

ADVANCES AND APPLICATIONS OF ULTRAMICROTOMY

By

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INTRODUCTION

Sponsler and Bath, in a recent paper (1949) on the electron microscopy of a minute green alga (1), have expressed the hope that "these chance observations may stimulate further submicroscopic studies of structure in the lower plant forms." The lower plant forms, however, have been one of the favored materials for electron microscope studies and have been the subject of numerous publications. A bibliography of electron microscopy up to 1950 (2) contains over ninety reports of observations on the unicellular plants. Bacteria have received, by far, the most study from the electron microscopists, but diatoms have also come in for a share of attention. On the other hand, electron microscopic investigations of the higher plants have been limited either to surface details or to bodies in tissue dispersions.

This hiatus in plant science is due to one of the limitations in the use of the electron microscope. The extreme opacity of most materials to electrons at the potentials maintained in the instrument presents a considerable problem in microtechnique. Dispersions of bacteria, diatoms, algae etc. when dried on a film supported on a specimen screen provide a convenient solution. In this condition they are sufficiently thin to permit electron

beam penetration and the structure is preserved to some extent. However, since much of our knowledge of plant structure is derived from the microscopic examination of thin sections it would be desirable to prepare similar sections for electron microscopy. These sections because of their electron opacity must be thinner than the sections used with the light microscope. It is generally assumed that a few tenths of a micron is the optimum section thickness and Wyckoff (3) finds that the maximum thickness of objects that can be examined increases approximately as the square of the accelerating voltage. For a voltage of 50 KV and for objects with light atoms this maximum is estimated to be about 0.2 microns.

Various solutions to the problem of microtomy for the electron microscope have been presented but few of them have been practical or of wide application. A number of investigators have attempted to use wedge-shaped sections, cut free-hand (4) or with a special microtome (5), to study the internal structure of material. The thin edges of such sections would occasionally permit observation of details. The high speed microtomes (6, 7, 8, 9, 10) were a more successful approach to the problem, but the operating efficiency of the various designs in terms of useful section production appears to be very low. These instruments are expensive and require relatively large expenditures of time to obtain even a few thin sections. By such methods tissue studies could be carried on only

with great difficulty and many of the reports seem to indicate that the cutting of a usable section, rather than the interpretation of observable detail, justified the publication. Comprehensive studies of tissues and cells, because of inherent variation, require numbers of suitable preparations and enough sections could not be made available by these methods.

In 1948 Pease and Baker (11) described a method for producing thin sections which used a modified laboratory microtome and double embedding of the specimen. Newman, Boryske and Swerdlow (12) found that advancing the specimen block by thermal expansion and using an embedding medium consisting mainly of polybutyl methacrylate permitted the ultramicrotomy of even difficult materials. Both of these methods, for the first time, provide comparatively simple means for obtaining the necessary quantities of ultrathin sections needed for histological and cytological studies with the electron microscope. Numerous applications of these techniques to animal tissues are in progress or have already been reported. Several workers (13, 14, 15) have used the technique of Newman et al. while others (16, 17) have followed the Pease-Baker methods. Still others (18, 19) have used a combination of the two.

The data and observations given here are intended to present some solutions to the problems met with in ultramicrotomy and to indicate the scope of the techniques when applied to plant materials. In the interest of clarity,

improvements and innovations in method will be presented first followed by application of the method to specific plant structures.

INVESTIGATIONS IN INSTRUMENTATION

THE MICROTOME

In the United States almost all recent work in ultramicrotomy has been accomplished through the use of the Spencer (American Optical Company) No. 820 microtome. The present rocking microtome of the Cambridge Instrument Company has a minimum feed of 2 microns, but the earliest model of this instrument had a feed of approximately 0.6 microns, and Bretschneider (20) has used one of these old machines to cut sections of 0.6 microns which are thin enough to be examined in an electron beam of 100 KV. He reports that owing to defects in the cog of his old instrument, only half of the nominal feed is given at some points of the cycle and that here sections of 0.3 microns are produced. Horio, Kobayashi and Kondo (21) have reported successful sectioning with both sliding and rotary microtomes whose smallest increment was 1.0 micron (rotary) and 0.5 microns (sliding).

