Electron Microscopy and Ultramicrotomy

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> "The old adage 'to travel hopefully is better than to arrive' scarcely applies to microscopy, because in a sense science never arrives, the road going on and on from any temporary stopping place. Moreover, hope alone is not enough except sometimes to counteract despair. We need tenacity and the will to cling on against odds to reach something we believe to be important."
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> Irene Manton, 1978 (1)

It is commonplace to recognize that the depth to which we explore ourselves and our environment is frequently determined by the development of new instruments and the creation of techniques for their use. Usually in such developments, one can recognize a time when fragments of information, acquired previously, are ready to be used to satisfy a concept or an urge to do or see what had not seemed possible before. So it was in the early 1930s that a group of physicists and engineers, mostly in Berlin, found conditions right to create an electron microscope. Max Knoll and his students, Ernst Ruska and Bodo von Borries, had available the knowledge that electrons would move through a vacuum and be deflected in their motion so as to be focused by solenoid lenses. It was mostly engineering skills that were needed to generate a microscope. Interest in the applications of the first microscopes naturally followed and, by the late 1930s, electron micrographs of recognizable value to biologists were being published.

Any consideration of the pace at which biological electron microscopy then developed must take into account worldwide events and constraints related to the outbreak and prosecution of World War II. Hitler invaded Poland on September 1, 1939. The very first Siemens & Halske AG electron microscope made for commercial sale was delivered in that year, only a few months before the War actually started (2). However, since the political alignments of Axis and Allied countries had been substantially established the year before, after the annexation of Czechoslovakia's Sudetenland, it is not suprising that not a single Siemens & Halske microscope was ever delivered to countries outside of Axis control. None was in Allied hands until one microscope was captured intact and brought to England after the 1944 Normandy invasion.

In the United States, the Radio Corporation of American was not ready to deliver its first commercially available electron microscopes, the RCA–EMB models, until 1941. The "Lend-Lease" program had started earlier in the year and developed quickly as a massive aid program to England. About one-third of all RCA-EMB instruments ever made were shipped to England.

About 40 Siemens & Halske microscopes seem to have been manufactured during the war years, and about 60 RCA/EMBs (2). On both warring sides, most of the applications of the new instruments were directed towards the research needs of the military. We are aware of only one RCA-EMB instrument in the United States that was available primarily for biological research: a microscope at the Massachusetts Institute of Technology in the laboratory of Cecil E. Hall, who was already recognized as an important pioneer in the original development of prototype instruments at the University of Toronto. A second EMB instrument, installed in the research laboratories of Interchemical Corp. in New York, was made available in 1943 to Albert Claude and Keith Porter at the Rockefeller Institute (3). Steward Mudd, a bacteriologist at the University of Pennsylvania, also had substantial access to RCA instruments at the RCA manufacturing plant in Camden, New Jersey, where Thomas F. Anderson worked on biological problems as an "RCA Fellow."

The situation in Germany for biologists during the early war years seems not to have been more advantageous. In April of 1940, Siemens & Halske AG sponsored an interdisciplinary meeting where the most prominent users of the new electron microscopes, as well as scientists who had had access to prototype instruments, reviewed the achievements of nonmilitary applications. R. Siebeck discussed medical applications in Germany (4), and there were other reviews of botanical and bacteriological applications. Biological work necessarily had been limited mainly to examining silhouettes of bacteria, viruses, fibrous proteins, and other organic objects that could be studied *in toto*. By this time, both bacterial flagellae and the repeating periodicity of collagen had been seen. Metallic mesh, suitable for grids, was available from photoengravers, and collodion support films had been introduced by Helmut Ruska in 1939 (5).

In the United States, Canada, and England, biological discoveries closely paralleled the German efforts at first. E. F. Burton and W. H. Kohl (6) reviewed the applications of electron microscopy that took place during the war years on this side of the Atlantic. Work with bacteria and viruses was soon underway, particularly at the RCA Laboratories, under the inspiration of L. Marton, Mudd, Anderson and W. M.

