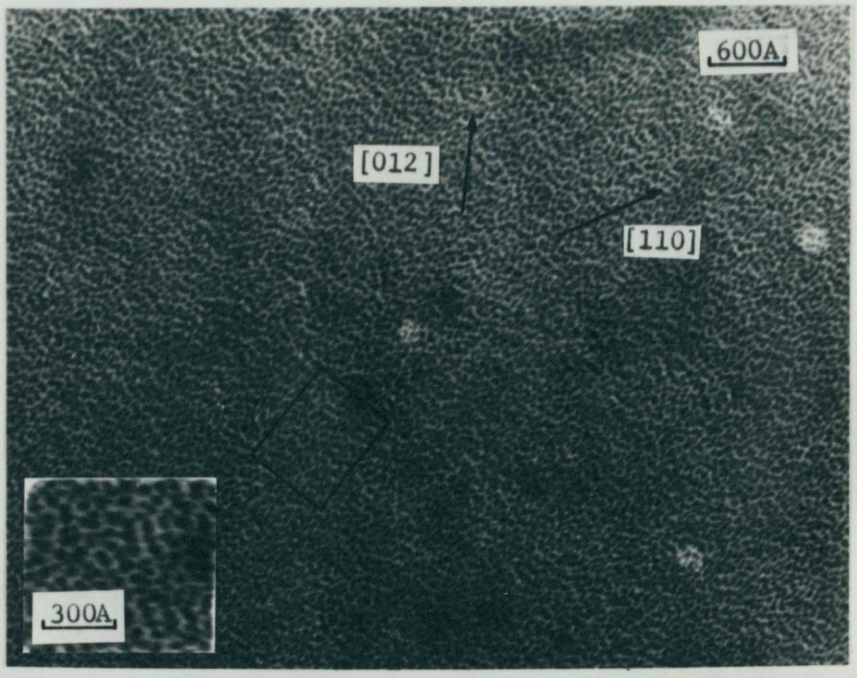

Figure 8. Photograph of electron micrograph area from which the optical diffraction pattern in Figure 9 was obtained with the insert showing the mask area. The arrows indicate the [110] and [012] directions.

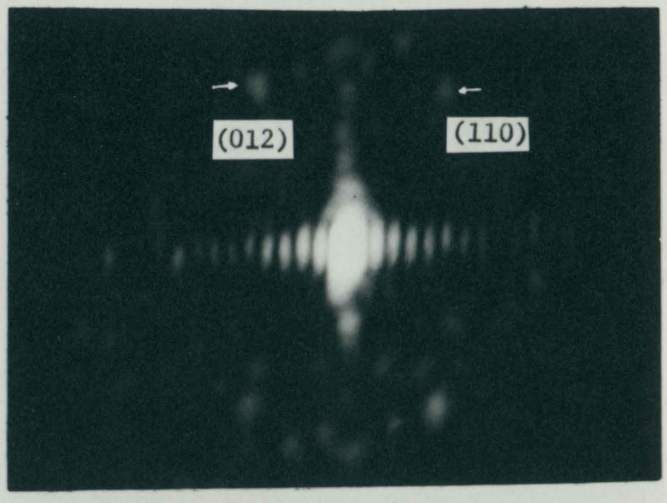

Figure 9. Photograph of optical diffraction pattern of β-Lactoglobulin showing the diffraction maxima generated by the (110) and (012) planes. Magnification 3.3X

pattern obtained from the insert area in Figure 8. The optical diffraction pattern has been rotated into the image using the diffraction spots arising from the mask dimensions to indicate the direction of the repeating structure giving rise to the diffraction spots associated with the repeat structure of β-Lactoglobulin. All the optical diffraction patterns recorded have been rotated into their respective images.

Figure 10 is a photograph showing the (001) and (100) planes for β-Lactoglobulin crystal surface. The surface appears to be both structured and periodic. The magnification of the image is 162,000X and the magnification of the insert is 324,000X. Figure 11 shows a photograph of the optical diffraction pattern associated with the mask area of the insert in Figure 10.

Figure 12 shows a photograph of the image from which the optical diffraction pattern in Figure 13 was generated. The magnification of the image is 143,000X and the insert 286,000X. This image shows the (010) and (101) planes for β-Lactoglobulin. The image appears to be structured and periodic.