The electron micrographs published by Horio and his co-workers (21) have been criticized by Hermans (22) but the fact that they were cut on a laboratory microtome set at an increment considerably above the estimated optimum is of interest. If the Spencer No. 820 microtome is set to the 1 micron increment and successive sections cut from a metha-

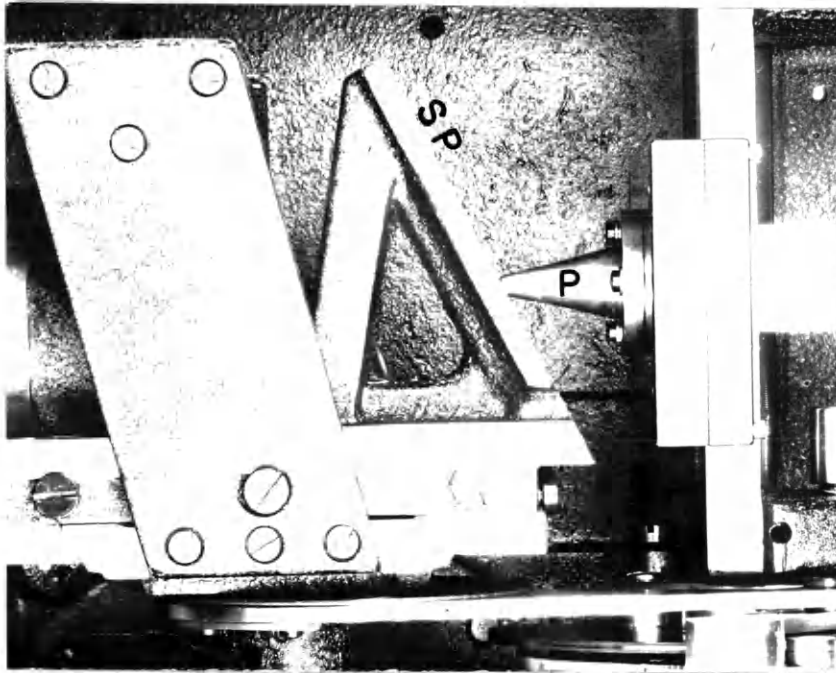


Figure 1. Top view of the American Optical Company (Spencer) No. 820 microtome. (SP) is the slide plane, (P) the propulsion point.

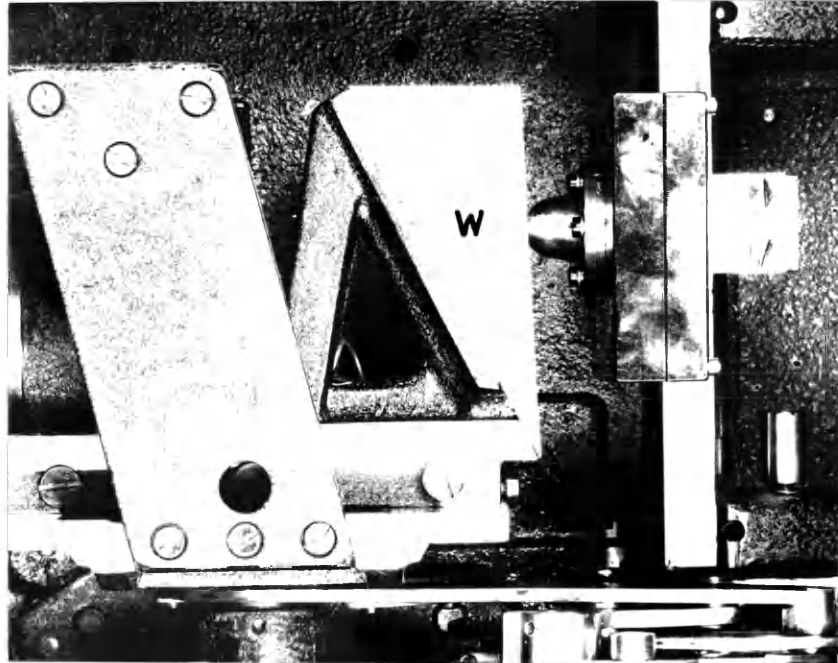


Figure 2. Top view of the American Optical Company (Spencer) No. 820 microtome modified for ultramicrotomy by the Pease-Baker device. Note the wedge (W) and the shorter propulsion point.

crylate block and floated on a water solution of dioxane it is obvious to the unaided eye that such sections are not of uniform thickness. Hillier and Gettner (23) find these variations are of the order of 0.2 microns but fail to reveal how they reached this figure. Visual estimates based on the comparative opacity of successive sections would indicate somewhat greater variations in thickness.