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Stanley. The M.I.T. Laboratory group, consisting at first of Hall, Francis O. Schmitt and Marie Jakus, particularly pursued studies of proteins that could be isolated by tissue fragmentation (collagen and muscle). But the war years also saw two important technical developments: first, in 1942, the replication of surface topography with Formvar films by Schaefer and Harker (7), and second, in 1944, shadowing by Williams and Wyckhoff (8). Then, in 1945, Porter et al. (9) demonstrated that whole-cultured tissue cells could be brought to the stage of the EM and examined profitably. While bathed in balanced salt solution (Tyrode's, pH 7.4), the cells in these early studies were fixed with vapors of OsO_4. The advantages of this reagent for the faithful preservation of cultured cells had been described in 1927 by Strangeways and Canti (10). For several years after 1945, and until thin-sectioning became a reality, these thinly spread cultured cells provided the only access to knowledge of cell fine structure, and contributed to observations on the endoplasmic reticulum and the intracellular presence of viruslike particles in cells from chicken tumors and mouse mammary tumors.

Quite apart from theoretical considerations, the limited availability in vitro of many kinds of tissue cells convinced biologically oriented electron microscopists of the need for ultrathin sectioning. In 1934, Marton (11) had examined osmium-fixed, 15-μm sections of plant material. Naturally he had little success, even at a magnification of only ×450. Later, von Ardenne (12) attempted to cut tapering wedges of tissue so that at least some parts of their areas would be adequately thin. Richards et al. (13) and Sjöstrand (14) continued this approach, but only the Richards' group had sufficient success to warrant publication of micrographs. A considerable historical hiatus in sectioning techniques followed, lasting until 1948, as the War and the recovery years took their toll. There was, however, one diversion during that interval into ultra-high-speed microtomy.

In 1943, O'Brien and McKinley (15) developed the hypothesis that, at high sectioning speeds, specimen inertia should restrict strain distribution so as to localize it very closely to the knife edge. They reasoned that there would be no time for plastic flow, and that thermal expansion would be negligible. They therefore designed a microtome with a steel knife supported just beyond the circumference of an 8-inch wheel. The wheel originally was driven at 12,500 rpm, and produced a cutting speed equivalent to 140 feet per second. The block was moved into this whirling blade at a rate calculated to deliver 0.1-μm sections. Their original article was not illustrated with micrographs of successful sections. However, according to a report by Gessler and Fullam (16), a year later, at the 1944 Annual Meeting of the Electron Microscopy Society of America, O'Brien and McKinley did show some micrographs of reasonably good sections, which inspired Fullam and Gessler to begin their own work with high-speed microtomy. (They also reported that by 1944, O'Brien and McKinley had almost doubled the original speed of their microtome to 22,500 rpm.) Subsequently, Fullam and Gessler (17) produced a microtome which operated at 57,000 rpm, delivering a cutting speed of 1100 feet per second. They used fragments of razor blades as knives, and sectioned a variety of plastics, as well as tissues. They demonstrated considerable success in cutting the plastics, and even some in sectioning tissues (3, 17). In view of this, it is curious that they did not explore the potential of embedding with plastics. Instead, they focused their attention on embedding media that would volatilize after sectioning was completed, and experimented with such substances as camphor, resorcinol, naphthalene, etc., and eutectic mixtures of these and related compounds, almost all with boiling points below 85°C, and some with boiling points as low as 32°C. They did try paraffin embedments, but recognized problems in its subsequent extraction from the sections. Their first article was illustrated with only one micrograph of a tissue section, that of liver fixed with osmium tetroxide. Fairly severe artifact was evident.

