LBL - 24477

MASTER

**IMPROVED METHODS FOR HIGH
RESOLUTION ELECTRON MICROSCOPY**

LBL--24477

DE88 005731

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April 1987

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This work was supported by the U. S. Department of Energy under Contract
Number DE-AC03-76SF00098

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ABSTRACT

Existing methods of making support films for high resolution transmission electron microscopy are investigated and novel methods are developed. Existing methods of fabricating fenestrated, metal reinforced specimen supports (microgrids) are evaluated for their potential to reduce beam induced movement of monolamellar crystals of $C_{44}H_{90}$ paraffin supported on thin carbon films. Improved methods of producing hydrophobic carbon films by vacuum evaporation, and improved methods of depositing well ordered monolamellar paraffin crystals on carbon films are developed. A novel technique for vacuum evaporation of metals is described which is used to reinforce microgrids. A technique is also developed to bond thin carbon films to microgrids with a polymer bonding agent. Unique biochemical methods are described to accomplish site specific covalent modification of membrane proteins. Protocols are given which covalently convert the carboxy terminus of papain cleaved bacteriorhodopsin to a free thiol.

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ACKNOWLEDGEMENTS

Perhaps it is fortunate that the hour is so late. Were I to acknowledge adequately and completely all those who have influenced and enriched my life during my years at Berkeley this document might be as long as the first Chapter of this thesis.

The major portion of such a chapter would be totally devoted to my wife Sally, as am I. Had I worked as hard and endured as much in the pursuit of my degree as she has in supporting me morally, financially, and in a hundred other ways, I would have finished years ago. Words of thanks on paper are not enough. But then there are other ways to show ones gratitude. I love her.

To my thesis adviser, Bob Glaeser I will forever be indebted for steadfast support and profound intellectual influence. I came to Berkeley to become an electron microscopist and it was my enormous good fortune to find the best in the world under whom to study. Together we have sailed stormy, uncharted waters. When I sailed my ship onto the rocks of chemical labeling Bob rescued me. When adrift in the Sargasso Sea of specimen supports, Bob gave direction with substantive ideas which became essential to the thesis. On a personal level I am particularly grateful for Bob for tolerating me when I was occasionally obtuse and impossible to work with. There were times when I should have been thrown out of Bob's office on my ear, but was not.

I am grateful to Alex Nichols and to Rudy Wenk for reading my thesis, and as well to Alex for moral support when I failed my french exam, and in the final days of running the thesis gauntlet.

I am greatly indebted to David Foster for his creative and elegant thinking which now is embodied in the chemical labeling experiments which comprise the second half of the thesis. David taught me about biochemistry in the real world, and about the meaning of excellence and expertise in biochemical methods. All this and good conversation to boot over many a lunch at Juan's Place.

To my work family Ken, Bing, Pete, Tom, Thomas, Paula, and Romy, I can only say that it's a rich man who works with such quality people. Special thanks to Ken and Bing who have always given so readily and so willingly of their time and their expertise, not only to me but to generations of graduate students. Along with Bob they set the highest standards to which to aspire. There is no way to repay them; they will have to settle for my enduring professional respect and personal friendship. Regarding the completion of the thesis, I am particularly indebted to Pete Walian and Ken Downing for being there in my hour of greatest need. When one has such friends, there is hope even when there is no hope.

Finally, I wish to thank my Mom, brothers John and Will, and the rest of the best family in the world for immeasurable moral support, often at their expense over long distance phone lines. Along with my wife, they are truly the bedrock on which my life rests.

CHAPTER 1 - INTRODUCTION

In 1935, Marton placed a shred of a leaf in the beam of Knoll and Ruska's newly constructed cathode ray microscope, and produced the first transmission electron micrograph of a biological specimen (Mulvey 1962). The image which they obtained revealed nothing new about the structure of leaves, but with this humble experiment, the science of biological electron microscopy was born. In the 50 years since, the electron microscope has contributed enormously to our understanding of the ultrastructure and organization of living things. None of this progress has been easily achieved. Schematic diagrams of cells and organelles, which now appear in high school biology texts and can be intuitively grasped by the bright student in a few lessons, represent the residue of countless hours of painstaking, careful experimentation and innumerable failed experiments. Scientific careers have been expended in pursuit of unattainable objectives and in defense of what proved to be plausible but incorrect hypotheses. What remains are hard fought and precious tenets of structural biology such as the lipid bilayer model of the biological membrane which seem, in the light of hindsight, so elegant in their simplicity.

My own introduction to this business came about at the controls of a 1960's vintage SEM, while scanning the surface of cultured human cells for evidence of viral (Swine Flu) infection. At 50,000X magnification, the virus buds for which I searched were only dimly visible as nondescript white blips; just at the resolution limit of our microscope. Like Knoll and Ruska's first images, mine as well were uninterpretable; noisy and full of electronic artifact. I found myself caught in the same grip of excitement and frustration which has been experienced by so many of my predecessors in this challenging science. What indeed, I wondered, might one see if only the magnification knob had a few more clicks beyond 100,000. This is the stuff of which microscopists are made.

I had wandered unknowingly into a frontier, not only of electron microscopy, but of structural biology in general. As a physicist, and a materials oriented electron microscopist, I was vaguely aware that the electron microscope had been of great importance in determining the ultrastructure of cells. It is somewhat ironic however that most of what has been learned about living cells by using the electron microscope has to do not with the outside of the cell but with the interior. As fundamentally important as this knowledge is, it is only a part of the whole story. The structural details which were the object of my maiden excursion into biological electron microscopy reside on and within the cells' outer membrane; which is often the site of specific receptor molecules which are recognized by the infecting virus. In general, the structure of the cell membrane is as crucial to a total understanding of the molecular basis of life as is knowledge of its internal organization. In the years since I have been working to develop methods capable of revealing the structure of the membrane and its component parts. This is the ultimate purpose of the work which is described in this thesis.

The thesis is divided into two parts; a reflection of our broad commitment to the singular objective of developing methods to determine the structure of integral membrane proteins. At the present time prospects for this type of research seem to be more or less equally divided between X-ray diffraction and electron crystallography. We do not know at this time which if either of these techniques will in whole or part ultimately provide the structural information which we are seeking. One does what one feels will achieve the aims of this kind of research and often the effort, however well defined and single minded unexpectedly bifurcates. Whenever some branch of crystallography ultimately reveals the structure of membrane proteins it will probably be by one of the paths shown below. The sections of the thesis are classified accordingly.

Resolution Limits - Beam Induced Movement

Before the invention of the light microscope by Leeuwenhook, man's ability to probe the structure of the natural world was limited to the observation of objects having a minimum dimension of about 0.2 mm; the resolution limit of the unaided human eye. ¹

Visible light microscopy is diffraction limited to a resolution of about 0.2 microns; of the order of the wavelength of light. The situation for electron microscopy is somewhat different. Although 100 KEV electrons have a relativistic wavelength of only .037

¹In the first compound optical microscopes, a tiny, roughly spherical glass bead was polished flat on one side and caused to form a primary image which was then further magnified by a second (ocular) lens. In fact the use of a single relatively weak lens or magnifying glass to allow the magnification of objects by allowing the lens of the eye to directly image objects at a distance considerably closer to the eye than the normal near point undoubtedly preceded the invention of the compound microscope. Thus it is probably not strictly true that the resolution limit was reduced from that of the unaided eye quite so abruptly as one would be led to conclude had the compound microscope been developed in one fell swoop. In fact a modern well corrected hand lens such as a Hastings triplet can easily achieve a useful magnification of as much as 30 diameters in a single optical stage. For a detailed discussion of these issues see Lacey (1986)

Angstroms, aberrations of state of the art magnetic electron lenses limit the numerical aperture to such a small value ($\leq 10^{-2}$ rad) that the resulting diffraction limit is $\approx 100 \lambda$ or about 3.5 Å. At the present time, instrumental limitations are not relevant to the question of resolution for objects of biological interest. In the case of such specimens, a critical distinction must be made between materials which are resistant to radiation and those which are not. For the former class of specimens, resolution approaching the theoretical resolving power of commercial microscopes has been achieved. For the latter class of beam sensitive materials which unfortunately includes virtually all biological macromolecules, the problem of radiation damage (and to a lesser extent the inherently low contrast of such materials) precludes the direct imaging of individual molecules.

The various processes by which energy is deposited and subsequently dissipated in radiation sensitive materials, and the manner in which these phenomena contribute to the destruction of the original order have been recently reviewed by Glaeser (1979-b).

The depressing litany of such mechanisms will not be repeated here except to say that many of the intuitively reasonable chemical events which might be imagined to occur in response to intense levels of ionizing radiation have in fact been documented to take place.

Interactions of the beam with valence shell electrons predominate; a fact which has been invoked to account for the anomalous radiation insensitivity of certain aromatic compounds which are characterized by relatively delocalized resonance orbitals. With the single significant exception of reduced specimen temperature, no methods which do not themselves alter the chemical structure of the specimen, have been discovered which can confer radiation resistance upon beam sensitive specimens.

To date there has been found only a single recourse with which to resolve the severe limitation imposed by the problem of radiation damage; the use of statistically averaged low dose images of 2-D crystalline arrays.

For electron energies in the range of 100keV, the critical exposures for organic materials range from typical values of $5 \text{ electron} / \text{\AA}^2$ for most materials (including virtually all of biological interest) to as much a $60,000 \text{ e} / \text{\AA}^2$ for some pure aromatic compounds. Since it is necessary to record images of beam sensitive materials at levels of exposure not greatly in excess of N_{cr} , problems arise in the acquisition of high resolution images. Even when the most sensitive photographic emulsions are used to record images, at these low exposures the images lack visually interpretable statistical definition. This effectively precludes imaging of individual molecules and leaves as the only recourse the averaging of multiple images as a means of recovering the structure of the molecule.

Fortunately, there exist in some instances two dimensional arrays of biological macromolecules of sufficient crystallinity to greatly simplify the computation of such an averaged structure from a single relatively low magnification image of an array of molecules. Were it not for the existence of such specimens, and the methods of averaging which can be applied to corresponding low dose images prospects for molecular resolution of biological materials would be grim indeed in view of the limitations arising from the problem of radiation damage.

For an image to be visually interpretable to a point to point resolution of d the usual relationship among the relevant parameters of contrast (C), and the exposure or electron dose (n) is known as the Rose equation:

$$d = \frac{1}{C} \frac{5}{\sqrt{n}}$$

This is an empirical relationship which expresses the intuitively reasonable inverse dependencies of the threshold of visual detectability of a statistically noisy image on; 1). the

specimen contrast (C) - defined as the difference between the intensities of a uniform disc on a uniform background divided by the average of the intensities - and; 2). the exposure or electron dose (n) which is presumed to contain random fluctuations having the usual statistical dependence on the square root of the number of independent observations or events. The Rose equation can be used to obtain an estimate of the number of molecules which must be average in order to obtain a given resolution in the averaged image. For an image containing N presumably identical individual molecules the equation becomes:

$$d = \frac{1}{C} \frac{5}{\sqrt{nN}}$$

Typical values of C and n for biological materials are:

$$C = .01; n = \frac{1e^-}{\text{\AA}^2}, \quad \text{so that:}$$

$$\text{For } d \approx 7 \text{ \AA}, N = 5000$$

One of the earliest applications of the averaging of periodic images to molecular biology was accomplished by DeRosier and Klug (1968) who employed conventional Fourier methods to extract a statistically averaged image (in this case the three dimensional structure of the helical tail of a bacteriophage) from which noise had been removed by spatial filtering. In this earliest work, images were limited by random structural variations in stained specimens rather than noise per se. Later work by Kuo and Glaeser (1975), and Unwin and Henderson (1975) involved images which were truly shot noise limited.

In the classic work of Unwin and Henderson (1975), a high resolution (7 Å) image of the purple membrane (containing the protein bacteriorhodopsin) was obtained. This

represented the emergence of the now relatively routine electron microscopical technique of electron crystallography in which intensity data from electron diffraction patterns obtained in the TEM are combined in a Fourier reconstruction with phases derived from computer processed low dose images.

More recently, real space methods of correlation averaging have been employed by Van Heel and Frank (1981) with some success to deal with specimens in which crystallinity is absent or poorly defined.

The work of Unwin and Henderson seemed to bring the science of biological electron microscopy to the very brink of truly molecular resolution in which the tertiary and secondary structure of protein molecules might be resolved. Such a capability seemed particularly auspicious at the time since among the first molecules obtained in the required form of thin two dimensional crystals were membrane proteins (like bacteriorhodopsin) which had previously been impossible to crystallize three dimensionally for analysis by x-ray diffraction.

It has now been over a decade since the elegant work of Unwin and Henderson appeared and the impending potential of electron crystallography to reveal the structure of integral membrane proteins has yet to be realized. Even the structure of bacteriorhodopsin is not yet known to a resolution significantly higher than the resolution claimed in the 1975 paper. This is despite some advances in specimen preparation and intensive efforts (some somewhat successful others controversial - see Hayward and Stroud (1981) for example) to extract more information from data which are essentially not much better than the data analysed in the original paper.

In the search for the Holy Grail of molecular resolution microscope images of biological specimens, when viewed in an overall historical perspective, ten years is not a very long time for progress to be stalled. Despite this realization however, particularly to investigators who have devoted their professional lives to the quest, this amount of time undoubtedly seems protracted. To some extent this is probably because most recently the search has taken place in the context of ever accelerating progress involving technology and scientific instrumentation, particularly computer technology. It may thus be more difficult to appreciate now than in years past, that the last of the great fundamental barriers which lie between microscopists and the ability to resolve the molecular structure of living systems have yet to be relegated to matters of passing historical interest. Thus modern technology notwithstanding, It may be fair to say that at the present time, that the science of electron microscopy needs better specimens (and specimen support films) more urgently than it needs better computers.

For several years immediately after the structure of bR was published to about 7 Å resolution, hopes were high that improvements in specimen crystallinity and refined methods of data analysis would soon bridge the gap between the resolution of data apparently present in diffraction patterns (3 Å) and the 7 Å phase data derived from images. Despite considerable progress in both areas however, the gap remains. It has been somewhat narrowed by the recent work of Hayward and Stroud (1981) and Henderson et al (1986) in which larger areas of specimen have been averaged. This has been made possible by the development of new data processing techniques, some quite elegant, to correct for instrumental aberrations, lattice distortions, and defocus ramps present in the low dose images. These improved methods reflect not only accomplishments in the area of data analysis, but have been made possible as well by the production by Henderson of specimens of purple membrane having coherent patch sizes nearly a factor of ten larger than those present in earlier specimens. The best specimens now available of purple membrane,

from which a 3.5 Å map has been obtained, are amazingly coherent considering the fact that they represent the non - covalent association of tens of thousands of molecules over in-plane dimensions which exceed the range of the forces binding neighboring unit cells together by many orders of magnitude.

It is difficult to imagine that two - dimensional specimens having significantly higher order will ever be produced. For that matter, it is questionable that the production of such super crystalline specimens would guarantee further improvements in resolution. What then are the factors preventing the acquisition of 3 Å images?

Most of the recent progress referred to above has focused on the problem of coherence - or long range order - in the specimen itself and in the image. The necessity to derive data from large coherent areas of a crystalline specimen results primarily from the low signal to noise ratio which one obtains in exposing radiation sensitive specimens at exposure levels not greatly exceeding the critical dose. Specimen quality is not however the only factor limiting the quality of the information which can be recorded in low dose images.

Judging from the presence of signal in diffraction patterns at resolutions better than 3 Å, the inability to obtain information at a similar resolution from low-dose images is not necessarily the result of insufficient specimen coherence. In fact, the missing high resolution information may simply be lost below the noise level. This raises a number of possibilities which until recently have received much less attention than issues of specimen quality. It has been known for some time that the signal present in low dose images of beam sensitive organic materials is only about 5% of the level which one might expect based on diffraction patterns and from theoretical predictions based on justifiable theoretical assumptions which include kinematical scattering, weak phase object, and isoplanatic imaging (Henderson and Glaeser 1985). The ability to record images having signal levels at

or near the theoretically predicted value (which is possible for some radiation resistant specimens) would greatly simplify data processing and improve considerably the prospect of extracting high resolution phase information from such images.

The factors potentially responsible for the severe attenuation of signal in low dose images of several beam sensitive specimens have recently been investigated in the work of Henderson and Glaeser (1985) who enumerated the following possible factors to account for the attenuation of signal: 1) The spatial and temporal coherence envelopes of the phase contrast transfer function, 2). The modulation transfer function of photographic film, 3) The non-optimum microscope transfer function at relatively low magnifications used to record low dose images, and 4) beam induced movement of the specimen or image during image recording. Partial spatial coherence was not theoretically significant at a resolution of 4 Å under the illumination conditions used to record the images which they analysed, while partial temporal coherence was estimated to reduce the predicted structure factor by about a factor of 0.5. An additional factor of the order of 0.5 was attributed to the exponential decay of the signal while recording the image.

The effects of the film and microscope MTF's (factors 2 and 3 above) were found to be more difficult to address exactly, both from a theoretical and from an experimental standpoint. The best results reported for imaging of vermiculite however, showed imaging contrast to be 26 % of theoretical. Since one would expect this best case figure to include the effects of the modulation transfer functions of both the film and the microscope, this would imply that further appreciable attenuation of the order of a factor of 5 less than the vermiculite case, as is invariably observed for beam sensitive specimens, can most reasonably be attributed to the remaining factor of beam induced movement of the specimen. It should be noted that the vermiculite result also suggests that beam induced movement of the image (due to stray fields for example) is probably not to blame either for

the poor results obtained with beam sensitive specimens. Thus Henderson and Glaeser were able to conclude that beam induced movement is responsible for attenuation of contrast in the image by at least a factor of 1/5 and in many cases is probably responsible for the total absence of detectable information in images of beam sensitive materials. The most important issue becomes, in the light of these findings, what if anything can be done in the way of improved experimental procedures to address the problem of beam induced movement.

The problem of beam induced movement is even more severe for specimens of biological importance than for more strongly diffracting beam-sensitive specimens such as paraffin because of the larger unit cell dimensions of macromolecular specimens which can reduce the signal to noise ratio of images obtained from arrays of practical size to less than unity. This phenomenon is in fact the latest in the long sequence of technical barriers which have confounded microscopists' efforts to push the resolution limit toward the realm of truly molecular dimensions for important biological specimens. At least in part, beam induced movement is the most recently recognized consequence of radiation damage, and it will have to be overcome before electron crystallography can determine the structure of specimens which are currently available and which appear to be of sufficient quality. Henderson and Glaeser discuss several possible solutions.

One possible approach involves what amounts to a continuation of work which has been going on since the science of electron crystallography was first developed. Progress has been made in the area of image processing slowly but steadily in recent years so that it is now possible to process larger areas of images than in the past. Larger areas of images contain more imaged molecules and therefore potentially improved statistical definition of the average molecule. The problem had been however that distortions were invariably present in the images of large areas which could not be dealt with utilizing existing

averaging techniques. Such image defects include those intrinsic to the specimen (lattice distortions), which are faithfully imaged along with the molecular structure, as well as systematic aberrations resulting from imaging such as pincushion and barrel distortion. Owing primarily to diligent work by Henderson and his collaborators at the MRC laboratory and elsewhere (Downing in our laboratory) methods are now available to correct for some of these systematic image defects. As a result of the application of these methods to specimens of the purple membrane - which were themselves of state of the art quality - a map of the purple membrane has been obtained which extends to a resolution of 3.5 Å. (Henderson et al 1986)

Although the averaging of larger arrays is a technical tour de force and represents real progress, it does not really address the problem of beam induced movement. To do so in earnest will require substantive modifications of experimental procedure. Henderson and Glaeser propose the following possibilities:

- 1). Averaging of images obtained with greatly reduced doses,
- 2). Exposure of the image utilizing a small illumination spot scanned in a raster, and,
- 3). Utilization of improved support films during imaging to better stabilize the specimen against beam induced movement.