These observations, however, lead to the conclusion that random cutting with a microtome set at the minimum increment will yield sections both thicker and thinner than that increment. Some of these sections will be thin enough for use in the electron microscope. Such a technique, however, is intolerably inefficient and more fruitful adaptations of available equipment were developed.

As first described by Pease and Baker (11) their modification of the microtome consisted of a metal wedge fastened to the slide plane (SP in figure 1) of the instrument. The slide plane in the standard microtome makes an angle of about 25 degrees with a vertical section normal to the long axis of the microtome. The wedge inserted by Pease and Baker effectively reduced this angle to a tenth of its value and this in turn was presumed to reduce the increment of advance of the specimen by the same amount. Figures 1 and 2 show the No. 820 microtome both with and without the wedge modification. It will be noted that it was necessary to reduce the length of the propulsion point (P) in order to fit the wedge in place. The angle of the slide plane has been

further reduced in the commercial model, the Spencer No. 821, said to be specially designed for thin sectioning but differing in no obvious details from the No. 820.

Hillier and Gettner (23) have used plate glass mounted in Woods metal to form their wedge which is calculated to give a minimum increment of 0.02 microns. These workers, however, have no illusion that their sections approach this figure in thickness. This slide plane angle is used to permit relatively large motions of the screw which tend to average out the aberrations in this part of the mechanism. It was also noted that the modified instrument was extremely sensitive to outside vibration since a finger lightly applied to the case or even traffic in the streets nearby caused significant variations in the thickness of the sections produced.

In some of the early attempts to section commercial fibers using the original wedge modification of Pease and Baker (11) at the National Bureau of Standards, the variation in specimen advance was one of the factors responsible for the relatively few good sections produced. A rather thick section would be cut in one cycle to be followed by the failure of the knife to meet the block in the succeeding cycle and then the production of another thick section. At the time, these irregularities were attributed to vagaries inherent in the screw but data now available indicates that external vibrations and thermal changes must have played a part. In addition, although the face of the wedge used was