Ernest F. Fullam's venture into biological microscopy followed an earlier acquaintanceship with Claude and Porter, who then were developing an interest in the potential of electron microscopy for cytological research. Claude wanted especially to identify the cytoplasmic origin of microsomes. In 1945, Claude and Fullam published a joint paper (18), illustrating osmium-fixed liver that was sectioned at high speed (49,000 rpm). Their embedment was specifically characterized as a eutectic mixture of camphor and naphthalene with a melting point of 32.5°C. Dry sections, which literally flew from the knife, were collected on a strip of copper mesh that was coated with a Formvar film. Areas of promising sections were selected with a light microscope, and then suitably positioned grids were punched out of the mesh. They strove for sections 0.3 to 0.6 μm thick. The published micrographs showed substantial artifacts, i.e., many artificially-created holes. The authors at least partially recognized this, and took the position that the major problems limiting effective biological ultrathin microtomy related not so much to the sectioning itself as to a need for refined fixation and embedding.

Although the War ended in 1945, and RCA was ready to begin marketing their newly designed EMU series microscopes at the end of that year, few instruments were available to biologists until well into the 1950s. By today's standards, those machines were rudimentary: the first of the new RCA microscopes did not even have a biased gun. Objective apertures were not introduced until 1950. Although Hillier and Ramberg (19) had recognized the need and means for lens correction as early as 1947, stigmators were not added to production instruments until 1953; externally controllable compensation was not available for RCA microscopes until Canalco Co. of Bethesda, Maryland, marketed a kit in 1956; and Siemens did not introduce its well-equipped, postwar "Elmiskop" until 1954. Thus, it took about a decade after the end of the War for electron microscopes to evolve to include features we now regard as absolutely essential for biological work, such as stable performance, astigmatic lenses, and excellent contrast. Only at the midpoint of that decade did ultramicrotomy and its associated techniques also mature; images of sectioned material were produced that would still be regarded as acceptable. Earlier, there simply were too many disparate problems to permit rapid progress towards a total solution.

Dr. Claude's 1945 experience with Fullam convinced him that very high-speed microtomy was not going to be essential. He therefore started work with Joseph Blum (then Director of the instrument shop at The Rockefeller Institute for Medical Research) to develop a microtome that operated at a more modest speed. The prototype, described by Claude (20), incorporated some of the design features of the earlier Fullam-Gessler instrument, but was operated simply by hand-turning a flywheel. The arrangement of the pulley system undoubtedly produced speeds that now we would regard as excessive. However, in this instrument, the knife did not move past the fixed specimen, as in the earlier high-speed microtomes; instead, the specimen was mounted at the edge of a turning and advancing

disk, which advanced by small increments toward a fixed knife. This permitted the use of a trough in association with the knife so that sections could be collected as a ribbon on a fluid surface. Claude's published report of this microtome was not illustrated with micrographs and he re-emphasized that "the task ahead is to find better ways for the preparation and preservation of the specimen."

In January, 1948, in New York, Claude delivered a Harvey Lecture on "Studies on Cells," in which he summarized his ongoing efforts, including his work on thin-sectioning techniques to improve electron microscopy of cells. The lecture reached mainly an audience from that city and the manuscript, unfortunately, did not appear in print until 1950. Thus, Daniel Pease and Richard Baker (21), working at the University of Southern California, had no inkling of the work in progress at The Rockefeller Institute when they published their own account of some success with low-speed microtomy. L. H. Bretschneider (22) in Holland was also attempting to produce half-micron sections, apparently unaware of the developments at The Rockefeller.

