Advanced Techniques in BIOLOGICAL ELECTRON MICROSCOPY

Edited by James K. Koehler

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With Contributions by

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With 108 Figures

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Preface

The past decade has seen a remarkable increase in the use of electron microscopy as a research tool in biology and medicine. Thus, most institutions of higher learning now boast several electron optical laboratories having various levels of sophistication. Training in the routine use of electron optical equipment and interpretation of results is no longer restricted to a few prestigious centers. On the other hand, techniques utilized by research workers in the ultrastructural domain have become extremely diverse and complex. Although a large number of quite excellent volumes dedicated to the basic elements of electron microscopic technique are now available which allow the novice to acquire a reasonable introduction to the field, relatively few books have been devoted to a discussion of more advanced technical aspects of the art. It was with this view that the present volume was conceived as a handy reference for workers already having some background in the field, as an information source for those wishing to shift efforts into more promising techniques, or for use as an advanced course or seminar guide.

Subject matter has been chosen particularly on the basis of pertinence to present research activities in biological electron microscopy and emphasis has been given those areas which seem destined to greatly expand in usefulness in the near future. It would be impossible to adequately cover the myriad technical developments available to cell biologists interested in fine structure within this modest volume, and the knowledgeable reader could cite many worthy areas that have not been touched upon. Nevertheless, the subject matter included will be seen to cover a number of subdisciplines within the ultrastructural area and is not restricted to a narrow range of interests.

Not only have considerable improvements been made in the design and use of conventional transmission electron microscopes, but entirely new instruments have appeared on the scene such as the scanning and high voltage electron microscopes which provide quite different kinds of information and require modified or new methodologies of specimen preparation and manipulation. The potentialities of these tools are explored in chapters by T. L. HAYES and K. HAMA, respectively. Alternatives to chemical fixation and staining have been devised, particularly those employing freezing methods of preservation. The freeze-fracture or freeze-etching approach has been particularly fruitful in this regard and is the subject of S. BULLIVANT'S contribution. Substitution methods, either with or without freezing involvement, have also turned out to be successful alternatives and complementary

approaches to traditional chemical methods of tissue preservation. These techniques and the unique inert dehydration method form the subject of the chapter by D. PEASE. Even the subject of embedding materials for fine structural studies (a field that many probably considered closed after the advent of epoxy resins) receives an imaginative reappraisal and infusion of new ideas in the article by J. LUFT. Although autoradiography has for many years been included in the repertoire of electron microscopy, the urge to incorporate more specificity and dynamic information into ultrastructural studies has recently caused many more laboratories to turn to this technique. Consequently, it may seem to be considered an almost routine part of the technology of fine structure workers. M. SALPETER and F. A. MCHENRY, however, indicate in their contribution the great care that one must exercise in optimizing considerations of resolution, specificity and morphology in addition to the many pitfalls which stand in the way of accurate interpretation of electron microscopic autoradiography. Finally, at the physical end of the spectrum, J. FRANK illustrates the possibilities opened up by computer processing of electron optical data and details the mechanisms by which such information can be obtained.

I wish to express my sincere appreciation to these authors for the time and effort which they have expended on these contributions and for their patience and understanding during the editorial processing. Dr. KONRAD F. SPRINGER and the Springer-Verlag have been most cooperative in this venture and have striven to achieve the highest quality possible in word and picture.

Seattle, February 1973 J. K. KOEHLER

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Computer Processing of Electron Micrographs. J. FRANK

Embedding Media — Old and New

JOHN H. LUFT

A. Introduction

Embedding media for microscopy have no other value than that of a convenient means to achieve a particular end, namely, to enable the object of interest to be cut sufficiently thin for the microscope to develop its full resolution. The embedding does not contribute to the staining of the object nor to the resolving power of the microscope. The best embedding medium permits thin sectioning with the least damage during specimen preparation and gives the least interference during microscopy. This is not to say that embedding is a trivial part of specimen preparation; the cutting of the tissue in the embedding matrix is a mechanochemical event which can be interpreted only in terms of sophisticated concepts of the properties of materials. The most fundamental approach to the problem in the biological literature is that of WACHTEL, GETTNER and ORNSTEIN (1966). Useful mechanical concepts are developed in various texts, such as NIELSEN (1962) or MCCLINTOCK and ARGON (1966) and mechanochemical concepts in the paper by WATSON (1961). Despite the advanced state of materials science, it has had little impact in improving our understanding of the mechanism of the cutting of embedded tissue, beyond what is intuitively obvious to biologists. It is clear that the embedding medium "supports and holds together" the tissue, but this phenomenon seldom is encountered in industrial processes where a detailed analysis is sufficiently important to engender research. It is possible that embedding material can be compared usefully to the matrix in composite materials, but that it functions in embedded tissue to produce an effect opposite to that intended for industrial laminates and composites.