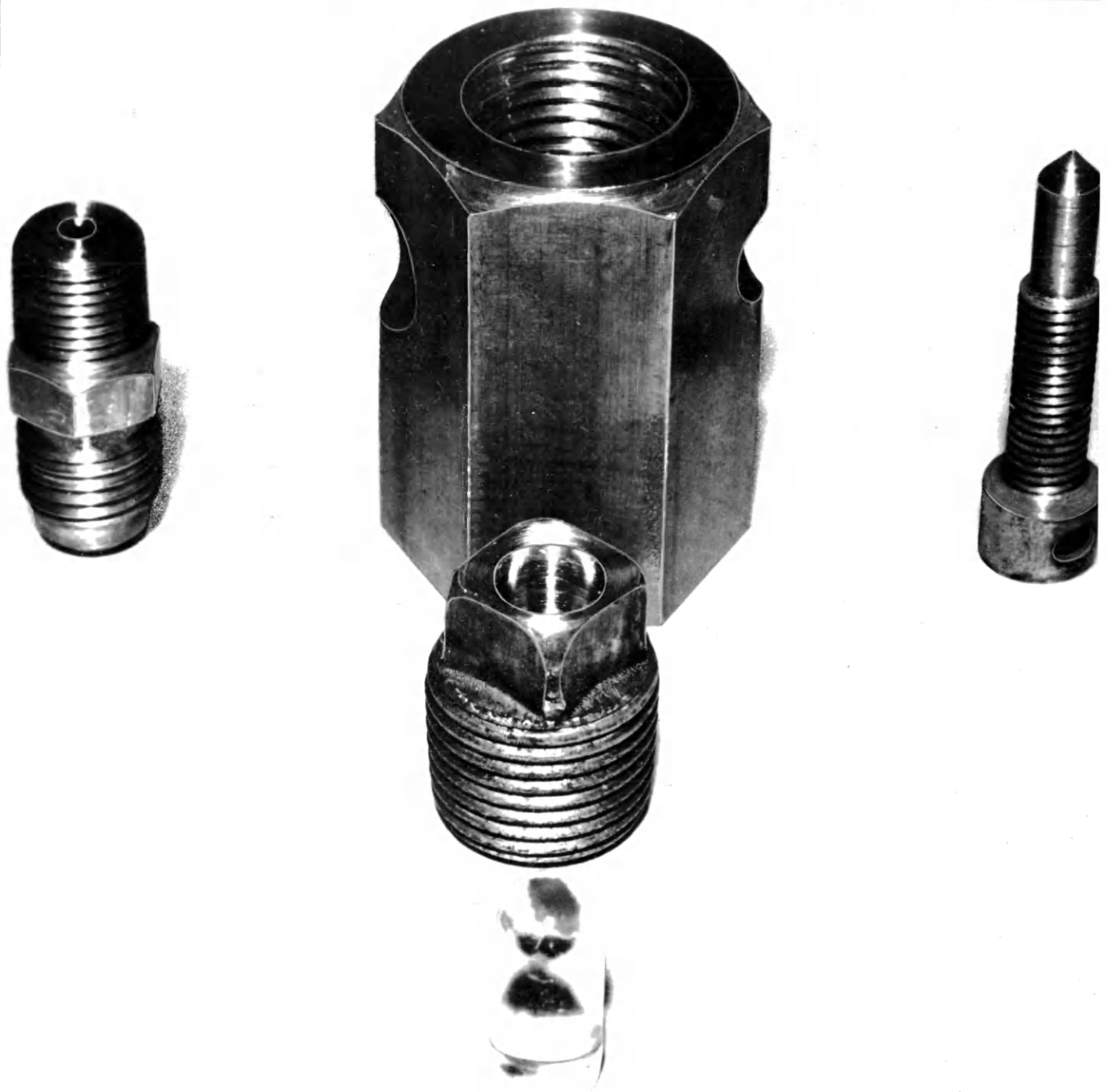


Figure 3. Disassembled view of the thermal expansion device. On the left is a Dole fitting, on the right a needle valve. In the center from top: the threaded block, pipe plug and embedded specimen.

surface ground it was not the equal of the optical flats and plate glass surfaces now in use. This may also have been the source of some difficulties. A steel wedge which Dr. A. W. Pratt of the National Institutes of Health used for little more than a year showed considerable wear visible to the naked eye. Since the frictional forces on the wedge are usually operating over a very small part of the surface such wear may occur in a short time and be another source of aberrations.

These considerations led Newman, Borysko and Swerdlow (12) to adopt a method of specimen advance based on thermal expansion. Their device is essentially a brass block with a hole drilled through the long axis and threaded at one end to receive a standard 3/8-inch brass pipe plug, which serves as the specimen mounting block (figure 3). A cavity drilled into the face of the plug provides a seat for the embedded specimen. A needle valve situated behind the plug is used to bleed in a coolant, compressed carbon dioxide gas. After cooling, specimen advance toward the knife is obtained by allowing the assembly to warm up again. The process involves the continuous thermal expansion of the metallic holder and specimen. This simple device can probably be adapted to any microtome. In fact, an even simpler strategem would be to use a metal block cooled in ice water and then clamped in the jaws of the microtome. This has been tried successfully, but the carbon dioxide coolant is somewhat more convenient.