Pease and Baker (21) were influenced in their efforts to obtain ultrathin sections for electron microscopy by a suggestion of Prof. F. Kiss from Hungary, who had been associated with Prof. St. Apathy. The latter, working at Cluj, Rumania, during the last years of the nineteenth century and the early years of the twentieth, contributed much to the development of conventional microscope techniques, including double embedments of paraffin and collodion. In a personal communication, Dr. Kiss indicated that it was almost commonplace for members of that school to section small, double-embedded blocks in the submicron range of thickness by using conventional microtomes at normal operating speeds. This encouraged Pease and Baker (21) to change rather simply the advance mechanism of a standard Spencer 820 microtome by a factor of ten so that the nominal increment of specimen advance was reduced to 0.1 μm. More important for success, however, were the realizations that an adequate embedment had to be much harder, and offer more support, than conventional paraffin, and that section size had to be reduced by at least an order of magnitude from that commonly employed for conventional sectioning. This led them, first, to infiltrate tiny tissue blocks with as much collodion as possible, and then, second, to add hard paraffin. Subsequently, Pease (23) hardened blocks still further by a triple-embedding procedure, which involved incorporating Damar resin between the nitrocellulose and wax infiltration steps. Also, the paraffin was hardened additionally with bayberry or carnauba wax.

At first, Pease and Baker (21) collected dry sections individually with a camel's hair brush, and so transferred them to grids. The sections then were flushed with xylol in order to remove only the paraffin component, thus leaving the nitrocellulose network in place to provide specimen support. (The partial extraction was deemed necessary for want of an effective stain to provide adequate contrast.) In retrospect, the residual collodion was inadequate to prevent fairly serious collapse of fine-structural detail. However, it seems fair to say that these results, when first published, finally demonstrated that adequately thin sectioning could be achieved with relatively simple instrumentation and with low cutting speeds. Thus, the work served as a stimulus for other laboratories, and within the next four years, a rash of modifications of old microtomes, as well as rather simply designed new microtomes, were announced. At the same time, Bretschneider (22) independently began efforts to achieve ultrathin sectioning without resorting to new instrumentation or high speeds. He realized that the unit of advance of the "Cambridge Rocking Microtome" might produce sections as thin as 0.6 μm or, with simple modifications, even thinner. The basic design of this fundamentally simple and mechanical instrument dates from 1885, and is attributed to H. Darwin (23). Later, the designers of the eminently successful Porter-Blum ultramicrotome unwittingly incorporated some of its design features in the mechanism whereby the specimen arm was suspended and advanced.

Bretschneider realized, as had Pease and Baker, that a principal problem with ultrathin sectioning lay in the softness of the conventional embedding media. He therefore used paraffin with a melting point of 65°C and operated his instrument at 10°C. The micrographs he published indicated successful sectioning in the submicron range. Unfortunately, he had not preserved his tissue with osmium tetroxide, but rather with the more conventional fixatives of the day, including Bouin, Champy, Carnoy, bichromate-formol, alcoholic sublimate, etc. Also, as a final step, he extracted the paraffin. Thus, although the specimens demonstrated some electron transparency, they were full of artifacts. At least two other European laboratories (Danon and Kellenberger [25] in Geneva, and Oberling, Gautier, and Bernhard [26] in Paris) also made serious efforts to use rocking microtomes for ultrathin sectioning, and had enough success to warrant publication. In 1952, Bretschneider (27) published a comprehensive review of ultramicrotomy, which included references in tabular form of what he thought to be the entire literature through 1951 on the results of ultrathin sectioning: the list included only 36 papers.

In the critical years immediately after 1948, other key developments permitted fairly rapid technological advances. The introduction in 1949 by Newman, Borysko, and Swerdlow (28, 29) of polybutylmethacrylate (and later, mixtures of butyl and methyl methacrylate) as an embedding medium served as a great stimulus, although the botanical material in their micrographs generally was not well preserved. Originally, these investigators advocated the extraction of the polymerized methacrylate by an organic solvent such as acetone, toluene, or amyl acetate. At the time, the latter step seemed necessary to provide adequate contrast in lieu of any effective "staining" procedure other than that provided by an initial fixation with osmium tetroxide.