The first of these strategies is not discussed in detail by Henderson and Glaeser except to say that it poses many of the same problems as the averaging of larger areas. The exposure of high resolution images of beam sensitive specimens utilizing small spot illumination has been investigated by Downing and Glaeser (1986) with some improvement in the image quality having been obtained. The third proposal, involving specimen support methodology, comprises the first part of this thesis.

For the purposes of this work, the single and essential working hypothesis has been made that beam induced movement of the specimen is a major limitation in the science of electron crystallography of beam sensitive materials. That this is the case is now fairly well recognized by workers in the field. Considerable experimental evidence supporting this assumption has just been discussed. The accumulation of additional evidence to support this assumption is not the main purpose of this research although it is quite plausible that as a result of this work such evidence may accrue. Rather, the explicit objective of this part of the thesis is to develop new experimental methods which might serve to reduce (hypothetical) beam induced motions of the specimen and thereby improve the signal to noise ratio present in high resolution low dose electron microscopic images of beam sensitive specimens. In particular, by "methods" is meant methods of specimen support which are the exclusive purview of this work.

It is the goal of this part of the thesis to optimize the properties, both chemical and mechanical, of the films used to support such specimens and to measure the effect if any on the signal obtained from the specimens under these optimal conditions. Specifically the optimization will focus on four areas:

- 1). The development of an optimum test specimen which can be reproducibly prepared and which can serve as a quantitative standard of comparison in evaluating the effectiveness of novel film preparations.
- 2). The preparation of carbon support films having optimum and highly reproducible chemical and physical properties.
- 3). The preparation, by a variety of techniques, of ultra fine mesh grids (microgrids) to support the carbon films.
- 4). The "cross term" i.e. the development of a technique to ensure that the microgrid and the carbon films are indeed in intimate contact.

Finally, we will measure the effect of this optimization of specimen support technique on the imaging capabilities of the films using as a figure of merit the $F(g)/F(0)$ ratio for the highest resolution reflection of the test specimen.

CHAPTER 2 - METHODS OF SPECIALIZED SPECIMEN PREPARATION

Microgrids

Thin perforated support films have been used by electron microscopists for many years. Most microscopists are probably familiar with the use of holey carbon films from their operational training days on the transmission electron microscope at which time many of us were taught to use the edges of small holes to correct astigmatism. Occasional holes also occur incidentally in many film producing procedures. Although the idea of imaging directly through such small holes may be quite old, the majority of film production methods designed intentionally to produce extremely holey films for use in imaging have appeared in the literature fairly recently. This may be primarily a consequence of the remarkable performance of routinely produced continuous support films - particularly carbon - which for most applications are so stable and electron transparent as to essentially obviate the need for improved technology for specimen support. Nevertheless, there is a body of literature dealing with perforated supports which have become commonly termed microgrids.

The literature which deals with microgrids has been reviewed by Baumeister (1978) who divides the techniques of microgrid fabrication into three classes:

- 1) Those based on the insertion and development of local faults in plastic films.
- 2) Those which employ the local destruction of plastic films by physical or chemical action.
- 3) Those based on the replication of perforated templates such as filters and eutectics.

Table 1 gives a summary of the techniques of microgrid production with some additional comments based on our observations and including some updated entries which have appeared in the literature since the Baumeister review was published.

Of all these techniques, the methods of Fukami et al and glycerol emulsion techniques are probably the most widely known. A generic emulsion technique for making microgrids is illustrated in Figure 1. Regarding actual practice, it may be fair to say that microgrid techniques in general are not presently in widespread use relative to more conventional support films. It bears repeating that this is primarily a tribute to the performance of conventional continuous films. It may also have to do with the only very recent recognition of beam induced movement as a major limitation in electron crystallography (Henderson and Glaeser 1985). Additional impetus may have to do with the recently recognized utility of holey films in frozen hydrated specimen preparations (Dubochet et al - 1985)

MICROGRID TECHNIQUES¹

<i>Method</i>	<i>Authors</i>	<i>Hole Size</i>	<i>Advantages - Disadvantages</i>
Electron beam writing methods	Boersch <i>et al</i> (1959) Martin and Speidel (1972)	.06 mm - 2 mm	Well defined geometries -Unsuitable for large scale production
Replica methods	DeSorbo and Cline (1970) Teschke (1973)	.1 mm - 8 mm	Well defined hole sizes -Unsuitable for large scale
Successive water droplet methods	Sjostrand (1956) Sakata (1958) Drahos and Delong (1960) Pease (1975) Williams and Glaeser (1972) *	.5 mm - 20 mm	Simple preparation -Difficult to standardize all conditions
Dew freezing method	Fukami <i>et al</i> (1972) *	.5 mm - 100 mm	Suitable for preparing large holes - Relatively troublesome
Emulsion methods	Harris (1962) Johnson and Reid (1971)	.5 mm - 10 mm	Relatively simple preparation, fairly controllable hole sizes
	Baumeister and Seredynski (1976) * Dowell (1970) Hoelke (1975) Moharir and Prakash (1975) Lunsdorf and Spiess (1986) *	.5 mm - 10 mm	
Etching methods	Moldner (1965)	.05 mm - 10 mm	Simple preparation -Hole size variation and polymorphism
	Lickfeld and Menge (1968)	.05 mm - 10 mm	
Film casting	Davison and Colquhoun (1985) *	.02 mm - .1 mm	Very flat films - Very fragile, holes may be too small

¹ Table adapted from Baumeister et al (1978) - pg 9. Updated for reprint.

* These references are given in the bibliography. For the remainder, see the bibliography in Baumeister et al (1978)

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Figure 1. Typical Microgrid Fabrication.

Microgrid fabrication usually begins with the production of fenestrated polymer films which are then reinforced with carbon or metal. Many techniques, including some of those investigated in this thesis, cast the holey plastic film from an emulsion of glycerol droplets in a volatile, non-polar solvent which contains a small amount of dissolved polymer. The emulsion is allowed to drain from a flat substrate forming a thin layer which entrains adsorbed glycerol droplets. The solvent evaporates leaving a thin plastic film which can be perforated in areas where glycerol droplets were trapped. The perforated film is then floated onto a conventional microscope grid, dried, and reinforced with carbon or metal.

Microgrid Methods Developed for this Work

Each of the various techniques of microgrid preparation present certain respective advantages and disadvantages. No particular attempt was made at the beginning of this work to rank the various techniques according to any of the numerous criteria which might be used to distinguish among them. Initially, the techniques were regarded as essentially equivalent and this is still pretty much the case. Each is reputedly capable of producing a perforated plastic film which can then be reinforced with other materials. What has emerged, however, is a single technique, which we now regard as somewhat optimal although at the same time still essentially representative. This has been the result of an evolutionary process by which the various methods were screened and selected primarily, and in some cases almost exclusively, according to the suitability of the final holey film in providing a substrate which facilitates low dose, high resolution imaging. A more or less chronological account of this screening process will be given after which it should be apparent why a particular technique has been settled upon and deemed optimum and exactly what it is about this final technique which renders it most suitable for the high resolution imaging of the test specimen. Several characteristics of the perforated films were deemed potentially consequential at the outset while other parameters were regarded as not so important. In the consequential category are: 1). hole size, 2). density of holes and 3). uniformity of hole distribution. These are not the only possible criteria of merit of course but represent those which seemed at the outset to be of *prima facie* importance in maximizing specimen support while at the same time being accessible to systematic experimental manipulation.

The following techniques of holey film preparation have been investigated during the course of this work.

- 1). Nuclepore filter replication
- 2). Dilute Formvar film casting
- 3). Ultrasonic emulsification of glycerol in Formvar/ Dichloroethylene
- 4). Preparation (2) supported on Preparation (3)
- 5). Brij 58 mediated emulsification of glycerol in Parlodion/ ethyl acetate.

Replication of nuclepore filters was the first technique employed to produce holey support films. It also proved to be the least successful. This strategy was chosen for several reasons; the most obvious being that by using commercially available polycarbonate filters one has immediately at ones disposal a predetermined range of hole sizes and distributions. In addition this approach seemed attractive because the same theme along with minor variations on it, has been utilized by a number of workers for various purposes including the production of a holey support film (Downing, personal communication). In this particular case a carbon replica, having been deposited by vacuum evaporation on the substrate filter material, was then removed by flotation in the manner in which carbon films are usually stripped from glass or mica substrates. Although Dr. Downing was at one time successful in this endeavor, neither he nor the author was able to repeat this success; despite the use of parting agents such as sodium meta-phosphate (sold under the trade name "Victawet" by Ernest Fullam Inc), and sodium chloride. Attempts were made to glue the coated filters to glass and mica to facilitate the stripping of the carbon film, but it soon became apparent that removing the carbon from the Nuclepore membrane was more of a

problem than at first anticipated. Even though stripping is quite probably not impossible, it was eventually decided to adopt a different approach.

The next generation of holey film preparation attempts was based on removal of the polycarbonate substrate (Nuclepore) from the evaporated replica film by dissolution in an organic solvent. This idea as well has precedent which includes a technique utilized routinely in Dr. Steve Hayward's laboratory at the California Department of Health (personal communication).

In Hayward's laboratory, samples of airborne particulates, which have been collected on Nuclepore filters, are carbon coated in the vacuum evaporator. Small wedges of the filter material are cut with a scalpel and placed on microscope grids which are then placed on wedges of filter paper. The paper wedges on which repose the grids are then supported on small cubes of polyurethane foam inside a shallow glass petri dish and the dish filled to the level of the paper (top of the plastic cubes) with an organic solvent capable of dissolving the filter membranes but not the plastic cubes. Carbon tetrachloride was recommended by those who routinely use the technique. The petri dish and its contents are then allowed to sit overnight in a fume hood thus allowing time for the filter material to dissolve. The result of this procedure is that the particulates originally on the plastic filter are now supported on an electron transparent film (composed primarily of carbon one would suppose) which is suitable for subsequent electron microscopic analysis of the particulate specimen. A by-product of this technique is a carbon replica of the Nuclepore filter complete with holes. If the removal of the plastic is allowed to progress a sufficient length of time, the holes are indeed holes rather than pseudo-holes (pore replicas over which remains a thin residual film of plastic) and the technique has achieved the replication of the holey filter membrane which proved to be so difficult by the previous method.

Having thus accomplished the avowed goal of generating a holey carbon film, a variety of different filter sizes were processed in this fashion and examined in the electron microscope to determine hole morphology. As a result of these experiments sufficient undesirable aspects of this particular method came to light to warrant disqualification of the technique for the experimental purposes at hand.

The production of replica films by the "solvent stripping" method had a number of drawbacks not the least of which was the considerable difficulty of achieving sufficiently complete removal of the substrate filter material to produce true holes rather than pseudo holes which predominated in many preparations. To compound this problem, it was found that commercially available Nuclepore membranes had a pore density or percentage of open area which ranged from barely adequate to clearly insufficient to facilitate high resolution imaging of specimens which themselves in most cases are apt to occupy a small fraction of the total area of the support film.

Custom filter membranes can be provided but we were advised by the manufacturer that off the shelf membranes are already close to the upper limit of percentage of open area which can be achieved by the batch manufacturing process used to produce the filters. As the filter material becomes increasingly populated with pores, the large sheets of film become too fragile to survive manipulation. This occurs well in advance of the point where random overlap of holes becomes significant in degrading the performance of the filter as regards the maximum pore size specification (personal communication with the scientific consulting staff of Nuclepore Corp.).

Yet another potentially serious problem with Nuclepore replicas was the hole morphology, which in all cases had the appearance a doughnut in projection. This was assumed to indicate that the holes were very likely surrounded by a raised rim. A distinct dark ring was

clearly visible around almost all holes in transmission electron micrographs suggesting either an increased mass of material surrounding the holes or a region of increased density. The former interpretation was consistent with the manufacturer's interpretation of this doughnut morphology which had been reported to them by other customers. Although it might be possible to verify this hypothesis by shadowing experiments, or by scanning electron microscopy, this was not done. In view of the plausibility that the holes had a raised rim and because of the previously discussed liabilities of this technique for the purposes at hand, the solution stripping phase of the project was abandoned in favor of other methods which held the promise of more uniform structure (flatness) in the potentially critical region at the periphery of the holes.

About the time that the Nuclepore replication experiments were drawing to a somewhat unsuccessful conclusion, the film casting method of Davison and Colquhoun (1985) was published. Several other techniques had been previously described which might serve adequately in lieu of the Nuclepore method as a means of producing holey formvar films. The film casting technique seemed especially appropriate at the time however since a primary alleged advantage of this new method seemed to address almost exactly what had been concluded to be the main weaknesses of nuclepore replication; the issue of film flatness. The claim was made by Davison and Colquhoun that the film casting technique was capable of producing a plastic film of exceptional flatness. Additionally, the production of a holey film could be accomplished quite easily by merely diluting the polymer solution sufficiently. Attempts to reproduce the technique of film casting were therefore undertaken with the goal in mind of eventually producing microgrid supports by this method.

Initial attempts to duplicate the production of holey formvar films by following the published protocol were unsuccessful. No films at all could be seen either on the surface of the water, or upon inspection of 400 mesh grids on which the films had presumably been

picked up. Occasionally, fenestrated remnants of what might once have been a holey film could be seen decorating the corners of a few otherwise open grid squares like tiny spider webs, but most specimens showed not even that much evidence of any film having ever spanned the windows of the grid. These discouraging results gave rise to several hypotheses to explain the failure of the technique to produce holey films; none particularly easily testable, and once again the primary hypothesis that microgrid supports might improve the information content present in low dose images remained untested pending the selection or development of a suitable technique to fabricate a quality holey film.

The claims of Davison and Colquhoun alleging to have cast holey films would eventually be corroborated; but not until perforated formvar films were produced by a different technique. The technique which first worked to produce holey films of reasonable quality in our laboratory turned out to be the method based on the emulsification of glycerol in a formvar / dichloroethane solution. Although this method has been around for along time, we had been somewhat reluctant to resort to it for reasons which hindsight has rendered rather feeble but which at the time seemed rather more persuasive. The glycerol / formvar technique had been attempted on a number of past occasions in our laboratory including once (for reasons which I have forgotten) by the author himself and the results of these attempts had been somewhat disappointing. The principal difficulty which both I and a coworker had encountered independently, and on separate occasions, dealt with achieving thorough emulsification of the glycerol in the nonpolar solvent. This had presumably been accomplished (Baumeister et al 1976) by ultrasonication but in our hands the step of ultrasonication merely caused persistent aggregation of the glycerol into buoyant, macroscopic white spheres rather than complete dispersal as claimed in the literature.

In addition, formvar films cast on glass substrates are not reputed to be particularly flat (Davison et al); nor are they particularly easy to float off with regularity. Holey films, we

assumed, might be even more difficult to remove due to their decreased mechanical integrity. On the positive side of the ledger however, is the allegation by Baumeister et al that the hole size can be tailored fairly easily by this method. Far more persuasive than that was our track record of back to back failures involving alternative methods so attempts were begun to refine our technique with the glycerol formvar method so as to get on with the business of finding out what, if any, effect microgrids might have on the limitations imposed by beam induced movement.

The problems encountered in the past with the glycerol formvar technique proved to be much less formidable than those which we had experienced with the two methods attempted previously. Two novel but relatively straightforward modifications of our technique were sufficient to advance the microgrid fabrication project to the next stage although the characteristics of the holey films which were produced at this point were not yet deemed optimum.

The first of these changes was the substitution of mica for glass as a substrate on which to cast the films. This not only solved the problem of stripping, but somewhat ameliorated our misgivings about the flatness of the films thus produced; this inasmuch as Davison et al allege films stripped from mica to be superior in consistency (and therefore conceivably flatter) than those cast on glass substrates. The second departure from conventional procedure in preparing glycerol formvar films was mechanical homogenization of the immiscible materials immediately prior to ultrasonication. This was accomplished using a Teflon piston homogenizer of the type used to resuspend pellets of membrane proteins after centrifugation and largely eliminated the white sphere phenomenon. The films produced after these methods were included in the protocol were still not as uniform in hole distribution as those illustrated in the Baumeister et al paper. They were good enough, however, to allow the next phase of the project to commence.

An apparatus was constructed to allow the use of a probe sonicator instead of the bath sonicator previously employed. The solution containing the emulsified glycerol is very volatile and would quickly evaporate if a probe sonicator were merely placed in a beaker of the solution and provision were not made to prevent free contact of the violently agitated surface of the solution with the atmosphere. The device designed to prevent evaporation consists of a teflon plug which forms a vapor-tight seal around both the sonicator probe and the rim of the vessel which contains the volatile dichloro-ethylene solution. The use of teflon was deemed advisable not only to form an adequate seal under moderate pressure, but also to prevent conduction of potentially destructive ultrasonic vibrations from the probe housing to the edge of the glass tube containing the emulsion. The device functions as intended and allows further improvement in the homogeneity of the preparations obtained. This procedure was used throughout the preliminary measurement of $F(g)/F(0)$ for paraffin.

Although the intent of the project was to use these microgrids to facilitate high resolution imaging, their very first application was not to support a specimen per se but instead another holey film! The method of Davison et al was repeated but the cast films were picked up on microgrids instead of 400 mesh copper grids as in previous attempts. The result of this hybrid procedure is shown in Fig. 2, in which the two different gauges of microgrids produced by the respective techniques can be clearly discerned. Apparently the holey films produced by the casting technique are too fragile to span the windows of a 400 mesh grid; although no mention of this potential pitfall is made in Davison's original article.

In addition to restoring our faith in the film casting method of holey film production, the fabrication of a microgrid having such a wide range of hole sizes proved useful in

Figure 2. Hybrid Microgrid

Two different gauges of fenestrated, metallized films can be seen. The larger holes were produced by a glycerol emulsion technique. This coarse mesh was then used to support a plastic film produced by dilute formvar film casting on an ultra-clean water surface. The fine mesh film, which is too fragile to be supported on conventional grids, is reinforced with gold. The coarse texture of the gold metallization layer is evident. Magnification is 10,000 X

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conducting a preliminary appraisal of the range of hole sizes with which to begin the imaging experiments. Although it is not apparent at the magnification of Fig 2, the smallest holes generated by the casting technique seem to cause the grain of the metal (in this case gold) used to reinforce the film to be more pronounced than on the edges of the larger class of holes. The reason for this is not clear. The structure and behavior of thin metal films cast by vacuum evaporation is a science in its own right and is beyond the scope of this work. What does seem fairly clear, at least from an intuitive standpoint, is that one probably would prefer to conduct preliminary imaging experiments using a microgrid having holes more in the larger size range than in the range of those in the figure which have such ragged edges. This was the choice which was made at the outset of imaging experiments with microgrids and as of the present time, we have yet to conduct any imaging experiments using films with holes as small as the smallest that can be seen in the hybrid preparation of Fig 2. At this point the production of what may well be the world's finest mesh microgrid remains somewhat more a technical tour de force rather than a useful scientific achievement. This may change at some later date, but for such a fine mesh microgrid to be of any use in imaging will probably require that the film be metalized with something other than the metals which we have surveyed as potentially useful in reinforcing microgrids.