In using the thermal expansion apparatus the embed-

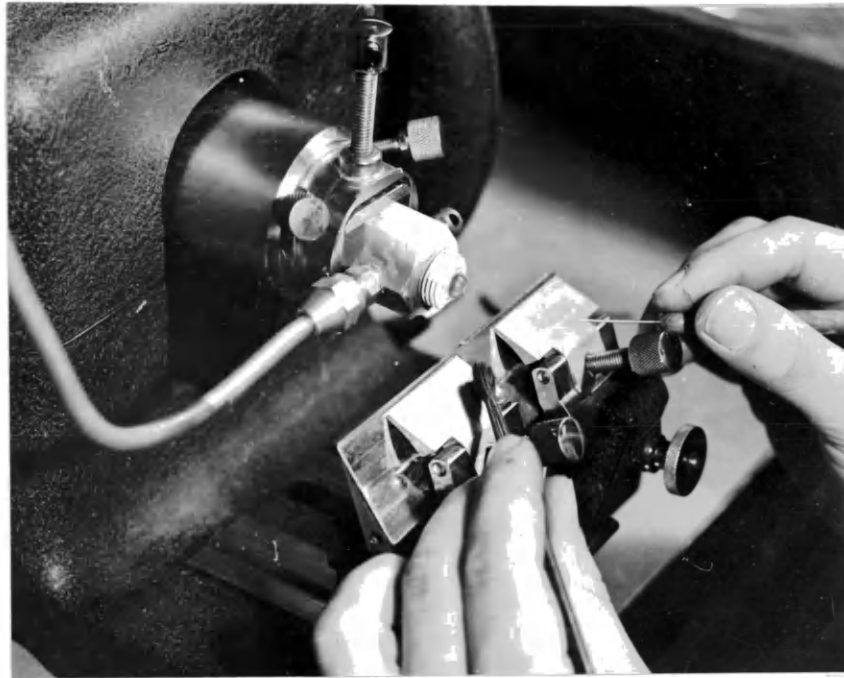


Figure 4. Assembled thermal expansion device in operation.

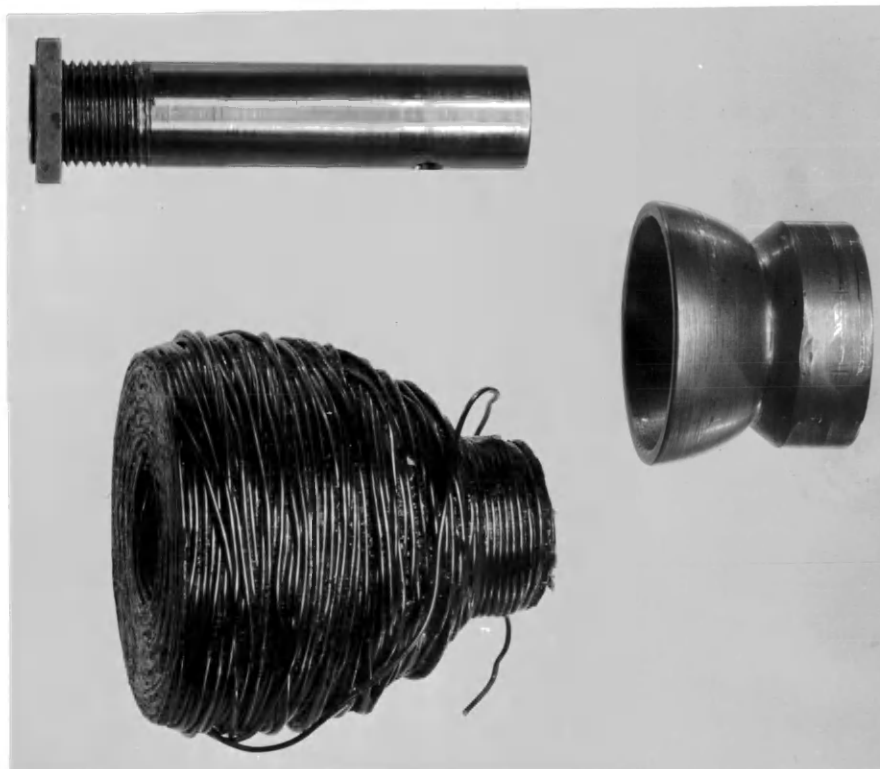


Figure 5. Disassembled magnetostriction device. At the left top is the nickel rod which forms the core of the coil (lower left). At the right is the microtome adaptor.

ded specimen is first cemented into the mounting plug. To do this, a small amount of a mixture of one part of gum rubber and twenty parts of paraffin is melted in the cavity by heating the plug. Paraffin alone would crack when the coolant is applied. The specimen embedded in methacrylate is then pushed into the cavity and the plug is allowed to cool. With the assembled device clamped in the jaws of the microtome, the needle valve is opened, allowing the compressed carbon dioxide to escape into the expansion chamber behind the mounting plug. The entire assembly contracts as it is cooled below room temperature.