B. Early Embedding Media

Before discussing the newer embedding materials, it would be profitable to examine the advantages of the older media as well as whatever faults may have prompted the search for substitutes. A glance at any edition of Lee's Microtomist's Vade-Mecum (LEE, 1928) suggests the variety of materials which have been explored for embedding. Included are "fusion masses" such as paraffin, soap and gelatin; and "cold masses" such as celloidin

(nitrocellulose), lead-gum, gum arabic-glycerin and shellac, as well as gums and sugar syrups to ptotect tissues during freezing. Of these, gelatin, celloidin and paraffin are worth examining in some detail along with the more recent methyl and butyl methacrylates.

I. Gelatin

Gelatin appears to have been the first embedding medium with a reference in LEE dated 1802. Later procedures in the 1880's consisted of soaking the fixed and washed tissue in warm gelatin solutions over a period of days to a week in increasing concentrations up to 25% gelatin and 10% glycerin, which after cooling, was hardened with formaldehyde and the gel cut wet. The defects were that the gelatin was a large molecule which gave viscous solutions requiring prolonged infiltration times, and that the gel was not strong enough to permit even moderately thin sections to be cut. However, it avoided temperatures above 35 °C and did not extract or displace lipids, since the tissue was never dehydrated. Shrinkage could be kept very low.

II. Celloidin

Celloidin embedding was introduced in 1879 and consisted of dehydrating the fixed tissue with absolute ethanol and then gradually infiltrating the tissue with increasing concentrations of nitrocellulose dissolved in an ether-alcohol mixture from 1—2% to 10—15% over a prolonged period. A small piece of tissue might take 2—3 days whereas an entire human embryo could require months. The most concentrated nitrocellulose solution was allowed to thicken further by very slow evaporation, and the nitrocellulose was converted to a firm gel by precipitation with chloroform, in which the nitrocellulose is insoluble. The tissue blocks were cut wet. The defects, again, were the high molecular weight of the nitrocellulose which gave very viscous solutions, and prolonged infiltration times. The ether-alcohol must have extracted most of the lipid. Somewhat thinner sections could be cut than from gelatin, down to 25—50 μ, and if the infiltration was done sufficiently slowly, large objects could be infiltrated completely and sectioned with remarkably little shrinkage.

III. Paraffin

Paraffin was introduced as an embedding agent in 1881. The tissue was fixed and dehydrated, and then "cleared" with some agent which was miscible both with the dehydrating alcohol and paraffin, such as benzene, chloroform, cedar wood oil, etc. (Some of these liquids by accident had a refractive index close to that of the tissue, so that the tissue became rela-

tively transparent or "clear" when the alcohol was replaced by them.) The tissue then was transferred to a warm mixture of the clearing agent with paraffin, and then into pure molten paraffin, each for an hour or so, whereupon the paraffin, together with the tissue was solidified by quick cooling. Paraffin had the advantages of speed — tissue requiring 3 days in celloidin could be embedded in an hour in paraffin (LEE, 1928) — and sections could be cut as thin as a few microns. Speed resulted from the low viscosity of the molten paraffin, but the heat produced shrinkage. The paraffin blocks could be cut dry, and the sections would adhere to form ribbons, which was a great simplification in preparing serial sections. The advantages of paraffin so far outweighed its disadvantages that it became the routine method for histology. With the introduction of electron microscopy in the late 1940's it became apparent that even paraffin could not deliver sections in the 1/10 micron range which was required for good electron imaging (PEASE and BAKER, 1948), and the search was launched for a substitute.

IV. Methacrylate

Methacrylate, and particularly butyl methacrylate, was introduced as an embedding medium by NEWMAN, BORYSKO and SWERDLOW (1949a, b). The procedure was almost too good to believe in terms of providing nearly ideal solutions to the difficulties of earlier media. For the first time a low molecular weight monomer was employed to infiltrate the dehydrated tis-

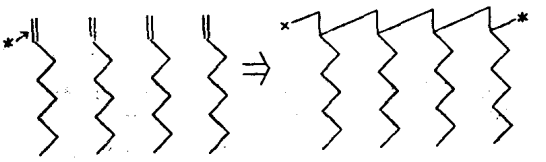

Fig. 1. Polymerization (curing) mechanism of methacrylate (⋀⋀⋀ =) with a free radical (*) as initiator

sue, with rapid penetration resulting from the low viscosity and rapid diffusion from the low molecular weight, all at room temperature or even at 0 °C if necessary. After infiltration, the monomer was polymerized to the final solid plastic by gentle heating, or by alternative methods polymerization could be accomplished at room temperature or in the cold. The mechanism of methacrylate polymerization is shown schematically in Fig. 1. Best of all, the resulting plastic was strong enough to be cut to the necessary thinness. The most serious fault was occasional injury to the tissues during polymerization which the authors identified from the first, suggesting that such damaged tissues be discarded since such blocks were easily

recognized. The chemicals required were relatively inexpensive, nontoxic, easily obtained, and the hardness of the final plastic block could be varied continuously and predictably by using different proportions of methyl (hard) and n-butyl (soft) methacrylates. In retrospect, it was obvious that the methacrylate blocks were easy to cut, and the tissue in sections one micron thick could be stained for superb light microscopic cytology. Although the plastic could be dissolved away from the tissue easily, it was soon apparent that the resulting loss of support of the tissue components by the resin seriously distorted the ultrastructure, so that the fine structure was better preserved if the plastic was left with the sections for electron microscopy. During observation in the electron microscope, the sections "cleared" and became more transparent as some of the plastic sublimed from the section, and the improved contrast which resulted enabled ultrastructural details to be recognized without special staining of the sections.