Virtually all of the problems which we experienced with the glycerol emulsification procedure used in the fabrication of our first microgrids can be averted by using a detergent to facilitate emulsification of the glycerol; an innovation developed recently by Lunsdorf and Spiess (1986). The following procedure, which we now use exclusively to produce fenestrated polymer films for subsequent metalization, is based on the method of Lunsdorf and Spiess, although some modifications, such as the use of mica and ultrasonication have been adapted from methods which we had previously utilized to make holey formvar films. The inclusion of the detergent Brij 58 in the protocol is an elegant innovation which seems

straightforward given the benefit of hindsight. It is probable that the detergent mediates emulsification by forming inverted micelles or vesicles as shown schematically in fig 3. A microgrid produced by this method and reinforced with nichrome is shown in Fig. 4.

Figure 3. Microgrid Emulsion

The final microgrid protocol which was used in these investigations is given on the following page. It is a hybrid procedure which includes methods derived from the work of Baumeister, and Lunsdorf and Spiess; as well as innovations developed in our laboratory. While there are obviously a large number of possible variations on the basic themes of this kind of procedure, we allege with reasonable confidence, based on several years of experimentation with various methods, that this technique represents an optimized procedure. This protocol can be practiced with a minimum of complications - i.e. steps which are apt to be irreproducible or which frequently might appear not to be working at all. This is no mean accomplishment inasmuch as such complications are typical of the prior art. Regarding the holey films produced by this method, it can be said that they are typical of the best films which can be produced by the methods from which the final protocol evolved. We do not allege that the films produced by this procedure are

necessarily any better (flatter); only that the technique represents somewhat of an optimization with regard to practicality and reproducibility.

Figure 4. Typical Microgrid

The holey plastic film used as a substrate was produced by the modified method of Lunsdorf and Spiess (1986). Metallization was done with nichrome as described in Chapter 2. Note the absence of granularity in the structure of the nichrome film in comparison with the gold film shown in Figure 2. Magnification is 5,000 X.

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Microgrid Protocol

- 1). Dry a small quantity of Collodion resin (Polaron Equipment Corp.) under a 60 Watt incandescent lightbulb for 5 min. (collodion is supplied moistened with ethanol to minimize risk of explosion)
- 2). Place 90 mg of the dry resin and a teflon stirbar in a brown glass screw top bottle.
- 3). Add 30 ml of anhydrous ethyl acetate, secure the cap tightly, and stir slowly until the resin is completely dissolved (several hours should be sufficient). The cap should have a metallic seal. Avoid closures which contain materials which might be dissolved by vapors of the ethyl acetate solvent. Store the solution in the dark.
- 4). Prepare a stock solution of 87 % (Vol/Vol) aqueous glycerol ($d = 1.26 \text{ mg/ml}$).
- 5). Prepare a stock solution of 10 % (Wt / Vol) Brij 58 (Sigma) in water.
- 6). Add 300 λ of the Glycerol solution and 50 λ of the detergent solution to the 30 ml of ethyl acetate / Collodion solution.
- 7). Tightly secure the cap and shake the solution vigorously by hand for 3 minutes. The solution should become uniformly cloudy.
- 8). Pour a small portion of the cloudy emulsion into a small wide-mouth glass bottle (we use a glass scintillation vial) to a depth of several cm.

9). Tighten the lid on the bottle and suspend in a bath sonicator for 5 min. A convenient method is to insert the bottle into a slightly undersized hole in a 1 cm thick piece of polyurethane foam packing which is then used as a flotation device. If this is done correctly a violent agitation of the surface of the emulsion should be visible during the ultrasonication process.

10). To cast the holey films, immerse strips of freshly cleaved mica (Pelco) about halfway into the solution in the vial for 10 sec and withdraw slowly (3 - 5 sec).

11). Rest the mica strips on edge (wet side at the bottom) upright against a vertical support and allow to air dry. The drying time is not critical; in fact, the film can be left for several days prior to stripping, although this is probably not advisable.

12). Float the films in the usual manner on distilled water. Slow immersion vertically can often strip useable films from both sides of the mica. Immerse at 45 degrees if stripping is more difficult.

13). Gently place 300 - 400 mesh copper grids in the center of the floating films - rough side down. If the prep is particularly good (highly fenestrated) the films will be very fragile and nearly invisible. To aid in locating them on the water surface, it is possible to stain the edges with a fine tipped permanent marking pen (Pilot SC-UF) prior to stripping. This is useful and feasible but not necessary if lighting conditions are ideal. The films can also sometimes be visualized because they bear dust particles which remain stationary with respect to each other as the film slowly drifts on the water surface.

14). Pick up the grid bearing film by blotting with filter paper. We find clear newspaper wedges to be most effective since it is much slower (by a factor of 5) than No. 3 or 4 filter

paper - an advantage with the very fragile fenestrated films. Under the best of circumstances not all films will survive blotting.

15). Dry the paper-borne films in a covered petri dish overnight prior to metalizing.

16). The films may be baked at 180 degrees Centigrade for 10 minutes to facilitate hole formation. Lundorf and Spiess recommend this but it does not seem to be necessary when employing our metalization procedure.

With the development of the preceding protocol, the task of optimizing the production of fenestrated polymer films was largely completed. Attention was then focused on the next phase of microgrid fabrication.

Metalization

There is a considerable literature dealing with vacuum deposition of metal films which should seemingly contain much information of potential value to a novel application like microgrid manufacture. Two classic books have been written; one by Strong (1938) and the other by Holland (1956). These books are worth looking at even if one is not particularly concerned with the details of vacuum evaporation. They are as dense in useful information as any references ever published in experimental science. Some information on the properties (mainly electrical) of thin metal films has been published (see particularly the reference by Bond, 1954). Unfortunately, none of these references deal with fenestrated films; which are considered pathological in most industrial applications. Almost all continuous film properties which have been tabulated apply to films which are very thick relative to specimen support films.

At the risk of belaboring a point already made, it is by no means obvious that the properties of thin metal films produced by the technique of vacuum evaporation are such as to render such films comparable in utility to macroscopic metal grids which are universally presumed to have idealized (but rarely measured), properties such as high conductivity and mechanical rigidity. From an experimental standpoint, there is little recourse but to cite the obvious utility of conventional metal grids as an argument for the plausible utility of microgrids. It is in this spirit that we proceed undaunted with the metalization of holey films; holding in abeyance a plethora of theoretical misgivings pending a final decision on the utility of metalized microgrids based upon the empirical criterion of the $F(g)/F(0)$ ratio for paraffin. To do otherwise would be to do nothing, or to embark on another experimental endeavor entirely; that of characterizing the properties of metal films produced

by vacuum deposition. While this may well be a subject which merits overdue scholarly consideration, it is explicitly beyond the scope of this thesis.

It has been assumed for this set of experiments with paraffin that the choice of metal to use should be based primarily on the ease with which an apparently contiguous (i.e. uniformly electron dense) film could be deposited on the fenestrated plastic substrate film. Gold was used in preliminary experiments but was found to be very "grainy" (see Fig. 2); a disadvantage particularly evident at the periphery of the holes. Copper, which was given consideration for its high conductivity, and to match the properties of the copper grid on which the holey films are supported, was found to be unsuitable because of an obvious tendency to oxidize upon removal from the vacuum. Experiments using nichrome showed promise from the beginning, based on the following criteria (more or less in order of importance): absence of pronounced graininess, ease of evaporation from tungsten filaments, apparent resistance to oxidation, and availability. Nichrome was found to be difficult to evaporate from commercial tungsten baskets in a reproducible manner, but this was deemed tolerable in initial experiments because of the obvious superiority of nichrome film ultrastructure relative to materials investigated previously. The problems of evaporation technique encountered when using nichrome were eventually solved with invention of the method of metalization which will be described later in this chapter. Like so many aspects of microgrid technology, the choice of metal has the potential complexity to consume several years of work if conducted thoroughly. In the interests of pragmatism and expediency, nichrome films were deemed to be of sufficient quality to allow the investigation of microgrids to advance to the next stage. Although arrived at independently the choice of nichrome to reinforce microgrids agrees with that made independently at the MRC laboratories in England (private communication with Peter Tullock).

Preliminary work using nichrome to metalize perforated formvar films led to the identification of distinct problems in the production of microgrid supports by vacuum deposition of metal on fenestrated formvar. They are :

- 1) Local flatness (on the scale of individual metal grains)
- 2) Global flatness (warping)
- 3) Meso-scale flatness (sagging into the grid squares)
- 4) Heat sensitivity of the plastic substrate.
- 5) Inconsistent evaporation conditions.

Assuming the choice of Nichrome to be optimum with respect to grain size in projection (a reasonable assumption based on the metals screened) , the problem of the ultrastructure of the metal film in the third dimension (thickness) may be unsolvable. It is certainly the most difficult of the problems just enumerated and could represent a potentially fatal limitation of microgrid technology at very high resolution.

Brute force attempts to mechanically flatten the microgrid in a press between wafers of freshly cleaved mica were unsuccessful. This technique produced a metal film unremovably bonded to the mica, and a badly deformed naked copper grid. Surprisingly, the mica was permanently deformed in this procedure by the copper grid bars.

Hypothetically, the problem of warping was thought to be related to the composite nature of microgrids, and to heat absorption during metalization; both by the copper, and by the plastic substrate. It was suspected that the microgrids might be undergoing differential thermal expansion during manufacture (and perhaps in the EM), and thereby undergoing a bimetal strip mode of global deformation. In fact, many if not all of points 1-5 might be coupled by the technique used to metalize the polymer films.

Consequently, an entirely new technique of metalizing perforated plastic films was developed. The technique employs the apparatus shown in fig 5.

This metalizing apparatus offers a number of advantages over the conventional tungsten basket technique of vacuum evaporation which was used previously in our microgrid work. Collectively these innovations bear directly on the quality of the films which the device is able to produce and their suitability as microgrid support films. The advantages afforded by the apparatus are:

- 1). By using multiple source arrays in mirror plane symmetrical configurations, the device makes possible the metalization of substrate films on opposite sides simultaneously. Different evaporation rates or different evaporant materials might be deposited on each side to obtain special structural characteristics (see discussion of point 3 above).
- 2). Sources used in the device have a novel design which allows them to be mass produced. This results in electrical characteristics sufficiently uniform to allow parallel arrays to be operated from a single power supply while maintaining nearly identical evaporant loading and emission characteristics. The design of the sources will be explained in detail subsequently.
- 3). The novel source design has a lower filament temperature (and total thermal emissivity) at the onset of metal vaporization due to increased thermal contact between tungsten and evaporant charge. This significantly reduces the potential for heat absorption by the target prior to metal deposition.

Figure 5. Legend on Next Page

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Figure 5. Vacuum Metallization Device.

Fenestrated plastic films supported on 400 mesh grids are metalized with nichrome using this rotary evaporation apparatus. Grids are placed in holes in a wheel which transports them rapidly into and out of a chamber which contains a mirror-plane symmetrical array of filaments. The wheel is driven by a spring wound mechanical motor at 100 RPM. Metallization takes about 30 sec. A projected 20 X image of one of the filaments is monitored visually to determine onset of metal evaporation.

- 4). Target heating is also reduced by shielding prior to evaporant vaporization; and by target exposure to the source in a 25 % periodic duty cycle.
- 5). Film uniformity is improved by continuous rotary shadowing during metal deposition.
- 6). Evaporant wetting of the filament can be detected and visually monitored by means of an optical projection system having a magnification of 20X.

The filaments, which load in cartridge fashion into the metalization device, are shown in fig 6. Each filament consists of 3 strands of 10 mil tungsten (annealed grade) wire and a single strand of 10 mil nichrome (evaporant) wire which are twisted together in about a 1/8 inch pitch (the trade term for this is 1/8 " lay). Each strand is 3.6 " in length before twisting. Electrical contact to the filaments as well as mechanical bonding for twisting purposes is achieved by inserting the ends of the untwisted wires in a 1/2" long sleeve of 1/16" outside diameter half-hard copper tubing (K D brand - available in the U. S. in most hobby shops in 12" lengths). The end sleeves are crimped three times along their length using a Buchanan Model C - 24 crimping tool, modified to accept the small diameter tubing. Shown in Figure 7 is paraphernalia used in filament fabrication.

Figure 6. Filaments

For use in the metallization apparatus of Figure 5. 3 strands of 10 mil tungsten, 1 strand of 10 mil nichrome.

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Figure 7

Paraphernalia for filament fabrication: (a) 1/16 " copper tubing, (b) Tubing shear (c) Hand drill for twisting, (d) Buchanan crimping tool (modified), (e) Vise for twisting (f) Completed filaments, (g) Wire length gauge, (h) Vinyl tubing section for pre-twisting (i) # 55 twist drill for reaming cut tubing sleeves, (j) Nichrome wires, (k) Tungsten wires

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This apparatus enables us to coat highly fenestrated parlodion films on 400 mesh tabbed copper grids without pre-coating with carbon. Grids left inside the coating chamber show severe damage from heating while those left outside prior to evaporation survive intact. Films to be used as microgrids are usually given two coats of metal. In applying more than two coats, one runs the risk of the metal film flaking off of the copper supporting grid; probably due to the development of large internal stresses which overwhelm the adhesive forces which bond the metal film to the grid. The metalized nichrome films produced in this manner seem to represent an almost complete solution to the problems designated 2, 4, and 5. in the preceeding discussion of microgrid non-flatness. Inasmuch as problem 1 is conceded to be outside the realm of experimental manipulation, this leaves the problem of sagging (3) the only obstacle remaining before microgrid optimization can be reasonably alleged. Unfortunately, this problem is quite serious since it bears directly on the crucial issue of carbon film to microgrid bonding. A discussion of our attempts to deal with this issue is best discussed in that context .

Paraffin Imaging

As a specimen with which to quantitate the imaging of radiation sensitive crystalline materials we have chosen to use the aliphatic compound $C_{44}H_{90}$. This is consistent with the choice of Henderson and Glaeser (1985), Downing and Glaeser (1986), Zemlin et al (1985), and a number of others who have done this type of work recently. Paraffins in this molecular weight range have received considerable attention as a test specimen in the study of high resolution low-dose imaging for two reasons. Both qualitatively and quantitatively, they exhibit radiation damage similar to that observed with more important biological materials such as the lipid constituents of biological membranes. Additionally, these materials can form highly coherent monolamellar crystals which are ideally suited to high resolution imaging and electron diffraction studies in the TEM.

Most of the credit for the now widespread use of these interesting and useful specimens belongs to Dr. Douglas Dorset who has been studying their properties, primarily by means of electron diffraction, for over a decade. Dorset (1985) gives a thorough review of the subject.

The techniques employed in this work, in the imaging studies of Henderson and Glaeser, and in the work of Downing all derive from previous work by Dorset and involve the deposition of paraffin crystals from solution directly on carbon films. The technique is straightforward although, despite its simplicity, it is not clear from the literature to whom or to which particular reference to attribute its invention. The reader determined to uncover its origin is referred to the review article by Dorset (1985) which contains an extensive bibliography.

Our version of the solution growth protocol goes like this:

- 1). Prepare a saturated solution of $C_{44}H_{90}$ in spectrophotometric grade heptane at room temp.
- 2). Dilute 500 λ of the saturated solution with 1 ml of heptane.
- 3). On a carbon coated microscope grid, place 5 λ of the diluted solution and allow the heptane to evaporate.

This completes the specimen preparation.

The abundance, and to some extent the thickness of the crystals which result from the evaporation of the solvent are determined by the degree of dilution of the saturated stock solution and the amount of the solution applied to the grid. The quality of the crystals is a more complicated issue. Given carbon films having the required properties, an educated guess about what dilution to use, and some persistence based on the prior knowledge that the technique will probably work eventually, one can usually produce in this manner (at least in cases of paraffins having even chain lengths of about 44 carbons), monolamellar crystals of the type suitable for direct imaging. In the process one frequently also produces a variety of crystal polymorphs which are not suitable for imaging. Nevertheless, the incidence of formation of crystals having the required properties is tolerable. In the context of the electron crystallography of biological materials, which is replete with tedious specimen preparations which have much less probability of success, the Dorset technique can be regarded as very easy; bordering on trivial.

When the Dorset technique works properly it is a study in simplicity. When it does not it is usually not possible to understand exactly why although the urge to speculate is usually irresistible. Speculation typically spawns several hastily conceived modifications of the unsuccessful protocol in an attempt to correct the hypothetical problem after which eventually (particularly if different carbon films are used) the technique suddenly works for reasons as inscrutable as those responsible for the original failure.

A more substantive criticism of the Dorset technique can be made with regard to its use in the investigation of support films properties as reported in this thesis. In most preparations the drop of solution applied to the grid can clearly be seen to wet both sides of the grid and there is no easy way to detect whether a particular crystal which one is examining resides on the front or the back side of the support film. Since these surfaces are potentially quite different (see section on hydrophobicity of carbon films), this poses a problem in formulating ideas about the nature of specimen - support interactions. A second criticism which may very well be related to the first one, is that the Dorset technique does not seem to work on microgrids.

For reasons which may forever remain the subject of speculation, the formation of monolamellar crystals on carbon films, using the Dorset technique, seems to be disrupted by the presence of a metallized microgrid. Had this not been the case (or had we been more readily inclined to accept this as fact), the preliminary work involving microgrids might have been completed more quickly. Exhaustive attempts to locate monolamellar crystals overlying holes in microgrid supports were extremely frustrating and virtually unsuccessful whenever the Dorset technique was employed to prepare the crystals.

It would be difficult to document this phenomenon quantitatively because to do so would require the inspection of the entire grid surface to determine the population density of

monolamellar crystals. This would have to include not only the portion of the film which overlies the holes, but also the region of the support film (which was quite significant in early microgrids) which is obscured behind opaque metalized regions. The former task would be difficult, particularly under conditions of low dose illumination. There is of course no feasible way to perform the latter assay in the transmission electron microscope. Were it possible, we suspect that one would find that a large portion of the paraffin originally applied to the grid in solution would probably be found in thick deposits behind metalized regions of the microgrid. This conclusion, although not directly testable, can be reasonably inferred from the repeated observation, when using the Dorset technique on microgrids, that the paraffin is not present in areas overlying holes to any extent reasonably approaching what one would expect to see based on the distribution of crystals which one would produce with the same specimen applied to a conventional 400 mesh carbon coated grid. The problems with the Dorset technique with microgrids were so severe and persistent that it was necessary to devise an entirely different method of applying the paraffin specimen to the microgrid which would factor out hypothetical interactions with the support film.

The Taylor Technique

For the specific purpose of preparing monolamellar paraffin crystals on microgrid supports, it was necessary to devise a novel method different from the direct application technique of Dorset. Although this new technique has since been refined to the point that it consistently produces better specimens than the Dorset technique, competition with prior methods was not what led to its development. The new method (hereinafter referred to as the Taylor technique) was necessitated by the inability of the Dorset technique to produce quality specimens of paraffin on metalized microgrids. A favorite theory to account for the shortcomings of direct application of the specimen to the microgrid was based on the

tendency (when employing the Dorset technique) of paraffin to form thick deposits adjacent to metalized grid areas and little or no paraffin crystals in "open" regions of the grid. This seemed to suggest that some interaction of the metal substrate with the evaporating solvent might be responsible for the consistently biased distribution of crystalline deposits. Any number of plausible mechanisms (different cooling or evaporation rates for example) might be invoked to lend more substance to such speculations but none of these ideas seemed particularly useful or easily testable except to devise a control experiment in which the metal grid is "factored out " of the process of crystal formation entirely. Thus a new technique was devised in which the solvent containing the paraffin is applied directly to the carbon film before the film is applied to the grid.

To accomplish this it was decided to apply the paraffin solution to the carbon film while the latter is still on the mica on which it was initially deposited by vacuum evaporation. This procedure has the added advantage that in so doing one produces crystals on only a single carbon surface. For this, and other reasons which shall soon be apparent, this new method is advantageous and offers considerably better prospects for control of the capricious process of crystallization.

