PROBLEMS IN METHACRYLATE EMBEDDING FOR ELECTRON MICROSCOPY*, ‡

BY DAN H. MOORE, PH.D., AND PHILIP M. GRIMLEY

(From the Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, November 16, 1956)

During an investigation of the effect of ischemia on striated muscle as seen with the electron microscope it became evident that all degrees of disorientation and destruction of tissues were taking place, and it seemed likely that much of the damage occurred during the embedding process. Such damage made it difficult to proceed with experiments in which comparison of normal tissue with any pathological or experimental condition was necessary. Since methacrylate embedding was introduced in 1948 (1), it has been recognized that tissue areas or certain tissue elements exploded, but it was generally assumed that these blocks were easily distinguished and could be discarded. The importance of embedding procedures in the preservation of tissue structure has been recognized by Borysko (2), who found that for *in vitro* cultured tissues, the use of prepolymerized methacrylate syrups and a hardening temperature of 80° gave best results. This procedure has not been found satisfactory, however, for excised tissue blocks. Except for Borysko (2); Maaløe and Birch-Andersen (3); and Glauert, Rogers, and Glauert (4) the microscopic derangement, destruction of membranes, granularity, and washed out appearance in electron micrographs of sectioned cells or tissues have been generally assumed to be the result of fixation or dehydration rather than of embedding. Although these factors as well as microtomy enter necessarily into the preservation of fine structure, it has become increasingly evident that many uncontrolled factors in tissue preservation as revealed by electron microscopy are primarily associated with embedment. A study of acrylic polymerization (5–7) reveals that the embedding procedure employed in most laboratories would not be expected to yield satisfactory results. This paper attempts to outline some of the factors which in-

* The material for this report has resulted from a conference held between the authors and a group of scientists of the E. I. du Pont de Nemours and Co. The authors are most grateful to Dr. Marcus Naylor, Dr. Carl E. Willoughby, Dr. A. L. Barney, and Dr. Anson R. Cooke for participating in this conference and to Dr. Crawford H. Greenewalt for arranging it.

‡ This investigation was supported by the Lillia Babbitt Hyde Foundation and by a research grant (G-4138) from the Division of Research Grants, Public Health Service, National Institutes of Health.

fluence polymerization and to describe procedures which have been tried and those which were found to yield better and more uniform results.

Theoretical Considerations

Butyl and methyl methacrylate have been the plastics most successfully used to preserve cytological fine structure. A useful embedding medium must permeate the tissue thoroughly, polymerize into an homogeneous plastic at a uniform rate with as little shrinkage as possible, and form a final product which is readily cut by the microtome knife. The embedding medium must be chemically inert with respect to the fixed tissue components. It is believed that a large measure of the tissue derangement now prevalent is due to incomplete penetration of the methacrylate, non-uniform polymerization, or even chemical interaction of the methacrylate with tissue components. If the 15 to 20 per cent polymerization shrinkage which occurs in the methacrylates were to take place simultaneously and uniformly throughout the tissue block or the particular cell area under examination, the tissue components would simply be brought closer together, but their relative spatial relationships would be unaltered. The cross-sectional area of a cell, for example, would be reduced by about 10 per cent, but the relative location of elements would remain unaltered. Non-uniform polymerization, on the other hand may cause uneven contraction and shifting of fine structures. The final ratio of solid matter to plastic in cells or cell structures is directly related to the original water content. The density of tissue thus affects the cutting properties and preservation of structure. Local variations in hardness due to the tissue density or incomplete polymerization may lead to distortion, compression, or "chatter" (8, 9) during microtomy.

It has been found that the plastic outside the tissue block is nearly always homogeneous and suitable for cutting regardless of the type of plastic-initiator (see below) mixture used. The situation within the tissue block may, however, vary considerably. It is the opinion of polymer chemists that a fine inert structure would not in itself physically hinder the progress of a polymerization reaction. A stretched out molecule of polymethacrylate would have a length of 1500 to 3000 A. These molecules are, however, usually arranged in random intermeshing coils. If the tissue structure alone is considered, the plastic molecules should not cause physical destruction during controlled polymerization. Brittle or charcoal-like areas are sometimes encountered which indicate that chemical properties of the tissue network may either prevent permeation or interfere with polymerization. Wetting properties or capillarity is probably an important factor in penetration, and the amount of bound osmium may also be involved.