Figure 14 and 15 are, respectively, an image showing the (110) plane of β-Lactoglobulin and the optical diffraction pattern generated from this image. The magnification of the image is 168,000X and the insert is 336,000X. The image appears to have structure in it, however, the density variation is great enough to generate a diffraction pattern which is difficult to interpret.

Because of the type of sample preparation only a limited number

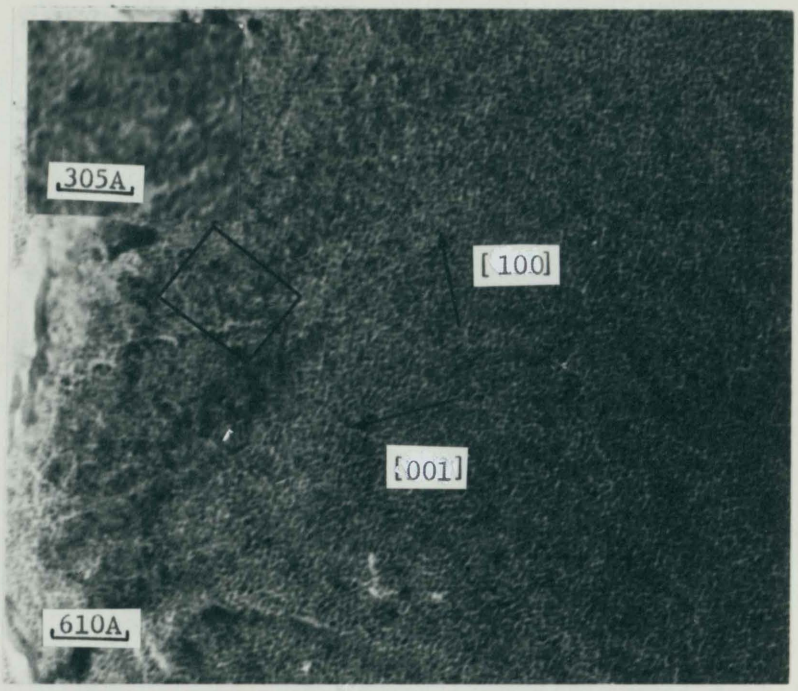

Figure 10. Photograph of the electron micrograph area from which the optical diffraction pattern in Figure 11 was obtained with the insert showing the mask area. The arrows indicate the [001] and the [100] directions.

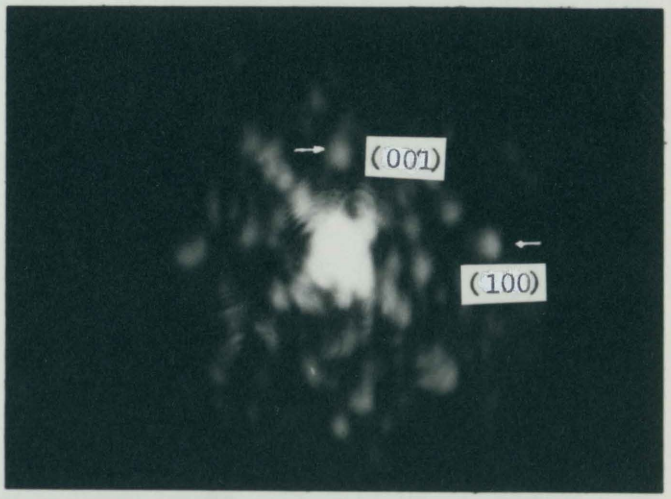

Figure 11. Photograph of optical diffraction pattern of β-Lactoglobulin showing the diffraction maxima generated by the (001) and (100) planes. Magnification 3.3X.