The primary reason to apply the paraffin crystals to the carbon film before application of the film to the microgrid was to avoid suspected, hypothetical influences which the grid might exert on the process of crystal formation. In addition, the film is more accessible to manipulation at this stage and this was exploited in the design of the new technique to effect better control over the conditions under which crystals are formed.

Phase changes generally occur, or are usually deliberately brought about, under conditions characterized by drastic and fairly rapid changes in temperature, pressure or the like; considerably outside the equilibrium realm where theoretical concepts can necessarily be

accorded their full significance. There may be no practical way to avoid such an excursion into the thermodynamic netherworld but as a general principle it is probably a good idea, if one is to maximize ones chances of exerting control (or understanding) over natural processes, to conduct experiments as close to equilibrium as possible. In its mildest form, this philosophy might be formulated roughly equivalently by saying that; starting from conditions at or near thermodynamic equilibrium, one should strive to vary the minimum number of parameters (degrees of freedom) , as slowly as possible over the minimum range necessary to effect the desired process to occur. This is a statement of scientific common sense with which few would be inclined to argue but to which a plethora of common techniques (among them the Dorset technique) fail to ascribe. By contrast, it was precisely in keeping with this spirit that the Taylor technique was designed. Basically, a single parameter - initial concentration - was chosen as the variable while all others were held as constant as possible. The " constant " parameters were primarily temperature, and solvent vapor pressure. Although it may be naive to attempt to control the temperature of the process which results in complete evaporation of the solvent phase, it was deemed worthwhile for reasons derived from the general principles as just espoused. As it turned out, temperature proved to be perhaps the most critical of the controllable parameters.

Figure 8. Legend on Next Page

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Figure 8. Oil Lamp Apparatus for paraffin specimen preparation.

A piece of carbon coated mica is held in a vacuum chuck which is enclosed in a cylindrical shroud. A Teflon tip is suspended above the mica forming an adjustable gap in which a 5 microliter aliquot of paraffin / heptane solution is held captive by surface tension. The heptane evaporates depositing monolamellar paraffin crystals on the carbon film. The carbon film which bears the crystals is then floated off and mounted on a metalized microgrid.

Prototypical versions of the apparatus (hereinafter oil lamp apparatus) of fig 8 were crudely fashioned to test the idea of using a teflon tip to confine the evaporating solvent to a small region of the substrate. These early versions of the oil lamp device lacked the shroud to confine the solvent in it's own saturated vapor. Despite this, they not only demonstrated the feasibility of the teflon confinement idea, but produced a region in the middle of the hyperbolic meniscus on which frequently (but not always) were deposited concentrations of high quality monolamellar crystals.

As an unexpected advantage, it was discovered that evaporation of the solvent from the captive meniscus produced not only thin specimen - quality crystals in the center of the film, but that the center of the film was usually surrounded by a set of concentric (more or less circular) rings of paraffin deposits which were visible to the naked eye and which were very useful for centering the paraffin bearing region of the film when mounting the film on the grid. Success with the oil lamp apparatus was quickly realized but relatively short lived inasmuch as the device was prone to the same sort of hit and miss track record in producing quality specimens as the Dorset technique. This was disappointing but provided much of the motivation to refine the apparatus into its present embodiment. Thus, in an attempt to control (primarily to slow down) the evaporation rate, a shroud and a means of accurately adjusting the gap were added. This provided much improvement in the consistency and size of the paraffin rings which were formed but little improvement in the quality or morphology of the central monolamellar crystals. It was only after a frustrating period of alternately switching between the two techniques that the importance of temperature was finally realized and that the specimens produced by the oil lamp apparatus improved so dramatically that it was possible to use this method of specimen preparation almost exclusively for work involving microgrids.

The discovery of the temperature effect came about as a result of a particularly unsuccessful series of experiments which seemed to be correlated with a period of unusually cold weather during which ambient temperatures in the laboratory were reduced significantly for a period of about a week. During this time it was decided, more or less out of a sense of frustration, to heat the entire apparatus on a heating convector immediately prior to producing a specimen. Such ad hoc experimentation is typically futile but in this case the results were dramatically successful. It was discovered that crystals having the morphology preferred for imaging form most readily at temperatures around 45 degrees Celsius.

Exactly what can be made to mean by the concept of temperature in this rather blatantly non equilibrium context is not rigorously clear but this phenomenon has since been firmly established as empirical fact, and heating the solution and the apparatus has become an essential aspect of the Taylor technique. Some time after the discovery of this effect, reference to the use of " hot " paraffin containing solvent was discovered in an article by Dorset. This was confirmed in a private communication with Dorset who attributes the formation of undesirable dendritic crystal polymorphs at reduced temperatures to the limited solubility of paraffin at these low temperatures. This is an indirect way of saying that crystal formation under conditions of supersaturation (low temperature) are apt to take place far from equilibrium. The solubility of paraffin in heptane increases dramatically with temperature around room temperature; a fact which can be easily verified by preparing a saturated stock solution consisting of 13 mg of C₄₄ paraffin in 10 ml of heptane. This solution will contain a substantial quantity of undissolved paraffin at room temperature which can be dissolved completely by warming the solution to 40 degrees Celsius. Whatever the exact nature of the paraffin/heptane phase diagram, it is clear that if one's aim is to do imaging of monolamellar crystals one should work with solutions somewhat hotter than ambient temperatures. The situation is still not cut and dried however inasmuch as we have observed dendritic and quasi-amorphous crystal polymorphs even at elevated

temperatures; particularly when using the Dorset technique although occasionally with the Taylor method as well.

Several hypotheses were formulated over the course of this work to explain the problems encountered with direct (Dorset) crystallization on microgrids. These ideas have been deemed no longer worthy of experimental verification. Since they bear on the properties of support films however, they warrant consideration if for no other reasons than to complete the historical narrative of our experiments. At first, it was believed that preferential nucleation in the regions of the carbon film overlying or adjacent to metal deposits might be causing the maldistribution of monolamellar crystals which was confounding our attempts to image paraffin on microgrids. This hypothesis seemed to offer little in the way of experimental recourse. More recently, an hypothesis has emerged which may bear on a more addressable aspect of microgrid fabrication; that of flatness. If one imagines that there is sufficient warping of the metalized microgrid that a vertical gap exists between the metal and the carbon film immediately above it, it is conceivable that when the specimen is applied using the Dorset procedure, the solvent might "wick" into the region between the metal and the film. This would result in these regions being the last to evaporate which could account for the (putative) presence of dense paraffin crystal deposits which appear to be associated primarily with metalized regions. In other words, the technique could be reporting incomplete contact between carbon film and microgrid probably due to insufficient flatness of the metal substrate on which the carbon film was deposited. As was the case with metalization of perforated films, the experimental issues seem to have converged upon the question of film flatness; a critical subject which will be addressed in a subsequent section.

The Modified Taylor Technique

The method of bonding carbon films to microgrids which is described in section 2(D) precludes the use of the Taylor technique because of the interaction of organic solvents used in the bonding procedure with specimen crystals which have been previously applied to the carbon film prior to bonding. This unfortunate turn of events necessitated returning to the Dorset technique for preliminary attempts to measure $F(g)/F(0)$. Initial work in this generation of experiments amounted to an instant replay of the frustrating and unsuccessful first generation of experiments which employed the Dorset technique on microgrids. Fortunately we were able to modify the Taylor technique for the purposes of preparing paraffin on bonded microgrids. The modified technique is a sort of hybrid procedure which is diagrammed in Figure 9. A bonded microgrid support assembly is grasped in anti-capillary tweezers which are placed on an elevated supporting surface. A glass bead about 2mm in diameter (fabricated by melting the tip of a disposable glass pipet) is suspended above the microgrid leaving a gap of about 1mm between grid and bead. The paraffin sample is then applied to the glass bead in a 5 λ aliquot. The solution forms an hyperbolic meniscus in the gap in a manner exactly analogous to the method previously described to apply paraffin to carbon coated mica. Evaporation is allowed to occur at room temperature with no provision being made to confine the solvent in its own saturated vapor. This is primarily a concession to expediency since it is difficult to imagine a simply constructed apparatus which would replace the tweezer method of holding the grid and could be enclosed in a small volume. Despite this somewhat ad hoc apparatus, the technique seems to work much better than direct application of the sample solution. We believe that the bead may actually be superior to the teflon tip in acting as a thermal clamp and as a reservoir from which the meniscus is maintained at a more uniform volume. Interactive control can be accomplished by using a stiff copper wire to suspend the glass bead in such a fashion that as the solvent evaporates the gap can be manually reduced; thereby minimizing the

amount of solution remaining to wet the grid when the meniscus ultimately collapses. This is an added measure to minimize the amount of solution which is presented to the back side of the microgrid.

Fig 9. Modified Taylor Technique

Crystals of paraffin produced by means of this technique are shown in Figure 10.

Figure 10 (a)

Ideal monolamellar paraffin crystals prepared on carbon films using the modified Taylor Technique. (a) Highly defocused, high dose, bright field image - 10,000X magnification. Small dark spots are defocused images of ice condensation, not holes in the carbon film. These crystals are of average size (for this specimen) and exhibit ideal morphology, indicating near equilibrium growth conditions and hydrophobic carbon films properties. Crystals of the same quality having linear dimensions over five times larger than those shown occur occasionally using this technique. (b) Typical highly over-focused diffraction image of the same specimen used for low dose scanning of the specimen to locate crystals for high resolution image recording.

Figure 10 (b)

Carbon Films

Evaporated carbon films are the most widely utilized specimen supports in the science of biological electron microscopy. Used alone or in combination with other types of films they predominate over numerous more specialized support film technologies which have been developed over the years. The field of support film technology is reviewed by Baumeister and Hahn (1978).

The predominance of carbon films in high resolution electron microscopy has most of its origins in the remarkable stability of these films under electron bombardment and their amazing mechanical strength which allows them to withstand rigorous manipulation. Some of the techniques described herein bear witness to this.

If carbon films were the perfect specimen support however this thesis would be superfluous. Obviously they leave something to be desired in their ability to stabilize radiation sensitive specimens against beam induced movement.

If carbon films, in conjunction with an underlying metalized microgrid support are to be effective in reducing beam induced movement, it will require the optimization of not only the microgrid support but of the carbon film itself. While it is true that carbon films are so useful that we have included them as a keystone in our attempts to devise improved support film technology; this is not to say that carbon film technology cannot or indeed must not itself be improved if the ultimate goal of eliminating beam induced movement is to be brought closer to reality.

This section of the thesis describes our efforts to optimize the carbon films which we will be using to support the test specimen over holes in the metalized microgrid. These efforts have concentrated in two areas in which it was our feeling at the outset that there was the most potential for improvement.

First, we have renovated, and to some extent redesigned our carbon evaporation apparatus to eliminate chronic problems which were resulting in inconsistent evaporation conditions. This will be discussed in detail presently. Secondly, we have developed a novel technique which allows us to produce carbon films which have more consistent, and for our purposes more desirable surface chemistry. This is considered particularly critical since the carbon film / specimen interface is the first link in the chain of interactions which we hope will result in a significant reduction of beam induced movement.. This link is probably a chemical one in which mechanical interaction will have to be derived from short range dispersion forces which may be highly sensitive to inconsistency and chemical inhomogeneity of the carbon film surface. These theories as well will be discussed later in conjunction with a description of the experimental strategies developed to address the problem of surface chemistry.

The first problem of carbon film production to be addressed was to improve the evaporation apparatus to allow better control of the evaporation rate and the thickness of the film ultimately produced.

The films are produced using a Denton Model DV-502 vacuum evaporator. This unit uses conventional oil diffusion pump technology to produce a vacuum in the 10^{-6} Torr range in a Pyrex bell jar, which houses various stations for the evaporation of carbon, evaporation of metals from heated filaments, and glow discharge treatment. Although carbon and metal film production technology which employs units of this kind is widely practiced, none of

the commonly used methods whereby such equipment is used to produce such films are entirely free of vagaries. Some of the problems of carbon film production have been already alluded to. To continue to discuss the litany of problems which one can encounter in routine use of a vacuum evaporation device of this kind would belabor the point. It should suffice to say that the commercial version of the apparatus to evaporate carbon with which the Denton evaporator was equipped was not entirely satisfactory for the purposes of this work.

In the apparatus as marketed, due to a combination of mediocre design and careless machining, the two carbon rod holding members of the device were easily misaligned through routine use. This resulted in frequently aborted film production runs and breakage of the thinnest portion of the carbon rod at random times during the brief critical period of carbon evaporation. As a result the final thickness of the films proved virtually impossible to control or to reproduce to say nothing of the deleterious effects which drastic variations in evaporation rate might have on the properties of the films thus produced. It was necessary to improve the alignment of the carbon rods in order to prevent breakage before better control of the film thickness could be achieved. This was eventually accomplished through a rather gradual and painstaking incremental improvement of the apparatus, primarily through remanufacture of parts to closer mechanical tolerances, rather than by a wholesale redesign which would probably have been more cost and time effective in the long run. An interesting alternative design for a carbon rod holder is described in Baumeister (1978) - pg 41. This device might be less prone to misalignment but this is only an opinion. Better attention to careful machining would have to be paid for this or any design to perform optimally than was originally given to the fabrication of the Denton apparatus. Whatever design one might choose to employ, the essential point is that the carbon rods must be aligned and biased together with considerable precision in order for reproducible results to be achieved.

Refurbishing of the carbon rod holder resulted in an improvement in the regularity with which films of sufficient thickness could be produced but both the quality and the consistency of the films still left much to be desired. Real time interactive control of the evaporation process remained a difficult and tricky proposition even after the problem of rod breakage had been largely overcome. A second and somewhat more creative and substantive modification of the evaporation apparatus was then devised which has proved to be quite useful as a means to allow the operator of the evaporator to monitor the progress of the evaporation much more closely during the crucial seconds during which the film is actually being deposited. The adaptation is relatively simple in concept and in use and consists primarily of an optical projection system outside the bell jar which is configured to project an enlarged image of the carbon source on the wall behind the evaporation unit. The system, which is shown in figure 11, employs an $f/3.5$ projector lens having a 4" focal length. Lenses of this type can be readily obtained from discarded 35mm slide projectors.

In use, the operator of the evaporator visually monitors the appearance of the carbon source while carefully (but not too slowly) increasing the voltage applied across the source. On our Denton unit, this is done by manually operating the variac which supplies the primary voltage to the low voltage transformer whose secondary supplies current to the carbon source. Some devices have been described which employ a control element such as a thyatron or control rectifier to better regulate the current in the source which tends to behave in a very nonlinear fashion as the critical temperature at which the source begins to vaporize is reached. This is probably not a bad idea but with the optical projector system considerable interactive control can be accomplished without the use of electronic control elements other than the variable transformer. At the moment of truth, just before the carbon begins to vaporize, a characteristic and fairly unmistakable change in the appearance of the image can be observed which allows the operator to begin backing off of the voltage just in

Figure 11. Legend on Next Page

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Figure 11. Modified Denton Vacuum Evaporator.

A 4", $f/3.5$ projector lens mounted above the bell jar projects a magnified image of the carbon evaporation source. Visual monitoring of the magnified image allows improved control of evaporation rate and film thickness. Hydrogen can be controllably leaked into the vacuum system so that hydrophobic carbon films can be produced by evaporation in a reducing hydrogen environment at a pressure of 10^{-4} Torr.

time to obtain a constant rate of disappearance of the carbon source. As the evaporation proceeds, the carbon rod can be seen to shorten at a uniform rate until the portion whose diameter has been reduced has been consumed and the current supply is insufficient to cause significant further vaporization. At this point the variac can be turned off promptly to prevent unnecessary heating within the bell jar. In an evaporation unit of conventional design which lacks the source projector unit other less direct methods to detect the onset of source vaporization must be employed.

Typical of more ad hoc prior art methods is the technique formerly used in our laboratory in which the operator increases the voltage across the carbon source until sparks begin to emanate from the region of the source. Such methods (of which there are many) which do not involve direct visual monitoring of the source have been found, at least in the authors hands, to be less than satisfactory in producing films having a consistent thickness. The source projection method on the other hand is readily mastered, and allows the production of films having less variation from batch to batch in thickness and, we have come to suspect, in properties which are less easily quantified but which may none the less be important in producing consistent specimen preparations for electron microscopy.

Film Properties

These modifications to the vacuum evaporator and to the techniques used to produce carbon films with this apparatus resulted in a noticeable improvement in the properties of the specimen support films which we were able to produce. The properties of our carbon films were, however, still not deemed optimal. Despite the progress just described, the preparation of hydrophobic specimens such as paraffin and purple membrane still seemed to depend upon inscrutable factors having to do with the batch or age of the films or some

combination of these factors. One particularly intransigent phenomenon was the persistent unsuitability of newly produced films for supporting well ordered hydrophobic crystalline specimens.

Experiments with paraffin were among the latest in our laboratory in a series of experiments dealing with hydrophobic materials which gradually gave rise to the suspicion that the carbon films which we were routinely producing were hydrophilic i. e. charged or polar as shown schematically in fig 12 below.

Fig 12. Hypothetical Hydrophilic Carbon Film

Carbon films typically become more suitable for paraffin imaging with age and to explain this curing phenomenon by postulating that the films start out as polar and spontaneously revert to a more non-polar character seems at first somewhat unreasonable. Such a seemingly spontaneous process which might involve the formation of covalent carbon - carbon bonds would seem contrary to the usual tendency of our chemical environment to favor natural processes which tend toward oxidation. It is possible however that the process by which juvenile carbon films become phenomenologically more hydrophobic is not a spontaneous redox progression but something mediated by an environmental agent other than oxygen. In an industrial society awash in exhaust fumes it is conceivable, for instance, that monolayers of adsorbed hydrocarbons are the culprit. Whatever the case, the curing of carbon films is well within the realm of recognized empirical fact and however

anomalous must be reckoned with. It is also quite a separate issue from the hypothesis that juvenile films are hydrophilic.

The latter postulate - adsorption of hydrophobic molecules - not only explains their observed behavior, but has the more conventionally reasonable possible explanation that the presence of dipolar groups on the films is the result of their exposure to atmospheric oxygen for the first time when they are freshly prepared and unprotected by a layer of oil or the like. This line of reasoning led us to wonder whether the properties of carbon films would be different if they were not immediately exposed to atmospheric oxygen. This led eventually to the development of a new technique for producing hydrophobic carbon films, but not before a relatively simple experiment was conducted to corroborate our suspicions that such a technique might indeed be successful.

Experiments with paraffin crystallization using the Dorset technique (which usually produces crystals on both sides of the film) often produces mixtures of crystals of different types; some well ordered as if reposing on what we have come to regard as a hydrophobic substrate, and others more typical of what is seen with polar (new) films. It seemed more reasonable to assume that these different crystals resided on different sides of the film than on adjacent areas of the same side which had come in some mysterious fashion to have different properties. This belief was based in part on the obvious difference in the way in which the two sides of the film had come into existence. In a film deposited on mica and stripped at some later time, the back side of the film has been sequestered for a prolonged period from exposure to oxygen. This could account for the two sides having different properties and seemed to provide a means whereby we could check our ideas by comparison of identical specimen preparations from the same piece of mica but with opposite sides of the film mounted upright on the grid.

In order to do this it was necessary to devise a different method of mounting which would flip the film over. This proved tedious but was eventually accomplished by placing grids directly on the floating carbon film and blotting the carbon films on a glass coverslip in a manner similar to the method used to mount holey plastic films. When the film mounted in this fashion, with the sequestered side up, was used to produce paraffin crystals using the Dorset technique, a reversal in the population distribution of crystal types was observed to occur. A fresh film which was mounted by the conventional method, and which produced only an occasional large monolamellar crystal, when flipped, produced many more such high quality crystals. Although this experiment may have other interpretations, the result was emphatic and encouraging enough to motivate the first production of carbon films which were deposited in a reducing (hydrogen) environment and cured in hydrogen prior to exposure to atmospheric oxygen.