Glauert, Rogers, and Glauert (4) reported that bacterial cells may be embedded in epoxy resin. An attempt was made to embed muscle and kidney tissue in these resins, but a successful technique has not been found. The highly viscous resin fails to penetrate more than a very thin shell of tissue making sectioning difficult. Since this material shrinks less than 2 per cent, it would be a promising medium for embedding, if the tissue could be sufficiently permeated with it.

Mixtures of butyl, methyl, and ethyl methacrylates have been tried in various proportions. None of these seems to give results superior to pure butyl methacrylate.

Kinetics of Polymerization.—The molecular weight of monomeric butyl methacrylate is 142. Addition polymerization may be caused by heat, light, short wave irradiation, or chemical initiators. Linear polymer chains are formed varying in length all the way from a few monomeric elements to several millions depending upon conditions; however, most of them fall within much narrower limits.

Initiators, though often referred to as catalysts or accelerators, are really neither. Unlike catalysts, these agents are consumed in the reaction and do not affect the rate of chain growth beyond the initial stage. Once a chain is initiated it goes to completion in approximately 10^{-3} seconds. Although the synthesis of an individual polymer molecule from unreacted monomer occurs within such a short time, the over-all conversion of monomer to polymer may require hours. At any stage the reaction mixture consists almost entirely of unreacted monomer and high polymer. The proportion of actively growing chains is ordinarily very small.

The commonly used peroxide initiators decompose slowly at temperatures of 40 to 100° C., with release of free radicals:

$$(RCOO)_2 \rightarrow 2\ RCOO \longrightarrow 2R + 2CO_2$$

Radicals released in spontaneous decomposition may attack other peroxide molecules. This does not result in an over-all reduction in the number of primary radicals available for initiation, but reduces the effective radical concentration at a given moment. Azo-bis-isobutyronitrile is less susceptible to this type of self-induced decomposition and other side reactions (7). Some initiators may be split by chemical action as well as by heat or irradiation; *e.g.*, dimethyl aniline may be used to split benzoyl peroxide into its two free radicals. The ideal initiator and conditions for its optimum use should be determined for each type of tissue. Luperco CDB (2,4-dichlorobenzoyl peroxide with dibutyl phthalate) has given good results in many cases.

The steps of polymerization may be described by the following equations, in which I = initiator, $I\cdot = R\cdot$ = initiator free radical, M = monomer, and $M_1\cdot = R + M\cdot$ (*i.e.*, a radical formed by the combination of initiator radical with monomer):

$$(1)\ I \xrightarrow{\text{U.V., heat, chemicals}} 2R\cdot$$
$$(2)\ R\cdot + M \rightarrow M_1\cdot$$
$$(3)\ M_1\cdot + M \rightarrow M_2\cdot$$
$$(4)\ M_n\cdot + M \rightarrow M_{n+1}\cdot$$

The rate of initiation is directly proportional to the concentration of initiator and the velocity of its dissociation as influenced by temperature, radiation, etc. The rate of conversion, however, is dependent upon the square root of the concentration of initiator. Thus degree of polymerization (chain length) may vary within wide limits since it is determined essentially by the ratio of the rate of conversion to the rate of initiation. Termination of chains may occur either by coupling[1] or disproportion-

[1] Coupling is where the active ends of two growing chains join.

ation.[2] The former is dominant. As the viscosity increases, the migration of chains is reduced, and coupling cannot readily occur. Chain growth continues, however, since the movement of monomer is relatively less affected. Chain length diminishes with higher temperature, and the percentage conversion to polymer increases, making for a more brittle plastic. Usually the active center remains on the same molecular chain. Chain transfer agents may remove this active center, shorten the polymer length, and at the same time start a new polymer. This reduces the degree of polymerization and a higher percentage of final conversion occurs. Chemicals with sulfhydryl groups such as the mercaptans may act as chain transfer agents, because the SH reacts with the growing radical as follows:—

$$M\cdot + SH \rightarrow MH + S\cdot$$

Transfer agents provide a means of controlling chain length.

Because polymerization depends upon the action of a small number of active molecules during a long period of time, it is extremely sensitive to traces of substances active as initiators or inhibitors. Agents yielding concentrations of active centers in the order of 10^{-12} moles may alter the course of the reaction (10). It is, therefore, imperative to maintain controlled standard conditions in the preparation of biological embedments. Much of the difficulty now prevailing probably could be eliminated through a more careful procedure. This includes the removal of dissolved oxygen and water. Oxygen may combine with low molecular weight polymer to form polymeric peroxide (R-O-O) which is slow to add monomer (11). If, however, the amount of oxygen available is small it may form an oxide with the methacrylate (R-O·) and become a source of free radicals which accelerate polymerization. Thus the presence of molecular oxygen, acting both as an inhibitor and accelerator, provides a very unstable and uncontrolled condition for polymerization.