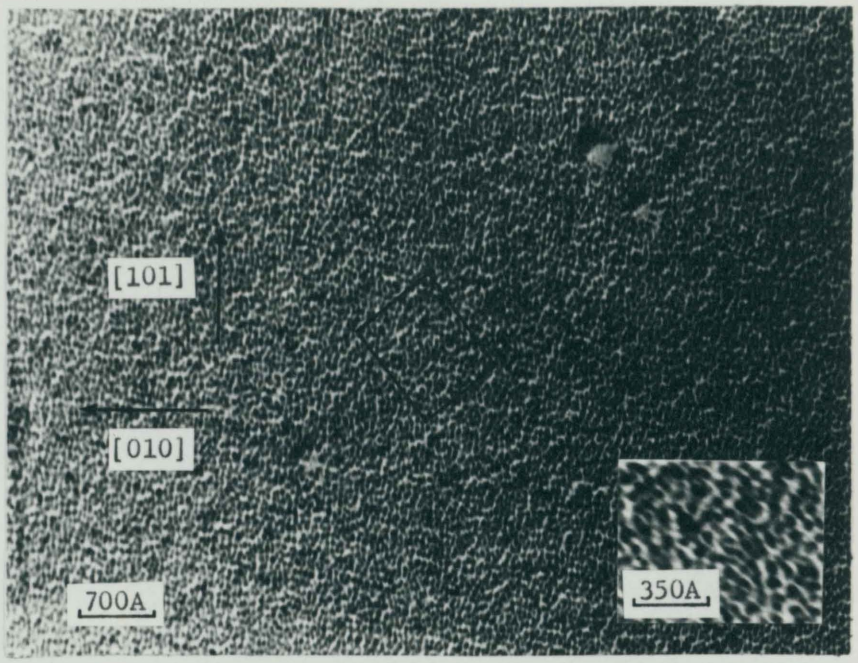

Figure 12. Photograph of the electron micrograph area from which the optical diffraction pattern in Figure 13 was obtained with the insert showing the mask area. The arrows indicate the [010] and [101] directions.

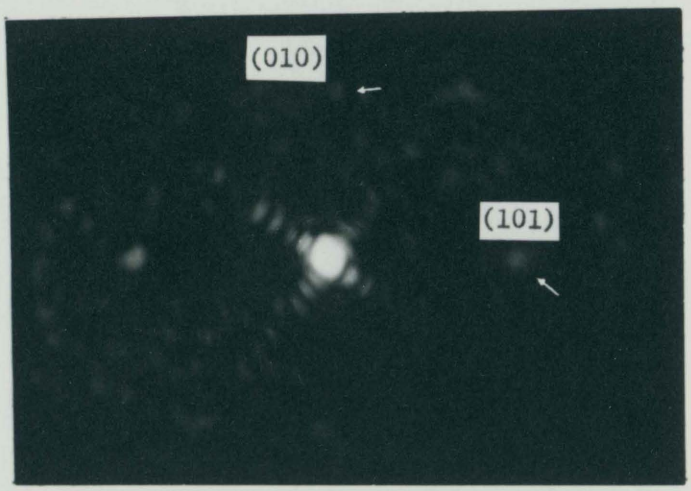

Figure 13. Photograph of optical diffraction pattern of β-Lactoglobulin showing the diffraction maxima generated by the (010) and (101) planes. Magnification 3.3X.

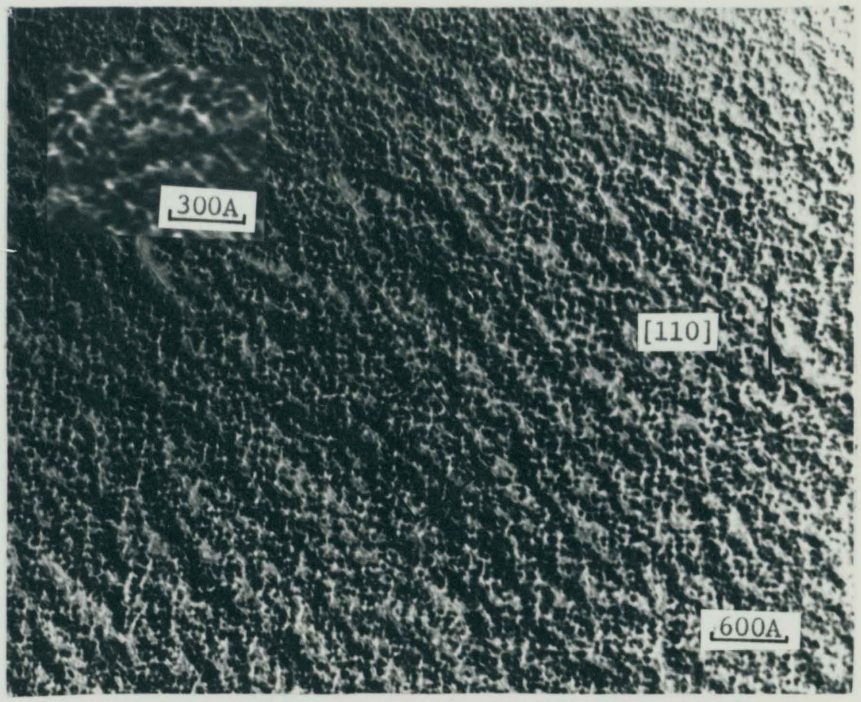

Figure 14. Photograph of the electron micrograph area from which the optical diffraction pattern in Figure 15 was obtained with the insert showing the mask area. The arrow indicates the [110] direction.