Another important advance was the 1950 introduction of glass knives by Latta and Hartmann (30). These immediately replaced the use of steel knives, which had always posed serious and largely unresolved problems. Apparently, most investigators had been using disposable razor blades which were ground with such an acute angle as to be undesirably flexible. Heavy knives, made for conventional microtomy, had to be resharpened before every use, at least if exposed to a trough fluid that visibly discolored (oxidized) edges within a few minutes. Very little had been published about how heavy knives might be sharpened easily and reliably (but see Hillier [31] and Ekholm et al. [32]). Perhaps this was because few investigators believed they had achieved anything approaching perfection. At best, the inherent grain structure of steel presumably would always have limited true uniformity and standardization. Fernández-Morán's (33) introduction of diamond knives in 1952 ultimately became an interesting success story, but these have proved to be more of a convenience than a necessity.

In 1950, Gettner and Hillier (34) formally introduced the useful and important technique of spreading and collecting

sections on and from aqueous surfaces in troughs attached to knives, although Claude (20) had suggested this technique earlier. During this period also, various laboratories experimented with heavy metal stains but had only limited success. However, it became obvious that phosphotungstic acid was useful as a stain, especially after OsO_4 fixation, and without the necessity to extract methacrylate embedments. The acid gained widespread use in an alcoholic solution. The usefulness of phosphotungstic acid had been partially realized and exploited earlier, notably by the group at the Massachusetts Institute of Technology, in work with whole mounts of fibrous proteins, etc. (However, it was not until 1955 that Hall [35] recognized, and deliberately used, phosphotungstic acid as a negative stain.)

From these early attempts at microtomy it became apparent that "single-pass" microtomes were a necessity, in order to take advantage of methacrylate embedments, sectioning with glass knives, and the collection of sections on fluid surfaces. Otherwise, sections often were lost on the return stroke of the microtome, or the face of the block was damaged. This influenced all subsequent designs of instruments made specifically for ultramicrotomy.

Many individual efforts to develop microtomes specifically for ultramicrotomy were made in the early 1950s. These included modifications of conventional microtomes, and also some ingenious original designs to minimize or eliminate problems with the bearings and lubricating films of moving parts. Thus, flexible rods and leaf springs were sometimes incorporated into the design to permit movements without bearing surfaces. Substantial efforts were made to increase the mass and decrease the elasticity of the machines. Design features that were finally to appear in commercial microtomes included, in addition to mechanical advance mechanisms, thermal expansion systems that originally were introduced by Newman, Borysko, and Swerdlow (28, 29). The list of ultramicrotome designs that have been published, but never reached commercial development, is long. In his 1955 paper, Sitte (36) appended an extensive bibliography of the pertinent information available at that time, and in 1956 Gettner and Ornstein (37) wrote a splendid review. Porter (38), in 1964, and Sjöstrand (39), in 1967, published considerable detailed information on the design features of early microtomes, particularly of those that reached commercial production.

For the truly rapid expansion of the developing field of ultramicrotomy to occur, a suitable, *commercially available* microtome was an obvious necessity. This was realized in 1953 with the introduction of the Porter-Blum instrument with a mechanical advance, manufactured and eventually marketed by Ivan Sorvall, Inc. of Norwalk, Connecticut (40). This was followed, also in 1953, by the Sjöstrand (41) thermally advanced microtome, manufactured by L.K.B.-Producter AB, Stockholm. For a time the latter microtome dominated the European scene (but eventually was taken out of production), while the Porter-Blum instrument became widely used in the United States and elsewhere. The simplicity and the reliability of the MT-1 Porter-Blum microtome soon made this the instrument of choice, and it is still manufactured to this day, despite the competition of second- and third-generation microtomes that are fully automated. As might be expected, this microtome went through several model changes before the commercial design was established. The most interesting of these incorporated a horizontal steel bar, which was suspended in a gimbel at one end and held the specimen in a chuck at the other. It had no mechanical advance, but relied on thermal expansion with heat from a reading lamp to move the specimen toward the knife. In its simplicity, it is still a charming and reliable instrument.