The disadvantages which led to the replacement of methacrylate were two: the "polymerization damage" to the tissue block, and the susceptibility of the plastic in the section to damage by the electron beam. The latter problem was inherent in the methacrylates; they were unusually sensitive to depolymerization by heat, so much so that, for example, a high yield of methyl methacrylate can be obtained merely by distilling scrap pieces of polymethyl methacrylate above 300 °C (RIDDLE, 1954, p. 39). Although attempts were made to reduce damage by protecting the section by layers of evaporated carbon, the final solution lay in adopting different plastics.

The damage during polymerization appeared as a swelling of the tissue block, in the worst cases to double its original dimensions (8-fold increase in volume) and sometimes was referred to as "explosion damage" because in the electron microscope the tissue elements appeared separated from each other as if blown apart. Methacrylate was accused of uneven and erratic polymerization and a variety of empirical procedures, some verging on witchcraft, were proposed to reduce or eliminate polymerization damage. In fact, methacrylate polymerization usually is smooth and predictable, and it was more valuable to understand the mechanism underlying polymerization damage than to curse it. The problem was first identified by BIRBECK and MERCER (1956) and by WATSON (1963), who suggested that the "explosion" was due to osmotic swelling due to unpolymerized monomer dissolving in the polymer which somehow had been formed rapidly within the tissue block. At the same time WATSON (1963) proposed the use of cross-linking additives to the methacrylate so that the polymer within the tissues would swell less. The reason that polymerization was accelerated within the tissue lay in the well-documented evidence that the polymerization rate in methacrylate esters is highly dependent upon the viscosity of the mixture. The maximum rate of polymerization, which occurs at about 20% conversion in methyl methacrylate, may be of the order of 10 times

the initial rate (BAMFORD et al., 1958). The fine texture of the fixed tissue itself apparently is sufficient to restrict the motion of the growing polymer chains, and thus to simulate the effect of viscosity in reducing chain terminations, which is the mechanism of the accelerated polymerization of the gel effect (BEVINGTON, 1961). The finer the texture of the fixed tissue block, the faster the methacrylate could polymerize within it with respect to the surrounding methacrylate, and the greater the osmotic swelling which could result. In retrospect, it is clear why viscous, prepolymerized methacrylate was useful to control polymerization damage (BORYSKO and SAPRA-

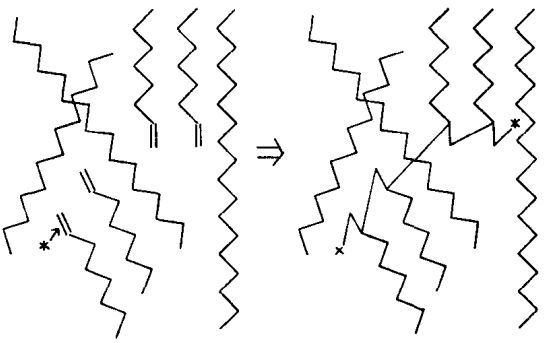

Fig. 2. Curing of prepolymerized methacrylate syrup, with completely formed polymer chains (∿∿∿∿∿) dissolved in monomeric butyl methacrylate (∿∿∿=). Note that polymer chains (∿∿∿∿∿) strictly should look like right side of Fig. 1. Free radical initiator at (*)

NAUSKAS, 1954): it more or less matched the rates of polymerization outside and inside the block by the increased viscosity outside, so that the osmotic gradient inside and outside the block was minimized. Polymerization of prepolymerized methacrylate is shown schematically in Fig. 2. Low temperature polymerization was no help because it gave more time for the osmotic damage to occur. High temperature polymerization, which was studiously avoided by NEWMAN et al. (1949b) for fear of damage, paradoxically gave better results over-all (BORYSKO, 1956): the tissue was stronger than the nearly molten plastic, and solution rather than swelling was the result. Polymerization by ultraviolet light, in ideal cases, should have produced rapid polymerization in the surrounding monomer but slower rates in the opaque black (osmium-fixed) tissue block, thus equalizing the rates in another manner, but the ideal was seldom realized.

Despite the heroic efforts which were expended in attempting to counteract these two defects of the methacrylates, the results were only partially successful at best. When the first electron micrographs of tissue embedded