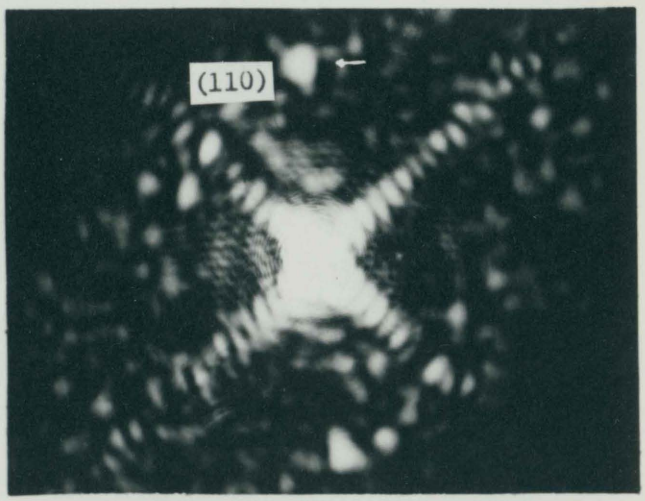

Figure 15. Photograph of optical diffraction pattern of β-Lactoglobulin showing the diffraction maxima generated by the (110) plane. Magnification 3.3X.

of diffraction patterns were obtained. The number was not sufficient to allow the combination of diffraction patterns with identical periodicities. However, since the structure of β-Lactoglobulin is known from X-ray diffraction analysis, the optical diffraction patterns can be used to determine the section of the three dimensional unit cell structure from which these diffraction patterns could be generated. The resolution of the replica will limit the number of possible planes, so that it is fairly easy to write down all the plane spacings and the angular relations. One then accepts those planes which are consistent with the known spacings and eliminates the others for any particular diffraction pattern. This process is repeated for each optical diffraction pattern. With three different two dimensional diffraction patterns, the unit cell structure can be reconstructed.

CHAPTER VI

DISCUSSION OF THE RESULTS

I. DATA ANALYSIS

Several problems had to be overcome in sample preparation. The electron images images obtained were not of as high quality as Dawson's (16). Since the crystalline material was not freshly cleaved, it is possible that there may have been contaminants on the surface of these crystals. In addition, there is some uncertainty in the lattice parameters because of the probable reduction in water content for the crystalline material exposed to the vacuum environment.

Both the density and spacing variation can lead to errors in the unit cell structure determination. This effect occurs because the optical diffraction method of analysis responds to all variations in the object without discriminating between them. The result is that the spacing between the zero order diffraction spot and the diffraction spot of interest will represent the reciprocal of an average spacing. The variations in the electron micrographs resulted in optical diffraction patterns which exhibited a great deal of noise or background intensity. The diffraction maxima were not as strong as they could have been, both because of the variation in the repeating structure and density and because of the limited number of scattering objects.

Because of the limited number of scattering objects, the elongation values ranged from 5% to 10% of the experimentally determined lattice parameters. No difficulties arose, related to elongation, concerning the selection of one plane as opposed to another for a particular Miller index because of the limited number of planes possible.

II. CONCLUSION

Electron micrographs and optical diffraction patterns were recorded which were found to be consistent with the structure of β-Lactoglobulin as determined by X-ray diffraction analysis. The unit cell dimensions were determined to be a = 58±4Å, b = 59±3Å and c = 102±12Å.

Sample preparation remains the most difficult problem. In the replica technique, the use of freshly cleaved crystals would probably reduce some of the problems.

The unit cell structure determination of a material whose periodicity is destroyed when it is removed from its native environment would be an interesting and challenging project. The development of a technique for unit cell structure determination of a material which has been embedded and sectioned would be useful information because it would provide a means of determining the three dimensional structure for some materials whose unit cell structure cannot be determined by other means. In addition, in ideal cases, it may be possible to determine the molecular weight of the material.

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