When the temperature of the block has been reduced five or more degrees centigrade, the knife is adjusted so that the specimen just misses it on the cutting stroke. The specimen is then advanced mechanically at 2 or 3 micron increments until the first slice is made. The mechanical advancing mechanism is then disengaged by setting it to zero and the gas flow reduced or stopped. After a few seconds the specimen, which is set above the knife edge, can be cut. Because the specimen is advancing continuously, a quick chopping stroke involving one complete revolution of the hand wheel is necessary. With a little experience one can soon judge the necessary time interval between cuts. The assembled and operating microtome is shown in figure 4.

Some control of the rate of specimen advance can be obtained by bleeding the carbon dioxide at various reduced rates into the expansion chamber. If, however, the tempera-

ture of the block is not too far below ambient temperature, the expansion rate is low and the needle valve can be closed completely. As cutting progresses, the cooling and warming cycle is repeated as necessary.

The one real disadvantage of this arrangement is the lack of control of the rate of advance. It would be impossible, for example, to determine the increment of advance by measuring dimension changes in the hollow block since the entire microtome is subjected, to some extent, to thermal stresses which affect the spatial location of the face of the block. Its successful use depends on the development of experience and a degree of judgment on the part of the operator. It has the advantage, however, of removing the screw, pawl, ratchet wheel and horizontal ways from the operation thus eliminating variations in section thickness due to mechanical and thermal aberrations in these structures. It also appears to decrease the sensitivity to external vibration since the device described has been operating efficiently while mounted on a table which moved visibly during each revolution of the wheel.

The desire to obtain a finer degree of control of the rate of specimen advance led Eden, Pratt and Kahler (24) to design a modified thermal expansion microtome. They used a heavy brass rod insulated on its surface with a thin sheet of mica. Turns of resistance wire are wound on this insulated surface. A specimen mount was machined into one end of the block. Power dissipated in this heating coil was

regulated by a variable transformer of suitable range. When an electric current of definite voltage was run through the coil the expansion of the block with time gave a linear relationship with little change in slope over part of the range studied. For the block used by these investigators the interval was about 4 microns, the specimen holder advancing about one micron for each degree rise in temperature. With this device the voltage can be applied and then each section could be cut at some predetermined increment of time. A time interval as long as 15 to 20 seconds could be obtained.

This device has rather obvious advantages over the unmodified thermal expansion microtome. However, it still leaves the operator at the mercy of the time interval. Fifteen or twenty seconds are sufficient to remove the section from the knife only if there is no intervening difficulty with static electricity or necessity for orienting the section on the needle. On the other hand, while the 4 micron range might not appear to compare favorably with the 30 or 40 microns available with the unmodified device, it must be admitted that the latter does not permit full utilization of this range because of the lack of control.

The search for more precise methods of controlled specimen advance led to an investigation of the use of the magnetostrictive and piezoelectric effect for this purpose. The phenomenon of magnetostriction is too well known to require more than a general definition here. The term magnetostriction refers to the change in dimensions of a ferromag-

netic material such as nickel when it is placed in a magnetic field. It also applies to the inverse effect; that is, the change in magnetization when the dimensions are changed by an external force.

Nickel has about the largest magnetostriction value available. This is approximately 35 parts per million when the effect is at a maximum, that is at saturation. The total motion that could be obtained through this force would be small amounting only to about 3 microns for a 10 centimeter length of nickel rod. However, the great problem in designing a practical magnetostriction microtome is avoiding heating effects from the copper wire magnet coil. Data given by the International Nickel Company (25) indicates saturation in a field of 800 oersteds. The curve of the magnetostriction effect plotted against strength of magnetic field, however, becomes almost asymptotic to a value of about 35 parts per million at a field strength of 300 oersteds.