During the winter of 1954, an extraordinary workshop on microtomy was held at the New York Academy of Sciences. Designers of microtomes from up and down the East Coast came to the meeting with their creations. Altogether, 10 or 12 different instruments were shown. Irene Manton, 25 years later, recalled the occasion as follows: "It was my privilege, soon after arrival in New York, to attend a meeting at the New York Academy of Sciences at which an array of devices for thin sectioning were displayed, some crude, others almost comically complex, but only the Porter-Blum behaved perfectly, cutting a clean ribbon of serial sections of the right thickness to order, from a methacrylate block (1)".

In 1955, H. Sitte designed a thermal-advance microtome, which then was manufactured and marketed by Reichert AG, Vienna; its derivative commercial models have enjoyed a continuing success. Four years later, A. F. Huxley (42) introduced a mechanical-advance microtome, which was first produced by the Cambridge Instrument Company, and a cosmetically improved and motor-driven version continues to be built and sold by L.K.B. In addition to these microtomes of early design that reached commercial production (most of which are still being manufactured), inevitably others were introduced, only to disappear without leaving an important heritage.[1]

[1] We know that the following microtomes for ultrathin sectioning were advertised as being in commercial production. Historically first, in the late 1940s, was the Fullam and Gessler very-high-speed microtome, advertised with the suggestion that the investigator could protect his investment by an easy conversion to an ultracentrifuge. The American Optical Co. of Buffalo then marketed a version of the adaptor for their "Spenser 820" rotary microtome that had been developed by Pease and Baker. After L. H. Bretschneider's use of the "Cambridge Rocking Microtome," the device was advertised specifically as an instrument suitable for the electron microscopists. "Minot" microtomes, redesigned according to plans by B. B. Geren and D. McCulloch, were sold for some time by the International Equipment Co. of Boston.

Other microtomes of substantially new design then began to appear on the market. A. J. Hodge, H. E. Huxley, and D. Spiro produced prototype instruments that were intended for manufacture by the Scientific Equipment Corp., Waltham, Mass. For a number of years, Ernst Leitz, of Wetzlar, Germany, marketed a succession of models based upon a design of H. Fernández-Morán, and its subsequent improvements. Philips, Inc., Eindhoven, produced microtomes designed by H. B. Haanstra. J. L. Farrant and S. E. Powell developed a microtome sold through Schuco Scientific Co., New York. B. von Borries, J. Huppertz, and H. Gansler introduced a microtome manufactured by Sartorius-Werke of Göttingen. D. Damon, at the Weizman Institute of Science, Rehovoth, Israel, marketed a commercial microtome through the Y.E.D.A.-Research and Development Co. associated with the Institute. M. E. Gettner made an effort to sell a microtome of his design through the Process and Instruments Co., Brooklyn, N.Y. Georg Jacob KG, Leipzig, offered a microtome patterned after an instrument first built by W. Niklowitz.

In addition to these designs of investigative scientists, the engineering staff of LKB-Produckter, Stockholm, anonymously developed a succession of substantially different designs. The Sorvall Division of DuPont Instruments Co., Newtown, Conn., is now beginning to do likewise, and, at least twice, the Japan Electron Optics Laboratory Co. of Tokyo has marketed ultramicrotomes without design credits. In addition, Jose Delville, Saint Germain-en-Laye, France, has recently introduced a new instrument. It is possible that still other microtomes of which we are unaware may have appeared in the market-place. It is evident,