This technique, represents perhaps the most significant result achieved in tailoring the properties of a support film to match those of the specimen since the technique of glow discharge was first employed to render carbon films hydrophilic. The apparatus to evaporate carbon in a hydrogen atmosphere is shown diagrammatically in fig 11. It consists of a conventional vacuum evaporator to which has been added a means of controllably (and safely) leaking hydrogen gas into the high vacuum region in which the films are produced at a pressure of the order of 10^{-4} Torr. The apparatus is straightforward and the results of its use are fairly dramatic. This technique is, so far as we know, unique in its ability to produce carbon films which, fresh from the evaporator, are capable of supporting high quality (very well ordered) specimens of paraffin and of purple membrane.

This result, whether future work should prove it more widely applicable or not, resulted immediately in dramatic savings of time and frustration in producing quality specimens for use in the investigation of the microgrid support films which comprise this part of the

thesis. In effect, the development of the reducing atmosphere carbon film was the last and probably most important step in the process of optimizing the carbon films used in our experimental investigation of microgrid supports.

Bonding

It has been stated in an earlier discussion (Chapter 1) of beam induced movement that our attempts to address this limitation were to be comprised of four explicit aims which were:

- 1) Development of a test specimen
- 2) Optimization of carbon films
- 3) Production of suitable microgrids
- 4) The development of techniques to insure the bonding of carbon film to microgrid.

A description has been given of the achievement of the first three of these aims. This has allowed preliminary measurements to be made of the effectiveness of these accomplishments in reducing beam induced movement i. e. in improving the signal present in low dose images. These preliminary results show no improvement. One possible interpretation of these results is that there may indeed be a problem with the interaction or contact between the components of the support film as was implicitly assumed to be possible when the fourth of these aims was appended to the above list of objectives. We have also during the course of this research seen some evidence, apart from the results of the image quantitation to be discussed, to substantiate this hypothesis. One such observation regarding the distribution of specimen crystals is discussed at the conclusion of the section which describes test specimen preparation. A more persuasive argument that bonding may pose a significant problem is the conspicuous non-flatness of metalized support films; particularly what has been dubbed " meso-scale " flatness.

Upon completion of the first three objectives, a solution to the fourth has thus assumed increased importance. Efforts to improve the bonding situation have been focused in two areas:

- 1) The development of a technique to achieve adhesion between the components of the support film assembly
- 2) The elimination of meso-scale non-flatness or sagging of metalized microgrids.

The following protocol contains novel provisions which we believe represent very significant progress toward the realization of these experimental objectives. Together with the microgrid fabrication protocol given previously, this procedure enables the production of microgrid supported carbon films which are essentially optimum according to the criteria defined by the four experimental objectives which were stated at the beginning of this research. The support technology represented by these novel protocols is the culmination of the work described in the first section of this thesis. We allege that these support films are the most mechanically stable and chemically well defined support films for the high resolution electron microscopy of beam sensitive specimens ever devised.

Final Protocol

- 1) Prepare fenestrated Collodion films on 400 mesh grids using the modified method of Lunsdorf and Spiess.

- 2) Screen the dried plastic films using optical microscopy selecting for further processing those with the desired degree and uniformity of fenestration. Reject films which show the plastic films to be torn away at the edges of the gridbars
- 3) Metallize the selected films on both sides using the multiple filament apparatus of Fig.5.
- 4) Screen the metallized films again sorting out torn or otherwise defective films.
- 5) Immerse the metallized microgrids for 10 sec in a solution of 90 mg collodion in 30 ml of ethyl acetate. Allow to air dry until slightly tacky.
- 6) Place the dipped microgrids in an EM-90 Airfuge rotor (adapted as shown below) between 1/8" dia discs of mica; holey film side facing away from the center of the rotor (see diagram).

Figure 13

- 7) Spin at 85,000 RPM (28 psig - 100,000 g) for 10 min. This procedure reduces the sagging of the metalized mesh and produces a very flat metal microgrid to which carbon can be bonded by the following additional procedures.
- 8) Remove the microgrids from the rotor and place them on filter paper in the bottom of a carbon film flotation apparatus.
- 9) Float a very thin carbon film (less than 50 Å) which has been produced by indirect evaporation¹ in a hydrogen atmosphere from mica and lower onto the microgrids. Blot on filter paper and air dry the films overnight in a covered petri dish.
- 10) Place the filter paper (bearing the undisturbed microgrid and carbon film) face-up on a 1/4 " cube of solvent resistant plastic foam (soft packing foam like polyurethane) in a small glass petri dish. Add enough ethyl acetate to saturate the foam and filter paper and wet the bottom of the dish.
- 11) Allow to dry in a fume hood until the filter paper is completely dry.
- 12) Remove the microgrid which now bears a plastic residue bonded carbon film from the filter paper. Place it holey film side down in the evaporator and evaporate a thin carbon film onto the back side.

¹ We strongly recommend the use of carbon films produced by "indirect" evaporation. In this technique the target substrate (mica) is shielded from direct line of sight exposure to the carbon source. The apparatus which we employ utilizes a geometrical configuration which maximizes the reflection of carbon atoms onto the substrate from the inside walls of a so-called "ricochet" cylinder. The geometry of such a device is described in Baumeister (1979) - pp 39-41. Thin carbon films produced in this fashion in a hydrogen atmosphere are superior in quality to any which we have ever seen as regards their integrity and apparent strength during floatation onto grids.

13).Thoroughly pre-irradiate the grid with 100 KEV electrons. This step is intended among other things, to convert the bonding polymer to a final stabilized residue.

14) Apply the specimen.

This completes the production of specimen supports which were used to measure the effectiveness of microgrids in preventing beam induced movement.

CHAPTER 3 - IMAGING METHODS

Image Theory

To define precisely what is meant by "improved" imaging, one could employ the familiar and useful concept of resolution - usually defined as the least upper bound of image data in the spatial frequency domain. In the case of beam sensitive specimens however, resolution is not the entire issue. Images of the purple membrane can now be routinely (though by no means necessarily easily) obtained which contain some structural detail at a resolution of less than 5 Angstroms (Henderson et al 1986). This by no means implies however that the imaging process used to obtain data at such high resolution is optimum. Nor does the existence of limited structural data at such high resolution in a particular data set guarantee that data are sufficient to allow the molecular structure to be determined to that resolution. For these reasons, lavish claims of high resolution in the discussion of electron crystallographic data may actually be misleading, and resolution per se may not be the most useful figure of merit to employ in a discussion of image optimization. A more useful alternative concept in this context might, for instance, be signal to noise ratio, which usually imposes practical statistical limitations on the significant of structure factors and crystallographic phases before an unknown structure can be computed to the absolute resolution cutoff.

The most appropriate figure of merit or index of image quality to use in discussing imaging depends upon the total experimental context at hand. Thus while resolution limit may be of some relevance in experiments directed at improving microscope performance or specimen coherence it may not be nearly as significant in evaluating the results of experiments having other objectives; the problem of beam induced movement being a case in point.

For the purposes of quantitatively comparing different support films it is useful to employ a

method which is based upon the comparison of electron diffraction intensities and intensities of "diffraction" obtained from corresponding images. The so called $F(g)/F(0)$ ratio has a number of advantages for this purpose principally derived from the fact that it is possible to define by this means an absolute figure of merit for the ideal case of theoretically optimum imaging. This is made possible by the theory of coherent imaging of weak phase objects.

Electron microscope imaging is a special case of quantum mechanical electron scattering for which a wealth of proven formalism has been developed. Consider the following integral equation which represents quite generally the scattering of an incident wave Ψ_0 from a localized scattering potential Φ .

$$\Psi(r) = \Psi_0(r) + \int G(r,r')\phi(r')\Psi(r')dr' \quad (1)$$

In this equation $\Psi_0(r)$ and $\Psi(r)$ represent the incident and scattered wave functions respectively and the function $G(r,r')$ is the Green's function which represents the contribution to the total scattered wave at the field point r which would be observed as the result of scattering of a plane wave of unity amplitude by a delta function scattering potential located at the source point r' . In the case of scattering in a region of free space which is devoid of static charge and polarizable material media, the Greens function which is usually used is:

$$G(r,r') = \frac{\exp(ik|r-r'|)}{4\pi|r-r'|} \quad (2)$$

which can be thought of as an outgoing spherical wave (Huygens wavelet) centered on the source point r' . The choice of Green's function is somewhat arbitrary in that the only essential requirement for the function to represent a solution to the wave equation is that it satisfy the differential equation:

$$\nabla^2 G(r-r') = 4\pi\delta(r-r') \quad (3)$$

This is necessary for the scattered wave as expressed in (3) to be a solution to the differential wave equation - a fact which can be verified by direct substitution (See Jackson - 1962).

The choice of the so called free space Green's function (2) is usually sufficient to deal with far - field electron scattering from weak potentials as well as for most problems in linear optical diffraction theory¹.

The integral equation (1) is a concise and elegant representation of the general scattering problem. Unfortunately, such a representation is not immediately useful for computing the

¹

The Green's function is a powerful mathematical device for solving otherwise intractable integral equations. Alternatives to (3) can be derived by path integration in the complex plane (see Mertzbacher - 1961). The choice of Greens function is somewhat arbitrary in a mathematical sense. Two requirements which must be met are the property expressed in (2) and that the chosen function satisfy certain boundary conditions; those imposed by the problem itself and others for mathematical convenience. For example, it is usually also required that the function G or it's normal derivative go to zero at infinity. This latter requirement is not rigorously satisfied by the free space Green's function although it is often used together with auxiliary mathematical arguments to justify ignoring integration over the infinite surfaces. Other Green's functions which have the property expressed in (3) and which do meet the boundary conditions at infinite radius are possible. One such function which is the basis of the Rayleigh - Sommerfield formulation of scalar diffraction theory is given on page 43 of Goodman (1968) This choice makes possible a more rigorous solution to the general problem of wave propagation using Green's theorem by requiring the imposition of boundary conditions which do not overdetermine the problem; as is the case when the free space Green's function is employed in deriving the Kirchoff formulation. This is an interesting example in classical physics in which the more intuitively satisfying formalism is mathematically disadvantageous. It should be pointed out however that the Kirchoff formulation is remarkably accurate in spite of its mathematical shortcomings. For further information the reader is referred to Goodman (1968).

scattered field from a given potential since the total scattered wave appears on both sides of the equation. The objective of electron microscopy represents a different class of problem in which one attempts to compute the unknown scattering potential (the object or specimen "structure") from limited measurements of the total scattered wave function; the incident wave being a partially coherent, quasi-monochromatic plane wave. The solution of both types of scattering problem would be greatly facilitated if the integral which appears on the LHS of the equation could be converted to an invertible form. This has never been accomplished for the general case but several approximations, which are applicable to scattering from thin biological specimens, can be made which will do so.

The Born approximation.

A major difficulty with the inversion of the scattering equation is the appearance of the total scattered wave function inside the superposition integral. For some scattering potentials, the use of such a rigorous formulation which takes into account so called multiple or "dynamical" scattering, is not necessary. In these cases, the scattered wave function in the integral can be replaced by the incident plane wave $\Psi_0(r)$. This is the so called first Born approximation, which clearly becomes increasingly accurate in the limit of weak (small angle) scattering and very thin scattering potentials (specimens). The domain of validity of the Born or kinematical approximation, as it is known among crystallographers, is discussed by Cowley - 1975, and most fortunately for electron crystallographers includes the case of scattering of 100 KEV electrons from monolamellar two dimensional crystals of biological macromolecules .

The Fraunhofer approximation.

Together with the kinematical approximation, a second set of simplifying assumptions can

be made which take advantage of the relatively large distances at which scattered electrons are detected in high resolution imaging relative to the dimensions of the specimen and the wavelength of high energy electrons. In terms of the coordinates which appear in the superposition integral, the magnitude of the field point vector r is large in comparison with the magnitude the vector $(r - r')$ so that for the purposes of approximating the scattering integral it is possible to consider the vector magnitude which appears in the denominator of the spherical wave Green's function a constant over the range of integration over the source point variable r' . Thus the Green's function $\text{Exp } iKr' / |(r - r')|$ can be replaced by the function $\text{Exp } iKr' / |r|$ and the integral (in the case of the kinematical approximation) reduces to:

$$f(q) = \frac{1}{4\pi r} \int \phi(r') \exp(-iqr') dr' \quad (4)$$

where $q = k - k_0$ and $f(q)$ is the scattered wave function

This can be recognized as an outgoing spherical wave whose amplitude, as a function of scattering angle (for small scattering angles) is proportional to the Fourier transform of the scattering potential. This is the familiar far - field, Fraunhofer diffraction approximation.

Since the Fourier kernel $\text{Exp } iKr'$ generates an integral transform (the Fourier Transform) which has the unique property of being its own inverse, taken together, the approximations of Fraunhofer diffraction and kinematical scattering render the superposition integral invertible.

Electron microscopic imaging of weak phase objects.

General theories which attempt to mathematically model the scattering of waves must be able to represent not only the scattering process but also the propagation of the scattered wave after it leaves the specimen. Such a thorough treatment is unnecessarily complicated for the purposes of modeling the coherent imaging of weak phase objects. Fortunately there is an alternative formalism which may be used to quantitate the coherent imaging of thin biological specimens which sacrifices little in either accuracy or elegance.

As we have seen, under certain circumstances the scattered wave forms a distribution of intensity which is related to the structure of the specimen through an invertible integral transform. In the electron microscope however the scattered wave immediately below the specimen is not simply allowed to propagate according to the laws of physics which govern the diffraction of electron waves in free space. Instead, the wave enters the magnetic potential of the objective lens and the different Fourier components of the modulated wave front are sorted into discrete positions behind the lens (in the back focal plane) according to angle which these diffracted plane waves make with the optical axis of the lens.

In the case of a phase object, the wave emerging from the specimen and entering the imaging system is a unity amplitude plane wave which has been modulated (in phase only) in proportion to the projected potential of the specimen.

$$(\text{Plane Wave Exp}) \times (\text{Phase Modulation Factor}) \quad (5)$$

The phase factor will be of the form:

$$\text{Exp} \{ i(\text{projected specimen potential}) \} \quad (6)$$

To simplify notation - and without loss of generality since the imaging process is a linear

system which transforms the wave according to a linear integral transform - consider a specimen which has a projected potential which consists of a single component which is a cosine wave which has a spatial frequency g .

$$\text{Specimen Potential} = \sigma \cos(2\pi g x) \quad (7)$$

Where $\sigma \ll 1$ is the amplitude of the phase modulation at the spatial frequency g .

It bears repeating that consideration of only a single "Fourier component" in this fashion is completely general since the response of the system to an arbitrary input can be constructed later by a linear superposition of the individual responses to the individual components (inputs) which comprise the spectrum of the arbitrary input. Returning to the wave function after it emerges from the specimen potential described by (7) one can write for the wave function:

$$\Psi(z, x) = (\exp\{ikz\}) (\exp\{i\sigma \cos(2\pi g x)\}) \quad (8)$$

This equation is still completely general in terms of the specimen structure. The assumption has been made however that the specimen potential alters only the phase and not the amplitude of the incident wave. In addition we have assumed that there has been no change in wavelength of the wave as it passed through the specimen. One can further assume that since the specimen is thin, the phase modulation is a small fraction of a wavelength. This so called weak - phase object (WPO) approximation is usually made to justify expansion of the second exponential term in (8) in a power series with the intention of neglecting all but the first two terms. This is a prevalent practice which results in a very clean mathematical framework for further development of the model. It is not however trivial to justify conceptually. We assume:

$$\exp\{i\sigma\cos(2\pi gx)\} = 1 + i\sigma\cos(2\pi gx) + \text{negligible terms in } \sigma^2 \text{ and higher powers.} \quad (9)$$

This then allows the wave exiting the specimen to be written:

$$\Psi(z,x) = (\exp ikz)(1 + i\sigma\cos(2\pi gx)) \quad (10)$$

In general, a truncated series expansion might not represent a solution to the wave equation. Fortunately that is not the case here. In fact, it can be shown that (10) is equivalent (within a constant phase shift) to the linear superposition of plane wave exponentials propagating at different angles to the incident wave. This is indeed most fortunate in that such an expansion into diffracted "beams" lends itself ideally to describing the propagation of the transmitted wave function according to the laws of optics which apply to simple imaging systems rather than more complicated theories of diffraction.

The Angular Spectrum

The validity of the WPO approximation (and for that matter the subject of Fourier optics in general) is derived from the fact that monochromatic plane waves of a single wavelength λ can interfere constructively and destructively to produce waves of any wavelength greater than or equal to λ in the fashion illustrated in fig 14. Shown in the figure are three plane waves and their corresponding complex exponential representations. The essential point of the diagram is that if one considers the waves only in the one dimensional domain

XBL 874-7663

Figure 14. Angular Spectrum Optics

Diffracted beams at $+\theta$ and $-\theta$, representing complex exponential plane waves, interfere along the x axis to form a standing cosine wave which represents the Fourier component of the specimen structure at the spatial frequency g .

orthogonal to the wave vector of the incident wave (the z axis) it can be seen that in this dimension (or in this plane if the diagram represented of a cross section of an imaging system) the diffracted waves superimpose to produce waves having a spatial frequency k_x less than or equal to the frequency:

$$k = \frac{2\pi}{\lambda} \text{ of the incident wave } \therefore k_x = k \sin \theta \quad (11)$$

Thus any plane wave of wavelength L travelling obliquely to the z axis represents a wave of spatial frequency $k \sin \theta$ along the x axis, and the set of functions given by the intersections of the set of complex functions $\text{Exp } iKz$ with the x axis form a basis set in which an arbitrary function $F(x)$ may be expanded in a Fourier series. The Fourier spectrum of the function $F(x)$ is therefore expressible equivalently as an angular spectrum in the variable θ ; each Fourier component representing a plane wave of wavelength λ propagating at an angle of θ to the z (optical) axis.

Because a lens can be thought of as a linear transformation which maps plane waves into points in its back focal plane, the angular spectrum expansion of an object function is ideally suited to the formal description of coherent imaging. These subjects are dealt with in more detail by Goodman (1968).

Returning to the problem of representing a weak phase object one can derive by using a vector formulation of the complex exponential, that three beams at $\theta = 0, +\theta$, and $-\theta$, represent a real standing wave in the object plane which has spatial frequency:

$$g = k_x = k \sin \theta \quad (12)$$

Noting that $i = e^{i\pi/2}$, and adding the "crystallographic phase" $\xi(g)$ for generality,

equn (10) becomes:

$$\Psi = (\exp ikz)(1 + e^{i\pi/2}\cos(2\pi gx + \xi(g))) \quad (13)$$

This allows the wave function to be written in the three beam form:

$$\left(1 + \frac{1}{2}e^{i\pi/2}e^{i2\pi gx}e^{i\xi} + \frac{1}{2}e^{i\pi/2}e^{-i2\pi gx}e^{-i\xi}\right) \quad (14)$$

In which the factor e^{ikz} which represents the "carrier" wave has been omitted.