It was decided to design a microtome for this value as the maximum field strength and using a 3/4-inch diameter nickel rod 10 centimeters in length. The formula for a circular coil of n turns, where the magnetic intensity at the center is

$$H \text{ (oersteds)} = 4\pi nI$$

was used as a guide. In this equation n is the number of turns per centimeter and I is the current in absolute elec-

tromagnetic units. Because of the space limitations and the shape of the microtome receptacle the finished coil had the shape shown in figure 5 instead of being a simple solenoid. To reduce the power loss through the coil the maximum electromotive force was held to 2 volts and a No.18 gauge copper wire was used, the largest gauge permitted by the limited space to give the needed number of turns. Even with these precautions, however, the coil caused a temperature rise of several degrees centigrade. Since the nickel's magnetostriction effect is negative it was effectively cancelled out by the thermal expansion of the rod. Some heating of the coil had, of course, been anticipated but it was hoped that the dissipation potential of the mass would make it insignificant. Experience indicates that the design problem of heating in this device must be approached in some other way to achieve good results.

The piezoelectric effect is the change in dimensions of certain materials when subjected to electrostatic fields and conversely the generation of electric charges on the surfaces of these materials when pressure is applied to them. In most natural crystals such as quartz the piezoelectric response is very small. Yet they would be desirable materials to use since they have little hysteresis and the piezoelectric constants are well known. Even the use of a "bimorph" quartz assembly, however, failed to magnify the dimensional changes sufficiently for this application. Quartz crystals of maximum utilisable size are difficult to obtain and it is easy

to compute that even if they were available they would not be satisfactory at practical voltages. Certain synthetically grown crystals like Rochelle salts have sufficiently large piezoelectric constants along some axes but they are hygroscopic, weak and unstable.

Before World War II it was discovered that certain of the oxides of titanium have relatively enormous dielectric constants and subsequently that certain titanates, notably barium titanate, exhibited a high degree of electro-mechanical activity. A recent U. S. Patent (26) covers transducers made of these materials for use in underwater sound detectors and other ultrasonic applications. In these transducers the barium titanate is cast in the desired form and fired at a temperature of 1350 degrees centigrade. Transducers of this type are available commercially in a limited number of shapes, and special forms can be ordered though at considerable increase in cost.

It was found that tubes about 1-inch in diameter and up to 3-inches in length with a wall thickness between 1.5 and 4 millimeters were needed for the microtome. Since these were not stock sizes and since it was assumed that a number would be needed, the manufacture of these tubes was undertaken in the laboratory.

Information on the casting of these relatively large pieces is difficult to obtain since it is either the result of private commercial development or of restricted governmental research. On the advice of Dr. G. R. Shelton of the

Ceramic Dielectrics Group at the National Bureau of Standards, a slip casting method was used. A three piece plaster of paris mold of a 1-inch diameter polystyrene bar was first prepared.* It is important that the three parts of the mold be made from the same mix and from the same batch of plaster to insure their having similar water absorption properties. The mix used was 25 parts of plaster to 18 parts of water.

A slurry or "slip" of barium titanate in water was prepared with constant stirring, the two constituents being added as needed until a thick creamy consistency was obtained. Three to five cubic centimeters of a commercial deflocculent per 100 cubic centimeters of slurry were then added which decreased the viscosity of the suspension. After constant stirring for about one hour the barium titanate dispersion was poured into the thoroughly dried plaster mold. A skin of solid barium titanate soon forms next to the wall of the mold as water is drawn from the slurry into the dry plaster. When the skin reached the required thickness the remaining liquid slurry was poured out leaving a tube of wet barium titanate in the mold. This was then allowed to dry for from 12 to 24 hours.

The barium titanate tube was found to have contracted slightly from the cast and was gently removed. In this condition the tubes are extremely fragile and it is preferable to permit them to dry out thoroughly, a procedure re-

* The writer wishes to acknowledge the technical assistance and advice of Mr. Louis Cosette in the manufacture of these tubes.