Purification and Handling of the Methacrylate.—Methacrylate is shipped commercially with hydroquinone inhibitor added. This material may be stored for many months at room temperature and longer at reduced temperatures. Before use, however, the inhibitor should be removed. This is accomplished by several washings with equal parts of 2 per cent NaOH, followed by at least three washings with equal parts of distilled water to remove the NaOH which may act as an initiator. The solubility of water in methacrylate is about 1 per cent by weight. The water can be removed by filtration through anhydrous Na_2SO_4, and subsequent storage over molecular sieve,[3] which is a strongly selective dehydrating

[2] Disproportionation is transference of a hydrogen atom from one growing chain to another, leaving the donor with a terminal double bond, while the receiver is terminated by the acceptance of the hydrogen atom.

[3] Linde Air Products Co., New York.

agent. Water tends to give the methacrylate monomer and polymer a clouded appearance. In order to remove all dissolved oxygen dried methacrylate should be evacuated to 10 mm. Hg and flushed with nitrogen in a desiccator jar. It may then be stored under dry nitrogen (<20 parts per million O_2) in a brown jar at 5°C. where it may be kept pure for long periods. Refractive index measurements with an interferometer indicate only slight changes after several months. The refractive index is a most sensitive indicator of polymerization. An increment of less than 3×10^{-4} is considered insignificant.

Embedding Procedure

The fixed and dehydrated tissue is soaked in two changes of pure monomer for 1 hour. The tissue is then placed in dry gelatin capsules which have been filled with a mixture of monomer and luperco.[4] This monomer-luperco solution was previously thoroughly dried and deaerated. The gelatin capsules are covered and a small hole is punched in the cap. These are then set in small test tubes and placed upright in a desiccating jar which is slowly evacuated over a period of 1 hour. This removes free oxygen from the tissue, the methacrylate, and the gelatin capsule. The vacuum is then slowly released by introducing nitrogen, and each glass tube is immediately stoppered. This leaves the gelatin capsules in a nitrogen atmosphere. The castings are cured (polymerized) at 43°C. or under ultraviolet light for 18 hours.

The unpredictable castings (embeddings), containing bubbles and plastic of varying hardness, previously obtained were found to be due to dissolved oxygen, water, and other impurities which readily enter the methacrylate unless precautions are taken at all stages of the process. Improper storage conditions may also impede standardization, since the monomer will contain unknown quantities of inhibitor, active centers, and polymer. Although the procedure described above has greatly improved the yield of well preserved tissue, much remains to be determined, including the optimal fixation and quantity of polymerization initiator to be used for each kind of tissue.

Grateful acknowledgment for able technical assistance is made to Miss Dorothy Gelber and Mr. John Weinstock.

BIBLIOGRAPHY

1. Newman, S. B., Borysko, E., and Swerdlow, M., *Bureau Standards J. Research*, 1949, **43**, 183.
2. Borysko, E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 3.
3. Maaløe, O., and Birch-Andersen, A., *6th Symp. Soc. Gen. Microbiol.*, 1956, 261.

[4] Methacrylate containing 4 to 6 per cent luperco CDB has been found to yield best results for embedding muscle tissues, whereas 1 to 3 per cent appears better for cell suspensions and tissue cultures.

4. Glauert, A. M., Rogers, G. E., and Glauert, R. H., *Nature*, 1956, **178,** 803.
5. Riddle, E. H., Monomeric Acrylic Esters, New York, Reinhold Publishing Corp., 1954, 29.
6. Schildknecht, C. E., Vinyl and Related Polymers, New York, John Wiley and Sons, 1952.
7. Flory, P. J., Principles of Polymer Chemistry, Ithaca, Cornell University Press, 1953.
8. Williams, R. C., and Kallman, F., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 301.
9. Morgan, C., Moore, D. H., and Rose, H. M., Effects of the microtome knife and the electron beam on thin sections of methacrylate embedded tissue, 13th Annual Meeting of Electron Microscope Society of America, University Park, Pa., October, 1955.
10. Walling, C., and Briggs, E., *J. Am. Chem. Soc.*, 1946, **68,** 1141.
11. Barnes, C. E., Elofson, R. M., and Jones, G. D., *J. Am. Chem. Soc.*, 1950, **72,** 210.