In the back focal plane of the objective lens (15) has the Fourier spectrum:

$$F(s) = F(0)\delta(s) + F(g)e^{i\pi/2}\delta(s - g) + F^*(g)e^{i\pi/2}\delta(s + g) \quad (15)$$

$F(0)$ is the square root of the intensity of the undiffracted beam, $I(0)$

$F(g)$ is the "structure factor"; $F(g) = |F(g)|e^{i\xi(g)}$, and $|F(g)| = \frac{\sigma}{2}$

From this point the theoretical image intensity and the theoretically expected $F(g)/F(0)$ ratio in the image and in the diffraction pattern can be derived according to Henderson and Glaeser who obtain, starting with (16), the following relationship which forms the basis for the evaluation of imaging by means of the $F(g)/F(0)$ ratio.

$$\frac{\hat{f}(g)}{\hat{f}(0)} = 2 \frac{F(g)}{F(0)} \sin \gamma(g) \quad (16)$$

Where $\hat{f}(g)$ is the amplitude (not the intensity) of the calculated Fourier coefficient of the image intensity, and $\hat{f}(0)$ is the amplitude of the zero-frequency term in the computed Fourier transform.

$\gamma(s)$ is the wave distortion due to spherical aberration and defocus given by

$$\gamma(s) = \frac{1}{2} \pi (C_s \lambda^3 s^4 - 2 \Delta Z \lambda s^2), \quad (17)$$

and $\sin \gamma(s)$ is the phase contrast transfer function.

According to equation (18), the highest possible ratio of structure factor amplitudes for the diffracted and undiffracted beams in the ideal image of a weak phase object is twice the corresponding ratio of structure factor amplitudes in the electron diffraction pattern.

Image Quantitation

Images were recorded on a JEM 100B transmission electron microscope. This instrument is equipped with a field emission electron source which can provide both higher intensity and greater coherence than can be achieved with a conventional thermionic electron source. Although these advantages are essential for some methods of reducing beam induced movement such as the small spot technique pioneered by Downing, the use of an FEG is not necessary for the recording of the low dose images of paraffin on microgrid supports. A thermionic source could have been used as it was in the work of Henderson and Glaeser who go into some detail regarding the coherence requirements for this type of investigation.

The microscope is also specially outfitted for low dose imaging; provision having made for the establishment of three different illumination conditions which can be quantitatively preset and independently and immediately accessed as required. The three condenser lens configurations normally used include:

- 1) A very low dose scanning mode for scanning the specimen in highly defocused diffraction mode in which a distorted, high contrast, relatively low magnification image of the specimen can be observed in order to locate crystals of interest,
- 2) A low dose mode used exclusively for the recording of low dose images, and
- 3) A high dose mode for use in focusing immediately prior to image recording. The same condenser aperture was used in all three illumination modes.

Image recording was done at 50,000X magnification with a dose at the specimen of from 1-5 electrons/A² (1000 counts/sec at 200,000X - 3-4 sec exposure). ²

The scanning dose was made as low as possible without making the detection of the

²1,700counts/sec at 20,000 magnification corresponds to a dose at the specimen of 1e⁻/Å²/min

crystals inordinately difficult and with sufficient illumination to allow the inspection of dark-field images to determine uniformity of diffraction from the crystals. Such a determination is prudent prior to imaging to select crystals having the required quality and thickness and to exclude the imaging of crystals which may be severely tilted. Tilt is evidenced by strong asymmetry in the brightness of the six-fold-symmetric dark-field images observed in defocused diffraction patterns when a selected area aperture is inserted to restrict diffraction to a small region of the crystal under examination. The scanning dose was also used to record defocused diffraction patterns for determination of $F(g)/F(0)$ ratio. Absolute illumination in the scanning mode was in the range of 1,700 counts/sec on the ratemeter at 20,000x.

Focusing was accomplished under illumination condition 3 which corresponds to the brightest illumination possible within constraints imposed by the available source brightness, first condenser excitation (spot size) and condenser stigmation necessary to produce a small uniformly illuminated region on the specimen in which the phase grain can be detected visually at 50,000X magnification. This was achieved with the illumination slightly overfocused to illuminate as small a region of the specimen as possible, as brightly as possible. Focusing was carried out by using the dark field deflection coils of the microscope to deflect the beam slightly away from the area to be imaged and onto a second off-axis fluorescent screen adjacent to the tilting screen of the microscope and used exclusively for focusing. With the beam thus deflected and with the condenser in illumination mode 3, focus was adjusted to minimize the phase grain. The beam was then blanked using an electronic shutter and the imaging mode selected for recording the image of a nearby region of the specimen using preset illumination mode 2 and the bright field deflection coils to position the beam back to the center of the column and onto the area to be imaged.

Images were recorded on Kodak SO-163 film (EIF). The film was developed for 12 min in full strength D-19 followed by 8 min in fixer.

Extraction of $F(g)/F(0)$ ratios - Image processing.

Defocused diffraction patterns were recorded at exposures of 0.8 and 8.0 sec. The shorter exposure was chosen to prevent saturation of the photographic film in the image of the undiffracted beam which was then determined from measurements of optical density by comparison with a calibration exposure series taken at the beginning of each session. The longer exposure was used to measure the intensity of the diffracted beam(s) which were then related to the intensity of the zero order beam through the tenfold difference in exposure times.

Images were screened optically using a convergent beam laser diffractometer to determine the presence of the prominent reflections at 3.7 and 4.2 Å which are characteristic of the orthorhombic paraffin lattice. At doses not greatly in excess of the critical dose, a requirement for low dose images to have optimum signal to noise ratio, no discernable detail can be detected in even the best images using the unaided eye. Even relatively poor images clearly show optical diffraction however, although the optimum dose is usually insufficient to reveal the phase contrast transfer function in the optical diffractograms. Fortunately, neither exact focus nor exact stigmatism are essential to produce contrast near the theoretical maximum. The possibility exists, due to random defocus among different micrographs, that in a few instances the characteristic reflections of the paraffin lattice might coincide with a zero of the phase contrast transfer function. If many micrographs can be recorded however, some should (by chance) have the prominent reflections coincident with a maximum of $\sin \chi$. These images should represent the best attainable $F(g)/F(0)$.

Direct measurements could theoretically be made of the strength of optical diffraction observed from images; however the preferred method to determine the value of $F(g)/F(0)$ for images is by computation. For this purpose the micrographs which showed the strongest optical diffraction were selected for further processing. The best images were scanned optically and the regions which showed the strongest diffraction were marked along with the axis corresponding to the strongest reflections. The selected areas were then scanned and digitized using a Perkin Elmer PDS flat-bed microdensitometer. Scanning was conducted by aligning the direction of strongest diffraction with the x-axis of the densitometer. A 512 X 512 array was then scanned using a 6.67 micron square aperture and a 5 micron step size. This corresponds to 3x the Nyquist limit for the spacing of the lattice which is 20 μm in the 50,000X images.

Scan data were processed using a VAX 11/780 computer to generate the Fourier transform of the image arrays. $F(0)$, the amplitude of the unscattered beam, was taken to be the amplitude of the computed Fourier transform at the origin. This required no integration since the amplitude there dominates the surrounding region of reciprocal space by several orders of magnitude. A correction was made for the fog level of the photographic film. The computed value of $F(0)$ was checked by comparison with the average value generated by the program multiplied by the total number of pixels in the array.

The structure factor $F(g)$ at 4 $\text{\AA}$ was computed by integration of the amplitudes of a 7 by 7 pixel array centered on the strongest diffraction spot present in the computed transform. Background correction was applied based on the average amplitude present in the vicinity of the diffraction spot. These calculations were performed using the program *mmbox*, which is part of an image processing software package obtained from the MRC Laboratory in Cambridge England.

CHAPTER 4 - RESULTS AND CONCLUSIONS - SPECIMEN SUPPORTS

Microgrids - The Flatness Problem

The table of microgrid methods given in Chapter 2, which is adapted from Baumeister et al (1978) is representative of microgrid related literature in general. Disadvantages of the various methods are tabulated, but no explicit mention is made that many of these techniques produce microgrids which are cosmetically acceptable but essentially useless for high resolution imaging of specimens supported on carbon films. We regard the recognition per se of problems associated with conventional microgrid methods as one of the foremost accomplishments of this work. By far the most serious of these problems has to do with non-flatness which, insofar as we can determine, is virtually ubiquitous in the prior art. As regards more specific results of these experiments, having identified these problems, we have devised techniques to alleviate them. Such techniques, which have the potential to greatly improve microgrid technology, and are reported in Chapter 2, include:

- 1) the development of novel metalizing technology; which facilitates the production of large quantities of metalized, fenestrated films which are potentially free of global deformations resulting from metallization.
- 2) We have devised a technique of further reducing meso-scale sagging which can be applied to the microgrids culled from the scaled up metalization procedure.
- 3) We have investigated the use of different metals and have determined the use of nichrome to be the best of those investigated. In a sense, this represents an optimization of the physical structure of the microgrid which may bear on the more general issue of flatness.

Microgrids - Other Issues of Quality

The potential utility of microgrids in high resolution imaging depends to a lesser extent on factors other than flatness which we have investigated in this work. We have screened numerous methods of producing fenestrated plastic films and, among those considered, have determined the (modified) method of Lunsdorf and Spiess to be the most easily practiced and the most effective in producing films which have uniform properties.

Carbon Films

As a prelude to experiments designed to determine the utility of microgrids in high resolution imaging, it was necessary to improve the procedures used to produce evaporated carbon support films for hydrophobic specimens. These important results, which may prove useful outside the area of microgrids, were reported in Chapter 2.

Paraffin Specimen Preparation.

When the Dorset Technique was found to be unsuitable for producing paraffin specimens on metallized microgrids, two new methods (also described in Chapter 2) were devised which enabled imaging experiments to be conducted using improved microgrids.

Bonding

After addressing the critical issue of microgrid flatness, improving the properties of evaporated carbon films, and devising new techniques of paraffin specimen preparation suitable for use with microgrids, we have developed a method to ensure bonding of the carbon film to flattened microgrids. Recent microgrids which have been bonded to very

thin carbon films utilizing the protocol given in chapter 2 appear to be of excellent quality with regard to the separation (or lack thereof) between carbon film and microgrid. Imaging experiments conducted with these supports have consistently demonstrated that the separation of the two films is undetectable in the electron microscope. When comparing the defocus at which one observes contrast reversal at the edge of the microgrid holes with the defocus at which the carbon film phase grain exhibits minimum contrast we have been unable to detect any difference in the two methods of determining focus. In fact, in recording high resolution low-dose images of paraffin crystals over holes in bonded microgrids, we have abandoned the method of focusing on the carbon phase grain in favor of the more easily judged criterion of whether one is in focus on the metal grains at the edge of the holes. Images acquired in this manner so far indicate no difference in success rate for obtaining in focus images between the two methods. This indicates that the film and the microgrid are consistently separated by no more than several clicks on the next to finest focus adjustment ($700 \text{ \AA} / \text{click}$). Low angle shadowing experiments are under way to further assess the degree of separation between the carbon and the metal films.

Microgrids - Imaging Results

Because of the question of specimen dependency, it is difficult to comment in general on the potential utility of microgrids for different applications. The crystallization of paraffin on microgrid supported carbon films probably approximates a worst case scenario for specimen preparation - microgrid interaction. This continues to be a source of unceasing frustration in our attempts to record high resolution images and measure $F(g)/F(0)$. Despite the fact that probably no more than one specimen in ten was of sufficient quality to offer the possibility of acquiring interpretable data, preliminary measurements were made of $F(g)/F(0)$ for paraffin on microgrids which were fabricated before the severity of problems of non - flatness was fully appreciated.

Out of many hundreds of holes photographed (and many thousands scanned), measurements were collected from only 12. None of these data were acquired using the Dorset technique of direct crystallization; although over half of the attempts to collect data were made using this technique. The 12 images for which $F(g)/F(0)$ were collected were all acquired using the Taylor technique of specimen preparation. Of these, none showed any improvement in the ratio.

In principle, these results represented the accomplishment of the goals set forth at the beginning of this part of the thesis. Although they indicated little or nothing conclusive about the ultimate potential effectiveness of microgrids, these experiments contributed greatly to our appreciation of the microgrid flatness problem. This spawned the second generation of microgrid techniques which are described in Chapter 2. Unfortunately, the Taylor technique, which was invented to circumvent problems associated with the use of the Dorset technique, could not be applied to bonded specimen supports. Resort to the direct application (Dorset) technique by default was met with no more success than in the past. This is not in the least surprising. While we are fairly confident that we are finally producing composite support films which included useable areas in which the films are in contact, the problems of microgrid flatness in other areas of the grid assembly persist.. In fact, non flatness -particularly sagging- probably persists to a degree that for the purposes of direct application of the paraffin specimen, the improved supports are not sufficiently different from previous generations that we would expect the Dorset method to be applicable where it was not previously. At this juncture, it was again necessary to improvise out of another cycle of specimen preparation problems associated with paraffin crystallization. This led to the development of the modified Taylor technique (pg 55).

Data collection using these improved microgrids has proved no less arduous than in the past. 5 high resolution images have been obtained from over 100 micrographs. All images were recorded at a magnification of 50,000X, at ambient temperature. These data are shown in Table 2. The Data were processed according to the procedures set forth in Chapter 3 - Image Processing. Prominent diffraction spots of the paraffin lattice were indexed on a hexagonal lattice for the purposes of entering data into the MRC format data processing program "MMBOX".¹ The indices of the reflections in the quasi-hexagonal system are given in the second column. The integrated amplitude of the computed diffraction spot, the maximum density of the computed transform, and the electron dose (calculated from the corrected $F(0)$), are given in columns 3, 4, and 5 respectively. From these, the values of $F(g)/F(0)$, given in column 6, were calculated according to the formula:

$$\frac{F(g)}{F(0)} = \frac{\text{Spot Integrated Amplitude (Bkg Corr)}}{(\text{Max Dens of FFT}) - (\text{Mean Dens of Bkg})}$$

¹ The paraffin lattice is actually orthorhombic, with systematic absences. The six prominent, quasi-hexagonal reflections, which have Miller indices (2,0), and (1,1) in the orthorhombic system, occur at resolutions of 3.75 Å and 4.1 Å respectively (Zemlin et. al. 1985). See also the footnote at the bottom of Table 2.

TABLE 2 - Microgrid Image Data

Scan #	H,K	Amplitude	FFT Dens. $\times 10^5$	Dose $e^- / \text{\AA}^2$	F(g)/F(0)
0929	1,0	540.2	2.14	4.8	0.0035
	0,1	502.6			0.0032
$\gamma = 112^1$	1,1	533.3			0.0034
0931	1,0	0.0	2.25	5.0	0.0069
	0,1	1117.0			
$\gamma = 112$	1,1	603.3			
0981	1,0	556.0	1.04	1.2	0.0142
	0,1	0.0			0.0091
	1,1	354.2			
0991	1,0	0.0	0.94	0.7	0.0149
	0,1	0.0			
	1,1	328.1			
1009	1,0	394.3	2.42	4.7	0.0026
	0,1	0.0			0.0019
$\gamma = 111.3$	1,1	291.0			

¹ γ is the angle between the a^* and b^* axes, quasi-hexagonal axes representing the first allowed reflections.

The best value of $F(g)/F(0)$ obtained from processing these images is 1.4%. This should be compared with the best value obtained in our laboratory of 1% for conventional flood beam illumination imaging of paraffin on carbon on conventional grids (Downing and Glaeser 1986). Although these results are not conclusive in view of the small number of micrographs, they do contain some intriguing (albeit highly preliminary) trends.

The best ratios of $F(g)/F(0)$ were obtained with the lowest electron doses. This suggests that future work should probably be conducted using a total dose in the range of $1 \text{ e}^-/\text{\AA}^2$ to see if this represents a persistent experimental phenomenon, or simply a random statistical variation resulting from a paucity of data. Ultimately, any explanation of the correlation of better results with lower doses would have to include the fact that $5 \text{ e}^-/\text{\AA}^2$ is too high a dose for paraffin at room temperature. While the diffraction amplitude $F(g)$ fades exponentially with increasing exposure above the critical dose, $F(0)$ increases linearly, thereby producing an apparent decrease in the $F(g)/F(0)$ ratio which is not related to beam induced movement. A correction factor to account for this effect is discussed in Henderson and Glaeser (1985). Doses in the $5 \text{ e}^-/\text{\AA}^2$ are not too high for imaging paraffin at low temperature. In addition to the preceding caveats concerning poor statistics and dose dependency, it should also be borne in mind that only by the acquisition and analysis of large quantities of data can one eliminate the randomizing effects of the phase contrast transfer function resulting from the random defocus of the individual micrographs (see the discussion of experimental methods in Chapter 2). Considerably more data than are presented in Table 2 would also be required to eliminate from consideration micrographs inadvertently obtained from crystals more than one monolayer in thickness, some of which might well exhibit an increased $F(g)/F(0)$ ratio relative to the best presently obtainable from monolamellar crystals.

Recent specimens produced with the modified Taylor Technique have also afforded compelling evidence that specimen preparations which involve crystallization of paraffin from solution are capable of producing extremely good monolamellar crystals over areas underlain by carbon film but almost nothing but thick poly-crystalline deposits over areas of metalized microgrid. These observations, which have been made most recently from areas of the same grid, are strongly indicative of two things. First, it seems clear that the choice of paraffin as a test specimen is not a good one in terms of offering the prospect for acquiring large quantities of definitive data with which to quantify the effectiveness of microgrids. Secondly, these results continue to offer evidence that problems of non-flatness persist with known methods of producing microgrid supports.

Future Prospects - Microgrids

Experiments are currently being conducted to evaluate the flatness of fenestrated parlodion films which have been reinforced with platinum by sputter coating. We do not expect data collection from paraffin specimens supported on these microgrids to be significantly easier, but will attempt imaging of paraffin if any improvement in flatness is evident. There is always the possibility of producing an anomalously good specimen using the modified Taylor Technique as occurred once with the generation of data reported in Table 2.

For specimen applications which involve the settling out of particles or membrane fragments from suspension, the outlook may not be quite so bleak. Although we have not yet investigated the imaging of such specimens (such as purple membrane) it is high on our agenda to do so. This will be complicated somewhat by the problems of locating a good specimen area, and focusing; prosaic but critical issues which, in general, are rendered more difficult by the presence of an underlying microgrid mesh. Based on our work with paraffin, we would expect such complications to be more severe as the density of holes

decreases. Unfortunately, as one increases the open area of the microgrid mesh (i.e. goes to more fenestrated films) the films become more mechanically fragile. This exacerbates the problem of non-flatness and may result in a decreased support capability. With regard to microgrid imaging of such specimens as purple membrane which, even under more auspicious circumstances are difficult to locate, the best approach would probably be to apply lots of specimen and choose holes through which to image at random.

Future Prospects - Carbon Films

It seems fairly clear at this point, at least in a qualitative sense, that thin carbon films produced by indirect evaporation in a hydrogen atmosphere at a pressure of 10^{-4} Torr possess superior properties for the purpose of producing crystalline paraffin specimens. In a very real empirical sense, such films represent a significant advance in the state of the art. These results stand on their own as a lasting accomplishment which, we believe, will stand the test of time; both in our hands, and eventually in other laboratories. We have, however, recently come to the conclusion that in at least one important respect, carbon films produced by conventional methods may still leave something to be desired; particularly with regard to the interpretation of specimen preparations such as those developed in this thesis, which assume that specimens are in direct contact with carbon support films. Recent experiments conducted in our laboratory by Downing have lent credence to a suspicion which the author has held for some time that carbon films produced in the usual fashion, utilizing conventional vacuum technology, are virtually never clean on a molecular scale. Such a hypothesis is plausible if for no other reason than because it is difficult to imagine a means of preventing contamination from forming on newly evaporated films. At a pressure of 10^{-6} Torr, it can be assumed that during each second, a surface exposed to the "vacuum" environment in the evaporator bell jar has impinging upon it approximately one monolayer of potentially adsorbable molecules. A fraction (probably of the order of 0.1% - 1.0%) of

this residual atmosphere consists of hydrocarbons derived from the fore vacuum pump and the oil diffusion pump. If contamination phenomena observable routinely in the electron microscope are any indication, such oil derived, fractionated material should probably be regarded as eminently adsorbable indeed; even if there is no electron beam to aggravate the situation as there is in the EM. Under such conditions, it seems reasonable to ask how one can expect carbon films, which are subject to no special handling measures designed to prevent contamination, can be expected to be free of contamination at least a monolayer thick.