As one reviews the published micrographs of the early years of ultrathin sectioning, it is apparent, in retrospect, that poor fixation—often bordering on the utterly inadequate—was a major source of difficulty. There were almost no guidelines except perhaps for that of Heidenhain; in a well-known essay in 1911 on "Plasma und Zelle," he had emphasized that osmium tetroxide was the only known fixative that preserved delicate tissues such as nerve axons without "enormous shrinkage" (43). Also, the faithfulness of osmium tetroxide had been dramatically demonstrated in 1927 by Strangeways and Canti (10) in their studies of cultured cells by dark-field light microscopy. It was this display that led Porter to use OsO_4 in the fixation of cultured cells in 1945. Certainly these considerations also influenced Pease and Baker's (21) original choice of this fixative. Subsequent uses of osmium tetroxide were influenced by the quality of those early preparations. Nonetheless, how to use it to best advantage in the fixation of tissues was not immediately evident. Its poor penetration through tissue was already notorious and it did not perfuse well. At first overly large tissue samples were used, which then were immersed in unbuffered solutions. The difficulties with this fixative were recognized and, in 1952, when Palade (44) first reported and demonstrated the value of pH control, the work was heralded as a landmark by all investigators in the field. In retrospect, no doubt the "Palade Pickle" worked as well as it did partly because he refined tissue-mincing to produce truly small blocks while the tissue was immersed in the fixative. In dissolving osmium tetroxide in Veranol buffer solution, Palade made no attempt to employ a physiologically compatible vehicle, for he did not think it was important. Most subsequent investigators have also ignored physiological compatibility, even when selecting other buffers. By contrast, in the mid-1950s, Rhodin (45), Zetterqvist (46), and Sjöstrand (47) advocated the use of a balanced salt solution with only minor buffering properties as the fixative vehicle, and also succeeded in fixing tissue remarkably well for the time. Unfortunately, since then this approach has been used only sporadically.

In the years 1952–54, the various U. S. National Institutes of Health came to recognize that the essential tools finally were available to utilize ultrathin sectioning techniques effectively to explore cellular structure and function. The NIH became generous in establishing new laboratories and in supporting existing ones. Electron-microscope installations proliferated. Many talented young investigators changed their research direction. In January 1954, a new journal, the first designed specifically to accommodate the expanding information relating to cellular fine structure, was launched under the aegis of The Rockefeller Institute. This was the *Journal of Biophysical and Biochemical Cytology* (JBBC), later to become the *Journal of Cell Biology* (JCB). In January of 1956, Keith Porter organized a "Conference on Tissue Fine Structure," which had the financial support of the Morphology and Genetics Study Section of the National Institutes of Health. This meeting produced an extraordinary volume, published in 1956 as a supplement to Volume 2 of the JBBC. The conference presented a good overview of what had been accomplished in the short period of time since satisfactory microtomes had become commercially available, osmium-tetroxide fixation had become reasonably well understood, and methacrylate embedding had become routine. One-hundred and nine investigators participated, including many from abroad, and 75 papers were presented. Despite the high quality of many of the micrographs presented, two papers spoke of impending problems. Borysko (48) had come to recognize "polymerization damage" that somewhat capriciously, but seriously, could change cellular fine structure. In addition, Morgan, Moore, and Rose (49) showed convincing evidence that the sublimation of methacrylate in the electron beam (previously recognized), could result in severe cytological artifacts, including damage to both cytomembranes and protein particulates. However, the full extent of the limitations of methacrylate embedments was not—and could not have been—fully appreciated until comparative evaluation was possible, after the development of cross-linked plastics as embedding media. Before that development, various palliative measures were devised to minimize tissue damage in methacrylate. These included the partial polymerization of methacrylate mixtures before initating the embedment (Borysko and Sapranauskas [50]), the use of more exotic catalysts than the original benzoyl peroxide (azodiisobutyronitrile, Shipkey and Dalton [51]); and the inclusion of traces of substances that could possibly serve as nucleation centers (uranyl nitrate, Ward [52]). In addition, to prevent, or at least to minimize, sublimation artifacts, Watson (53) proposed sandwiching methacrylate sections between two supporting films.