One way to produce a substrate which, conceptually at least, can be assumed to be molecularly clean, would be to deposit a layer of molecules from solution and immediately, from the same solution, deposit a second layer of the same kind of molecules on the first layer. This in fact is exactly what happens when paraffin crystals grow from solution on carbon films to form crystals which are more than one monolayer thick. Recently, Downing has utilized low angle shadowing with platinum/carbon to obtain transmission micrographs which dramatically indicate that the surfaces of monolamellar paraffin crystals on carbon substrates have a rough texture, whereas the surfaces of thicker layers of the same crystals, which are underlain by paraffin, are virtually featureless. There are interpretations of this observation other than the obvious hypothesis that paraffin does not crystallize well on contaminated carbon films. For instance, monolayers of paraffin might simply be inherently less well-ordered in the z direction than are truly 3-D crystals.

Further substantiating the contamination hypothesis however are results from the relatively new technique of scanning tunneling microscopy (STM). At this time, the belief is widely held by workers in the field of STM, that substrates such as graphite (which is fast becoming the standard specimen for the technique of STM), which have been freshly cleaved in oilless vacuum systems at pressures less than 10^{-10} Torr, are nevertheless

contaminated with a non-graphite film of adsorbed material. Some evidence exists that this ill-defined contamination layer may actually be on the order of tens of Angstroms in thickness! (John Clarke's laboratory - personal communication). It is not unreasonable to suspect that specimens prepared at poorer vacuums, particularly oil pumped systems, are even more severely contaminated. Thus it seems clear that in future thinking regarding specimen supports for transmission electron microscopy, we may have to alter our conceptualizations of specimen supports to account for the emerging recognition that specimens probably rarely, if ever, come within Van der Waals bonding range of carbon, or perhaps anything chemically resembling pure carbon. Exactly what this bodes for future attempts to address beam induced movement is not yet clear.

We believe a high priority should be given to the investigation of techniques designed to produce clean (uncontaminated) carbon films and to apply low angle shadowing to determine if monolamellar paraffin crystals grown on such films have a surface texture which resembles the smooth texture of paraffin on paraffin. Such films might well stand a better chance of reducing beam induced movement.

CHAPTER 5 - INTRODUCTION - LABELING

One endeavors to produce better crystalline specimens for imaging with the recognition that we do not yet have at our disposal the technical means to derive truly molecular resolution data from images of such specimens should they ultimately become available.

Nevertheless, we should persist in these worthwhile attempts while simultaneously developing the techniques necessary to overcome the fundamental limitations which presently limit the science of electron crystallography. It was precisely this kind of essential technical development which was reported in the first section of this thesis.

We now report the results of experiments directed toward developing methods to obtain molecular resolution structural data from 2-D crystals of membrane proteins without resort to imaging.

The protein which was utilized in these experiments is bacteriorhodopsin (bR) which has become in the last decade quite famous in the biophysics community. The reasons to study this particular substance are many. bR is easily obtained, it is quite stable, and it is of immense historical (evolutionary and scientific) and biological significance. The reader determined to seek more information about bR is directed to the review articles by Stoeckenius (1979, 1982) and Henderson (1977).

In addition to the usual reasons of expedience cited above, we have chosen to conduct studies of bR because it is the quintessential exemplary specimen in the emerging field of electron crystallography. Specimens of bR were among the first ever analysed by means of this technique. Specimens developed by Henderson in the years since are presently far superior in quality to any other 2-D crystalline arrays; yet the structure is still not known to sufficient resolution to understand the manner in which the protein accomplishes its

biological function. Thus this specimen has come to embody the essential conondrum of electron crystallography.

The central methodology of electron crystallography can be consisely summarized in the following manner.

Intensities from diffraction patterns + Phases from images = The structure of the 2-D specimen

This should be contrasted with the competing technique of X-ray crystallography in which (for large molecules like proteins) the corresponding equation reads:

Intensities from diffraction patterns + Phases from Multiple Isomorphous Replacements¹
= The structure of the 3-D specimen.

Resolution in electron crystallography is presently limited by the resolution of the phases derived from images (probably because of beam induced movement). There is however no theoretical reason why phases form electron diffraction patterns could not be combined with phases obtained by MIR phasing provided that the required isomorphous chemical modifications of the 2-D specimens could be accomplished. The preparation of such chemical derivatives of bR provided the primary motivation for the experiments which are now to be reported.

¹The definition of a MIR and of MIR phasing is too complicated to be fully explained here. The reader is encouraged to consult Cantor and Schimmel, or any good textbook of x-ray crystallography for an explanation if in doubt as to what this important technique involves.

Site Specific Labeling of Bacteriorhodopsin

Attempts to apply MIR phasing in the science of electron crystallography have been infrequent and have met with little success; at least as regards the ultimate determination of high resolution phases. What little work of this kind has been advanced in the scientific literature has appeared under the general description of labeling. (Dumont et al,1981; Wallace et al,1980; and Katre et al,1984). If MIR phasing was not the ultimate avowed intention of the investigators involved in these experiments, it is probably safe to assume that the potential for extending phase information by heavy atom labeling was not far from their minds. The results obtained in these various schemes are somewhat mixed, but all reflect several fundamental limitations which are inherent in any attempt to locate heavy atom labels in electron crystallographic studies.

The vast majority of labels which have utility in X-Ray work (see Blundell and Johnson 1976 for a comprehensive if somewhat dated survey of labels) are of insufficient scattering density to serve as labels in electron diffraction experiments. Figure 15 illustrates this important point, which is due to the fact that electrons are scattered primarily by the shielded coulomb potential whereas x-ray cross sections depend upon the total electronic charge density. As is evident in Fig 15, the total electronic charge density $\rho(r)$ increases more or less linearly with atomic number while the scattering factor for electrons increases roughly at the rate at which electrons populate outer shell orbitals with increasing atomic number (note the superimposed periodicity). There are a few multi-atom complexes however which may be dense enough to use for MIR phasing with electrons. One such compound is tetrakis(methylmercury) (TAMM) which was used by Wallace and Henderson (1980). Another electron - dense substance which has not been tried is tetraiodomercurate which has a distinguished track record in X-Ray work which spans almost the entire history of the technique itself. The HgI_4^{2-} complex like TAMM is potentially sulfhydryl

specific (Clarke et al, 1979). It is not quite as electron dense as TAMM but under favorable conditions might be adequate. A major practical advantage of HgI_4^{2-} is that unlike TAMM, it is available commercially and does not have to be custom synthesized immediately before use. In order to use HgI_4^{2-} as a heavy atom label one would ideally require at least the following several things:

- 1) A sulfhydryl group on the protein molecule (native bR has none) in a meaningful place or places in the case of an MIR experiment;
- 2) Very high "crystallographic" occupancy (say 90%) to overcome the relatively low prediction which one would make for the Delta F change (4%) in a difference Fourier in which one HgI_4^{2-} group is bound per unit cell (Crick and Magdoff - 1956);
- 3) A generous aliquot of good fortune that the label binds covalently to the target sulfhydryl tightly enough that the sample can be handled and data collected but not so tightly that all of the Iodines fall off of the Mercury. There is some evidence from x-ray work that the complex is stable enough to retain tetrahedral Hg-I coordination in a diffraction experiment. The work of Clarke et al represents what is hopefully a kind of worst case scenario viz. linear Hg I coordination.

Point number 3 is not really something we can do much about. Points 1 and 2 however can be addressed with a well conceived chemical protocol.

XBL 806-3391 A

Figure 15

The ratio of atomic scattering factors for heavy atoms (f_z) to that for carbon (f_6) is shown as a function of atomic number, z . The upper curve shows this ratio for x-ray scattering, while the lower curve applies to electron scattering. The electron scattering is less strongly dependent upon atomic number, and also shows more pronounced fluctuations due to electronic shell structure.

Proposed Labeling Protocol (Foster and Taylor 1984)

- A Start with native bR
- B Covalently block all solvent accessible carboxyl groups
- C Proteolytically cleave the protein quantitatively (e.g. with papain)
thereby generating a new carboxyl group
- D Convert this new and unique carboxyl group quantitatively to
a Sulfhydryl group.
- E Attach a thiol specific label

The proposed labeling protocol was inspired in part by the use of water soluble carbodiimides as protein modification reagents in the literature on bR (Renthal et al, 1979). Carbodiimides (hereinafter referred to generically by the acronym EDC which is the abbreviation for the most commonly used reagent of this type) are potentially COOH specific reagents which have been used to assay the number of carboxyl groups present in proteins (Hoare and Koshland, 1967). Thus the proposed protocol is potentially quantitative while simultaneously addressing the issues of label specificity, and interpretability of label location; which have been major weaknesses of previous attempts to interpret the results of bR labeling experiments. With these encouraging advantages in mind, experiments based on this solution chemistry labeling protocol were undertaken in the fall of 1984. These experiments eventually culminated in the development of the labeling protocol illustrated in Figure 16.

XBL 877-3293

Figure 16. bR Solution Labeling - Generation 3

(a) Solvent accessible carboxyl groups are covalently blocked with AES which conserves the net negative charge of the protein molecule. (b) The blocked molecule is proteolytically cleaved with papain removing the 17 residue carboxy terminal polypeptide and generating a new, unblocked carboxy terminus. (c) The new carboxy terminus is covalently reacted with a disulfide reagent generating a chemical moiety at that location which can be reduced to a free thiol (step not shown) suitable for labeling with sulfhydryl - specific heavy atom labels.

CHAPTER 6 - BIOCHEMICAL LABELING METHODS

Methods devised to accomplish the labeling scheme described in Chapter 5 will now be described in detail. The protocols are presented in chronological order, a reflection of the evolution of the experiments in response to various technical obstacles encountered throughout the approximately 18 month period which preceded the final experiments in which stoichiometric labeling was finally achieved. In all protocols, the steps are described in sufficient detail that they could be conducted without reference to other biochemical literature. To facilitate an understanding of the biochemical rationale behind these admittedly somewhat complicated procedures, and to preface the discussion to be presented in Chapter 7 of results, the protocols are classified as belonging to one of three distinct generations of experiments. Within each generation, protocols are presented in the sequence in which they are actually to be performed.

Throughout the labeling experiments, numerous technical problems were encountered which resulted either in aborted experimentation, or in products in which stoichiometry was highly suspect. Many of these problems, which had to be overcome before quantitative conversion of the carboxy terminus of cleaved bR to a free thiol could be accomplished, are discussed in Chapter 7 under the heading of "results". To appreciate the evolution of the experiments into distinct generations however, it is sufficient to appreciate only one aspect of this type of work. Bacteriorhodopsin is a membrane protein. As membrane proteins go it is very well behaved and relatively easy to work with. It is nonetheless a membrane protein and as such it is not very soluble; relative to proteins for which virtually all covalent modification techniques have been previously invented. bR is frequently manipulated chemically and physically but only in one of two forms: as a suspension of membrane fragments (purple membrane), or as detergent solubilized monomers. In neither of these two forms can it be said, or should it be assumed, that the bacteriorhodopsin protein

molecule is truly in solution in a classical sense. With this perspective clearly established, it should come as no surprise that initial attempts to apply aqueous solution phase protein modification techniques to a biphasic (hydrophobic/hydrophilic) system were plagued by problems of keeping the protein, and its sundry chemical derivatives "in solution" i.e. accessible to dissolved reagents.

Generation 1

Generation 1 experiments consisted of initial brute force attempts to react the purple membrane suspension in all phases of the labeling protocol with dissolved reagents in aqueous solution as if the protein molecule was in solution; like a globular protein such as haemoglobin. These experiments, particularly those involving derivatized and proteolytically cleaved protein, were constantly menaced by the membrane falling out of suspension into a precipitate, or a very hard pellet in the bottom of a centrifuge tube.

Generation 2

To address chronic problems of solubility encountered in the first generation of solution phase experiments, Generation 2 experiments were spawned (see Fig 18) in which the covalent derivatization of the detergent solubilized bR molecule was attempted on a "solid phase" support - a thiol-agarose chromatography gel. To our knowledge this is the first time that so called "covalent chromatography" has been attempted on a membrane protein.

Generation 3

Technical problems (this time not exclusively related to solubility) with Generation 2 experiments led to return to solution chemistry with purple membrane in suspension. These experiments employed reagents chosen to yield a derivatized bR molecule having maximum potential solubility in aqueous solution (maximum net negative charge on the derivatized protein molecule).

Protocols will now be given for the labeling of bR. Generation 1 was not particularly successful so only the last two sets of experimental methods are given. Generation 1 protocols were similar enough to those of generation 3 that they could be reconstructed from the generation 3 methods with reference to the discussion of results for generation 1 which is given in Chapter 7.

Protocol 1 - Generation 2

Generating an S-S-NH₂ Column from Affi-Gel 401¹

- 1). Withdraw the required amount of Affi-Gel which is supplied in a reducing buffer which is 10 mM Tris, about 10 mM BME², and 0.2% Na azide. A typical quantity for the small disposable columns which we use is 6 ml of slurry which is comprised of about 75% bed volume. Lots are processed in a 15ml plastic tube.
- 2). Allow the gel to settle and after discarding the supernatant wash the bed thoroughly with 10mM Tris, pH 7.5, containing .05M DTT (MW - 154.3) and .001M EDTA (MW - 336.2). (771 mg DTT + 34 MG EDTA to 100 ml) This removes the BME.
- 3). Resuspend the gel in the DTT/EDTA buffer and mix gently for an hour or so. Replace the supernatant then repeat the mixing. For our experiments the mixing was performed by tumbling a sealed tube end over end at 4 RPM at 4 degrees C with 1/2 hour allowed for settling between each cycle. This should reduce the gel-bound SH groups completely.
- 4). After the second wash allow the bed to drain in a column and wash thoroughly with pH 7.4, 10m mM NaAc. Periodically collect 10 drops of supernatant and mix with an equal volume of DTNB solution. Continue the wash until no free SH is detected in the effluent.

¹ Affi-Gel 401 is a thiol-agarose affinity chromatography gel available from Bio-Rad Laboratories, Richmond, CA. The gel contains free SH when properly regenerated in a nominal concentration (based on total gel volume) of 5mM.

² Chemical abbreviations: BME, β -mercaptoethanol; DTT, dithiothreitol (Cleland's reagent); DTNB, 5,5'-dithiobisnitrobenzoic acid (Ellman's reagent); EDTA, ethylenediaminetetraacetic acid; DMSO, dimethylsulfoxide;

For 3-4 ml of bed this should take about 5 refills of the column which holds about 12 ml total volume.

5). At this point the gel can be assayed for free SH content qualitatively with a little DTNB or quantitatively according to the protocol SH.DET (given next). The gel can be used immediately or stored in DTT/EDTA.

6). Prepare 100 ml of 10 mM NaAc buffer, pH 7.4 containing 20 mM cysteine ethyl ester-HCl, .05M NaCl, and 2 drops of trace elements stock solution¹. This buffer is made by adding 371 mg of cysteine ethyl ester -HCl (MW - 185.7) to 80 ml of buffer, adjusting the pH back to 7.4 with 1N NaOH and bringing the volume to 100ml after adding 290 mg of NaCl. The trace elements solution contains divalent metal ions which catalyze the oxidation of thiols to mixed disulfides. Upon addition of the trace elements to the final 100ml of buffer, a purple caste will appear in the previously colorless solution. This is normal and represents, we believe, the reduction of Mn from the +3 to the +2 oxidation state.

7). Chase the column with one wash of the step 6 buffer then mix the gel with about twice the volume of said buffer in a new plastic tube. Tightly cap the tube and rotate as in step 3 for an hour. The old tubes could not be rinsed free of the smell of DTT which is the reason for using a new one. Allow the gel to settle then discard the supernatant.

8). Repeat step 7 but retain the supernatant over the gel and cure tightly capped in the refrigerator for several days prior to use.

¹ For a 100ml stock of trace elements solution dissolve .0218 gm of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (MW 197.9) in 100ml of distilled water then dissolve .358 gm of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (MW 198.8) in that order. Sterilize by filtration or autoclave. This solution is also used in the growth medium for *H.halobium*.

SH.DET

This protocol is referred to in step 5 of the preceeding protocol. It utilizes DTNB to assay for free thiol.

Solutions Required:

Soln A: 10 mM Tris, pH 8.5 - 100ml

Soln B: 5 mM DTNB (MW - 396.4 Pierce) - 20 mg in 10 ml of 10 mM NaAc, pH 5.0.

10 ml of buffer can be made by combining 2 ml of .05M NaAc and 8 ml of water.

Soln C: Soln A also containing 50 mM DTT (MW - 154.3 - Calbiochem; 771 mg for 100 ml of solution.

Soln D: Soln C also containing .001M EDTA- disodium salt (MW - 336.2 Sigma) 3.36 mg for 10 ml of solution)

All solutions except solution A should be prepared immediately prior to use.

- 1). To a 1ml aliquot of reduced/washed gel, add 8 ml of Solution A.
- 2). Add 1ml of Solution B.
- 3). Mix the reactants then allow gel to settle or centrifuge briefly at low RPM.
- 4). Withdraw 1ml of the supernatant and record the OD at 412 nm.

NINHYDRIN ASSAY OF DERIVATIZED AFFI-GEL FOR FREE AMINE

Ninhydrin should be capable of detecting the presence of free amino groups covalently bound to the gel after oxidative coupling to cysteine esters and subsequent washing to remove unbound amines. The literature indicates that accurate quantitative detection of amino acids in aqueous solutions e. g. in chromatography column effluents may be complicated by several factors. Color development resulting from the reaction of ninhydrin with free amine is due to the presence in ninhydrin solution of the fully reduced form of the substance hydrindantin (Moore 1968). The amount of this form present in aqueous solution is a function of the overall redox environment of the reaction mixture and therefore may dependent on the quantity and characteristics of reducing agents included in the solution; deliberately or otherwise. Not surprisingly, in view of this, the amount of dissolved oxygen has been found to effect the color yield significantly (Moore 1968). These factors are compounded by the fact that the reduced form of ninhydrin is almost insoluble in water. This has led to the development of ninhydrin reagents which are currently available commercially (Sigma) which contain DMSO as a solubilizing agent. Accurate amine quantitation should probably employ such a commercial preparation. For a rough preliminary assay of the course of the oxidation of our thiol agarose gel (a destructive and relatively controlled procedure), it should be possible to employ the ninhydrin preparation usually reserved for the detection of amino acids and the like on thin layer chromatography plates. This consists of 0.2% ninhydrin dissolved in absolute ethanol. Although ninhydrin is more commonly used to detect primary amines, the detection of amino groups bound to the affi gel as well as the detection of amino groups on the amino acid cystine on thin layer chromatography plates have both been accomplished using the following protocol.

Standardization:

- 1). Dissolve about 20 mg of ninhydrin (MW 178.14) in absolute ethanol to 10ml volume.
- 2). Dissolve 4.2 gm of citric acid monohydrate (MW 210.14) in 40 ml of 1N NaOH and bring to 100ml with distilled water. Adjust the pH to 5.0 .
- 3). Add 34 mg of cystine dimethyl ester -HCl (MW 341.3) to the solution from step (2). This should produce about a 2M amine concentration.
- 4). Mix 1ml of each of the solutions from steps (1) and (3).
- 5). Heat to develop the ninhydrin color.¹ Boil the solution in a Kimax screw-top test tube for 20 min in a water bath to develop the color. Read and record the OD.