It was, however, the work with epoxy resins, begun by Maaløe and Birch-Anderson (54), that finally disclosed the full limitations of methacrylate embedding. It became apparent that these cross-linking resins do not liquify or decompose in the electron beam as does polymethacrylate, and that potentially destructive surface-tension forces could be avoided entirely through their use. Originally, Maaløe and Birch-Anderson used an unspecified "highly viscous epoxy compound," with diethylene triamine as the "hardening" agent. Soon after, Glauert et al. (55) introduced Araldite M. At about the same time, Kellenberger et al. (56) started to explore cross-linking polyester resins as embedding media, which led Ryter and Kellenberger (57) to settle on Vestopal W as their final choice. All of this original work was concerned with improving the preservation of bacteria, and it might have had a more immediate impact if more complex cellular morphology had been presented. A particular difficulty arose with Araldite M, for the American-made product turned out to be different from the English one, and there were problems with its infiltration into tissue. This also delayed its general acceptance as an embedment, and it really was not until Luft (58) introduced Epon 812 in 1961 as the resin of choice that electron microscopists worldwide had an easily obtainable and reasonably reliable cross-linking embedment.

Investigators faced another problem when they started to use epoxy resins, that of inadequate specimen contrast. A principal difficulty was that cured epoxies are themselves very dense substances, and there is no sublimation of material during electron bombardment to enhance a contrast differential. Furthermore, cured epoxy resins are quite hydrophobic, so that the aqueous, heavy-metal stains that had been used successfully with polymethacrylate did not always penetrate well and did not produce adequate contrast. Fortunately, Watson (59, 60) introduced an alkaline lead stain in 1958 that proved to be highly effective with epoxies, and, with its variants, is still by far the most valuable general-purpose stain.

however, that very few ultramicrotomes have had long competitive existences, and even these, with time, have undergone extensive modifications. The "improvements" have added to automation and to costs, but not necessarily to the ultimate quality of the sections they have produced.

Actually, Watson did all of his original work with methacrylate embedments. It seems to have been simply fortuitious that the alkaline-lead stain worked so well with the epoxy embedments. These embedments and an effective staining technique were the next-to-last step toward reaching the goal that we now recognize as "standard operating procedure."

The final step was to be fixation. In spite of the cytological detail that obviously could be preserved with osmium tetroxide, it was suspect for a number of reasons. Its chemical reactivity, particularly in relation to proteins, was poorly understood, even though Porter and Kallman (61) and Bahr (62) had reported on numerous model experiments. These had made it clear, however, that by no means all cytoplasmic macromolecules were rendered sufficiently insoluble to withstand leaching in subsequent processing steps. Furthermore, it was generally recognized that OsO_4 destroyed essentially all enzymatic activity, so that cytochemical reactions could not be demonstrated after its use (Sabatini et al. [63]). Thus, protein configurations were recognized as being severely damaged. Also, and quite unfortunately, there was no other fixative known in the 1950s that could be used for comparison with OsO_4 to help evaluate the quality of its ultrastructural preservation. Thus, formaldehyde had proved to be completely inadequate in methacrylate embedments, and although acrolein, as introduced by Luft (64), was recognized as an improvement, its noxious toxic properties discouraged its widespread use and delayed experimentation.

The "discovery" of glutaraldehyde as the primary fixative of choice by Sabatini, and Bensch, and Barrnett (63) immediately demonstrated consistently good and uniform tissue preparation, particularly of proteins. Cytological structures not generally seen before, such as cytoplasmic microtubules, now were routinely observed. Many tissues could be readily perfused because glutaraldehyde does not contract vascular smooth muscle as did OsO_4. A first approximation of the protein chemistry involved in glutaraldehyde fixation appeared to be relatively simple and understandable. To a considerable extent, proteins and other macromolecules often were so gently denatured that histochemical and immunological specificities were preserved. Fortunately, glutaraldehyde could be used with osmium tetroxide, as well as with uranyl salts, so that double fixation with the addition of heavy metals proved to be possible, and demonstrated particularly well-preserved cytomembrane systems. With this somewhat belated recognition of the great value of glutaraldehyde, ultramicrotomy finally could be said to have completed at least the first phase of its historical development.

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