Gel Assay:

- 1). Prepare 100ml of solution of citrate buffer as in step (2) above.
- 2). Wash a 2 ml bed of the Affi-gel free of DTT with 10 mM Tris, pH 7.5 until no free sulfhydryl is detected in the effluent with DTNB.
- 3). Resuspend the gel in about 2 ml of Citrate buffer.

¹ For a first attempt heating the solution appeared to be having no effect so the solution was poured on filter paper and the paper dried on a hot plate. The characteristic purple color developed quickly. This procedure would be difficult to quantify but it restored faith in the chemistry involved in an hour of great need. The procedure set forth in step (5) above was then developed.

4). Mix the gel with an equal volume of ninhydrin solution from step (1). Allow the mixture to settle then develop the color. Read and record the OD and calculate the ratio. Compare to the standard to compute the concentration of the unknown.

COUPLING OF TRITON SOLUBILIZED bR TO AFFI-GEL 401 - GENERATION 2

1). Drain the cysteine solution from 7 ml of amino coupled gel and wash the bed free of free amine with solution A.

Solution (A) 0.05 M NaCl (290 mg)

.33 % Triton (6.6 ml)

Adjust pH and volume to 4.5 and 100 ml.

2). Prepare the following solutions:

Solution (C): EDC (MW 191) - 200 mg (100:1)

NaCl (MW 58) - 300 mg (.05 M)

5% Triton ¹ - 6.6 ml (.33%)

pH to 4.5 (HCl) and volume to 50 ml

Solution (B) 30 ml of solution (C) diluted to 60 ml.

3). Wash the bed with the 30 ml of solution B just prepared. Stop the wash with the meniscus just above the bed.

4). From 15 mg of bR in 1 ml 5% Triton, withdraw 7mg. Typically, this will be roughly 1ml in volume.

¹ All Triton should be scintillation grade. Old or impure polyoxyethylene detergents may contain peroxides.

- 5). Dilute it to 3.5 ml (1/2 the bed volume). The bR should now be at a concentration of about 2 mg/ml.
- 6). Mix it with an equal volume of Solution C (this can be done conveniently in the vessel in which soln B was made up).
- 7). Place the reactants over the gel bed and allow them to flow in until there is almost no bR soln remaining above the gel.
- 8). Stop the flow and store the column at 4 degrees in the dark for two hours to allow coupling but hopefully a minimum amount of cross-linking to occur.
- 9). Terminate the reaction by draining and washing with pH 4.5 acetate buffer. Collect the effluent for possible study by PAGE.
- 10). To recover the derivatized protein, wash the gel with the following reducing buffer.

Elution Buffer:

50 mM NaPi pH 7
50 mM DTT (MW - 154)
.1 % Triton
1mM EDTA (MW - 336)
50 mM NaCl

PROTECTIVE BLOCKING OF SURFACE CARBOXYLS OF bR -GENERATION THREE

This procedure is used to block all solvent accessible carboxyl groups prior to generating and labeling the carboxy terminus of papain cleaved bR. No sucrose density gradient purification is done. After harvesting and repeated centrifugation to remove impurities, the membrane is stored over sodium azide. Just prior to use, the azide should be removed by washing 3 times at 35,000 RPM for 30 minutes.

- 1). Resuspend the pellets from the final wash in several ml of distilled water.

The reaction conditions are:

pH 4.75 ¹

EDC : bR = 1000 : 1

.05M NaCl

bR conc = 1 mg/ml

AES conc. = 10X the EDC conc.

- 2). Prepare 100 ml of .05M NaCl and 100 ml NaOH and HCl

- 3). To about 40 ml of the NaCl solution, add 2.6 gm of AES ² (MW 125.1). Adjust the pH with a few drops of 1N NaOH then .01N NaOH to 4.75.

- 4). Rinse the bR into a single tube with another 20 ml of the NaCl solution.

¹ See Hoare and Koshland (1967)

² AES -aminoethanesulfonic acid

5). To the remaining 40 ml of the NaCl solution, add 400 mg of EDC¹ and adjust the pH to 4.75.

6). To start the reaction, combine the solutions in a small beaker and adjust the pH to 4.75. This should require only 1 drop of .1N HCl. Place the beaker on ice and continue the reaction for 2 hours. Check the pH and adjust as required at 15 min intervals during which the solution should be stirred briefly with a magnetic stirrer. There should be an initial drop in pH of about .1 unit in the first 15 minutes then stability.

7). After 2 hr place the reactants into 4 centrifuge tubes and spin at 35,000 RPM for 30 min. The pellets will be very hard.

8). Dislodge the pellets from the tube using a disposable pipette which has the tip melted into a glass bead. Rinse them into a teflon piston homogenizer with 50 mM NaCl. After homogenization and recombination into a single tube, sonicate the bR in an ice water bath for 5 min.

9)² To 40 ml of 50 mM NaCl solution add 1.5 gm of AES; adjust the pH to 7.4.

10). To this solution add 200 mg of EDC and reset the pH.

11). Combine this with the resuspended bR to start the second reaction sequence.

¹ 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide

² Steps 9 + are an optional second reaction sequence to insure quantitative reaction of all solvent accessible carboxyl groups. Although it might not be necessary, a second blocking reaction was performed in the experiments for which we report results of generation 3 labeling.

- 12). Continue the reaction as above and terminate it after 2 hr in the following manner.
 - 13). After centrifugation, remove the supernatant and rinse the pellets with distilled water.
 - 14). Soak the pellets overnight in a small quantity of pH 4.5, 10 mM NaAc kill buffer.
- Resuspend them immediately prior to papain cleavage.

PAPAIN CLEAVAGE OF BLOCKED BACTERIORHODOPSIN

- 1). Suspend purple membrane at a concentration of 0.5 mg/ml in cleavage buffer:

Cleavage Buffer - 50 ml stock:

Slightly less than 50 ml of 10 mM Tris, pH 7

.044 gm cysteine - HCl (MW 175.6)

.1 ml of 100mM beta mercaptoethanol - make afresh in the hood by

weighing 9.8 gm of distilled water into a vial and adding 143 λ (microliters) beta mercaptoethanol¹ (MW 78.13). Increase the volume to 50 ml.

- 2). Add papain (MW 21,000) to a final concentration in the reaction mixture of .0065 mg/ml (normally around 5-7 λ for reaction volumes of about 50 ml when the papain is supplied in suspension as from Sigma).
- 3). Incubate for 2 hr at room temperature or a little above.
- 4). Centrifuge at 18 ,000 RPM for 50 min. Discard the supernatant. The pellet will be very hard so it can be rinsed several times with a few ml of distilled water. It is very important to get the papain out of the membrane suspension as soon as possible. A few residual molecules of papain can do extensive undesirable proteolysis if left in with the membrane for periods longer than a few hours - not to mention days of storage. Although an organo-mercurial inhibitor (PHMB) is included in the following steps of the protocol, because

¹ BME is a liquid at room temperature. Pure BME has a "concentration" of 14.26 M.

papain is very difficult to permanently inhibit, the following procedures are designed to remove as much residual protease as possible.

5). Resuspend the pellet in distilled water; using homogenization and ultrasonication as was done in the blocking protocol if necessary.

6). Withdraw a gel sample ¹

7a). Dialyse twice against 1 liter of 10 mM Tris at pH 7.0 containing 0.3mM (100mg) parahydroxymercurobenzoate (PHMB - MW 360.70) or:

7b). If the pellets can be resuspended relatively easily, wash 4 times by centrifugation at 18,000 RPM for 40 min. This is preferable to attempting to inhibit the papain with PHMB which isn't an irreversible inhibitor. In addition, because PHMB might seriously interfere with subsequent steps of the labeling protocol, it is preferable to avoid its use entirely. There are to our knowledge no reliable, proven papain inhibitors.

8). Resuspend the pellets from the final spin in a few ml of distilled water. If the membrane is not going to be used in the next stage of the labeling protocol immediately, flash freeze it in liquid nitrogen. Storage in this form is additional insurance against unwanted additional proteolysis by residual papain.

Glassware exposed to papain should be washed in chromate. Papain has been known to survive other methods of cleaning and ruin subsequent experiments. Plastic centrifuge tubes should be avoided, discarded, or retained for use exclusively in these stages of the

¹ Use a non-reducing gel buffer to avoid reactivating residual papain.

protocol. This reaction does not use EDTA in the papain cleavage buffer as is customary. This was done to obviate the possibility that the purple membrane, which has already been washed or dialysed repeatedly, might undergo a transition to the divalent cation - deficient blue form¹. The use of cleavage buffers which do not contain EDTA has recent precedent (Liao and Khorana, 1984). PAGE studies in our laboratory show no inhibition of the proteolytic action of papain as a result of the elimination of EDTA from the cleavage protocol.

¹ bR which has been stripped of non-covalently-bound divalent cation counter ions undergoes a reversible conformational change to a species which appears blue instead of purple. This conformation is not believed to be sufficiently drastic to expose "buried" carboxyl groups to the aqueous phase, but it is obviously prudent to avoid such a transition in the middle of the labeling protocol.

SOLUTION PHASE CYSTAMINE COUPLING - GENERATION 3

This procedure covalently attaches a disulfide reagent (cystamine) to the carboxy terminus of papain cleaved bR which can subsequently be reduced to generate a free thiol.

Conditions: bR Conc : 0.5 mg/ml - $.192 \times 10^{-4}$ M

pH: 4.75

Reaction volume: 28 ml

EDC Conc: bR x 1000

Cystamine Conc: 2 gm in 28ml

.05 M NaCl

- 1). Prepare 50 ml of 0.05 M NaCl and pH it to 4.75 with .01N HCl
- 2). To 10 ml of this add 100mg EDC and readjust the pH with HCL
- 3). To 14 ml of 1). add 2 gm of cystamine; pH with NaOH cystamine is supplied as a di-HCL salt so the pH goes down to 3.4 or so upon addition of the cystamine.
- 4). Combine 2. & 3. and adjust the pH.
- 5). Add 4 ml of bR (native purple membranes in distilled water)
- 6). Monitor the pH of the reaction mixture which will require periodic (very slight) adjustment upward for the first half hour or so.

7). Run the reaction for two hours at room temperature then centrifuge the membrane out at 25,000 RPM for 20 min.

8). Wash the membrane five times to remove EDC and such.

The membrane can now be reduced with DTT and washed to deprotect the sulfhydryl groups prior to Hg labeling

DETERMINATION OF MONOTHIOIOL CONTENT OF MODIFIED bR

- 1). Take 0.2 ml of purple membrane containing about 300 micrograms of protein and mix it with an equal volume of 1% SDS (sodium dodecyl sulfate).
- 2). Add 0.1 ml of 50 mM Tris-HCl, pH 9.0 and 0.1 ml of 3 mM dithiothreitol.
- 3). Incubate for 60 min at room temperature.
- 4). Add 1.0 ml of 0.2 M Tris-HCl, pH 8.1 and 1.5 ml of 5mM NaAsO₂.
- 5). Incubate 5 more minutes at room temperature.
- 6). Add 0.1ml of 3 mM DTNB in 50 mM acetate buffer, pH 5.0.
- 7). Record the absorbance at 412 nm at intervals of 1 min for 15 min.
- 8). Determine by extrapolation the absorbance at the time of DTNB addition. Subtract the value for a sample containing no protein.

This assay - taken from Zahler and Cleland (1968) - is a spectrophotometric determination of carboxylatonitrothiophenolate (CNTP) anion released by reaction of DTNB with monothiois. The molar extinction coefficient of this anion is 13,600.

CHAPTER 7 - RESULTS AND CONCLUSIONS - LABELING

Summary of Generation 1: Blocking reagent: Glycine Ethyl Ester

Kill Method: Acetate or PHMB

Sulfhydryl protection: Disulfide

Coupling reagent: Cystine Dimethyl Ester

Purity assay by thin layer chromatography -ninhydrin detection.

Summary of Generation 1 Results:

SUCSESSES:

HgI is highly soluble
in excess NaI

Electron diffraction
results promising (Fig 17)

pH shocked proteins
turn blue - purple¹ even
after modification

FAILURES

Severe aggregation / resuspension
problems; post blocking
and post coupling.

pH shocks during coupling and labeling
are difficult to avoid. Most buffers interfere with
EDC reactions.

Hg assay on final product was
ambiguous

¹ Evidence of "normal behavior" i.e. little or no denaturation. See footnote 1 on pg 124.

Purple membrane which had been through the multi-phase modification procedure still showed high resolution electron diffraction. High resolution diffraction patterns from frozen hydrated specimens were fairly reproducibly obtained and showed one provocatively interesting feature. As is shown in Figure 17, almost all patterns obtained manifested strong mirror symmetry consistent with the interpretation that the patterns represent diffraction from membrane patches in intimate face to face crystallographic register. This type of bonding could be quite plausibly attributed to the formation of disulfide or S - Hg - S bonding between reacted sites on bR molecules in adjacent membrane patches which correspond to the target of the labeling attempt (the papain cleavage site near the carboxy terminus).

This was regarded as highly encouraging. Not only did the membrane's crystallinity survive the extensive manipulation necessary to accomplish the covalent modification; but evidence was obtained as well that the procedure had achieved results detectable in an electron diffraction experiment. A typical diffraction pattern is shown on the following page.

The technical obstacles encountered in the initial solution chemistry labeling protocols were probably no worse than might have been predicted for attempts to employ aqueous protein modification techniques on a system as hydrophobic as bR. Insofar as the ultimate goals are concerned however, particularly that of near unity labeling stoichiometry, at least one problem—that of aggregation—should probably be realistically regarded as potentially fatal. Not inconsistent with this pessimistic view were the results of a preliminary Hg assay which, while by no means hard, suggested a very low yield. With these liabilities in mind, a more sophisticated labeling scheme directed at the objective of achieving quantitative labeling was hatched. The scheme was born of the idea that one might be able to effect simultaneously the aims of high reaction stoichiometry and modification reagent sulfhydryl

Figure 17

Computer processed diffraction pattern obtained from covalently modified, papain cleaved purple membrane. Native purple membrane diffraction patterns exhibit obvious handedness. By contrast, note the strong centro-symmetry and register of reciprocal lattices evident in this pattern, which indicates coherent patches in register, face-to-face.

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group protection by means of the following scheme. Coupling experiments of this kind come under the heading of covalent chromatography.

Figure 18. Covalent Chromatography of Bacteriorhodopsin

RESULTS: Second Generation experiments

After some initial problems having to do with regenerating the sulfhydryl agarose gel were worked out the covalent attachment of native (uncleaved) triton solubilized bacteriorhodopsin to the gel was achieved. The attachment of the protein can be easily and immediately be determined because of the presence on the protein molecule of the covalently attached retinal chromophore. Thus the gel was observed to become purple after the coupling reaction. This color was subsequently observed to persist despite repeated washes and to elute (and be recovered) upon treatment with reducing buffer containing excess dithiothreitol. These facts among others were taken to confirm the feasibility of the COOH specific solid phase reaction scheme and the labeling of papain cleaved bR was attempted.

At this point two problems occurred which for the time being spell doom for the solid phase generation of experiments. First, the coupling of native bR, which was intended to serve as a control for future experiments, proved difficult to reproduce. It was found that the covalent coupling was fairly reproducible but that elution under reducing conditions was not. Secondly, it was found that papain cleaved protein would not couple to the gel in the time required to achieve reaction of the native (uncleaved) protein (12hr). Reaction times in excess of this produce a pink solution which is believed to represent the result of the reaction of "buried" carboxyl groups which are reactive to hydrophilic reagents when the protein is detergent solubilized.

Dr. Bing Jap who is experienced with hydrophobic DCCD modification of bR is in agreement with the plausibility of this hypothesis. In fact these are not the only problems with the solid phase scheme which while perhaps conceptually elegant is apparently more difficult than originally anticipated.

Shortly after the solid phase generation of experiments were concluded a new solution phase plan was devised. The new coupling scheme was inspired by the appearance in the literature (Wu-Chou et al - 1984) of a reagent - amino ethane sulfonic acid (AES), which can be used to block the carboxyl groups of bR in a carbodiimide reaction scheme. Unlike other blocking reagents used in the past by others (Renthal for example); and unfortunately by us, AES carries a permanent negative charge. Thus it can be used to block carboxyl groups while conserving the negative surface charge associated with the native membrane. It was decided that the use of this reagent in the protocol of the early solution phase labeling might substantially improve our chances of achieving high yields in the blocking and coupling reaction by reducing the persistent aggregation which so severely plagued the early experiments. This was in fact found to be the case and the third generation of labeling protocols was developed.

Third Generation Solution Phase Labeling - AES Blocked:

The initial experiments in this generation ended on an encouraging note. The rather badly aggregated product of the SH coupling reaction was observed to resuspend more easily after the addition of DTT. Labeling was not immediately attempted, because of the difficulties encountered earlier in obtaining hard results on the amount of mercury bound.

Summary of Generation 3:

Blocking Reagent:	AES
Kill Method:	Repeated Washing
Sulphydryl Protection:	Disulfide
Coupling Reagent:	Cystamine

Cystamine - which is the compound cystine without the carboxyl groups - was used instead of cystine dimethyl ester for the following reasons. When the solid phase coupling reactions began to produce coupling to the support which was not cleavable with disulfide reducing agents, an explanation was sought. We suspect that the main component of this non-cleavable -apparently covalent -reaction is due to the reaction of residual free sulphydryl groups on the gel with the protein. We think it probable that the oxidative conversion of the gel from free SH to free amine is not always quantitative and is difficult to drive to completion reproducibly. Free sulphydryl groups compete with about the same kinetics with nucleophilic amino groups in carbodiimide reactions ; necessitating sulphydryl protection in the final step of our covalent modification scheme. While formulating this hypothesis however, we consulted Dr Alex Glazer who raised the question of whether the esters which were present on our reagents were stable to hydrolysis under the conditions

employed in the coupling reactions. At the pH (4-5) used in the reactions we do not believe this is a problem (the use of esters as blocking reagents is not unprecedented- see e.g. Renthall et al). It was however a point with which we did not reckon at the outset, and we therefore elected to obviate the question by choosing reagents which don't have esters appended to them for no reason other than to protect superfluous carboxyl groups. Fortunately, there is no problem with respect to the solubility of cystamine which is in fact probably more soluble than methyl and ethyl esters of cystine.

The results of generation three labeling are shown in Figure 19, which indicates essentially complete conversion of the papain cleavage site from carboxyl to sulfhydryl. With this accomplished, labeling of the new thiol group with sulfhydryl specific heavy atom labels will be undertaken.

We believe that these experiments should be repeated on bR which has been sucrose density gradient purified. Labeled specimens should be submitted (e.g. to General Activation Analysis Inc. in San Diego) for Hg assay by neutron activation. The approximate cost per sample would be around \$200. Our investigations of presently available methods of Hg analysis have left us with the strong conviction that NAA is by far the best technique for this analysis. No matter what technique is employed (Atomic absorption is the only viable alternative) the business of mercury in biological materials is not something which should be attempted by amateurs, no matter what facilities might be available on a do-it-yourself basis.

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Figure 19

Monothiol assay of covalently modified, papain cleaved bacteriorhodopsin in the presence of the reducing agent dithiothreitol and sodium arsenite; according to the procedure given on page 127. The upper curve shows the color development of the unknown modified sample starting with the addition of DTNB at time $t = 0$ minutes. Extrapolation to time $t = 0$ gives the contribution to the absorbance which is due to the free thiol present on the modified protein. The bottom curve is a control done with native purple membrane. Comparison of the molar concentration of thiol on the protein with the known protein concentration indicates labeling stoichiometry of 0.99 SH per mole of protein.

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