Figure 6. Unfired barium titanate tube. In this condition the tube is powdery, fragile and has a faint pink color.

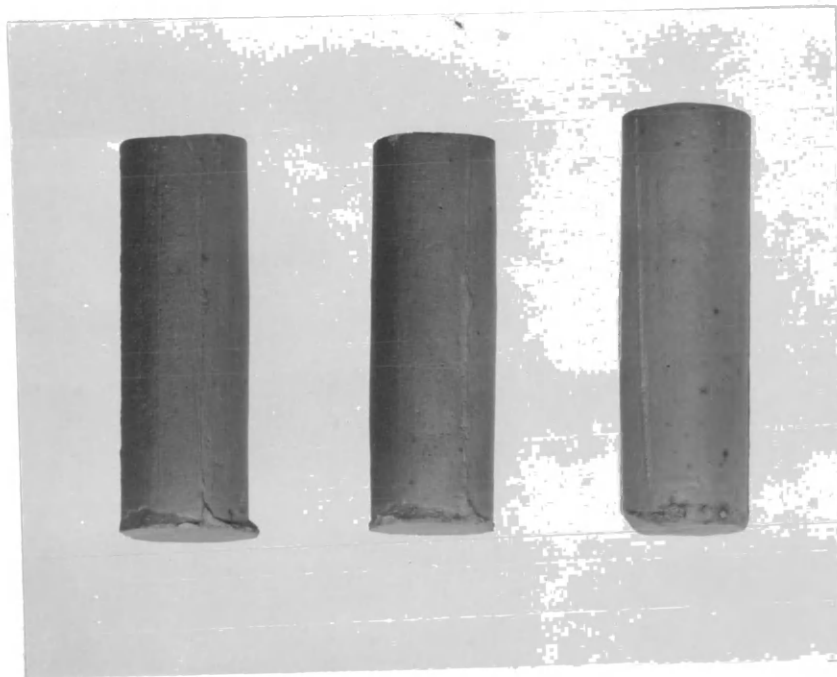


Figure 7. Fired barium titanate tubes. These tubes are a rugged, heavy ceramic.

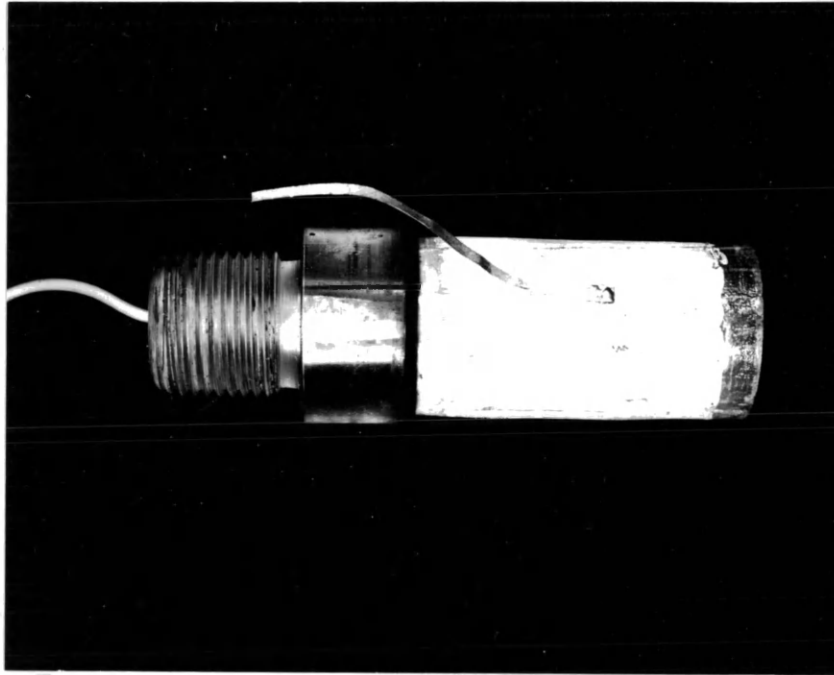


Figure 8. Fired and silver coated barium titanate tube. The tube is fastened to a plastic base. Leads are soldered to the silver coating. The inner lead comes through the base.

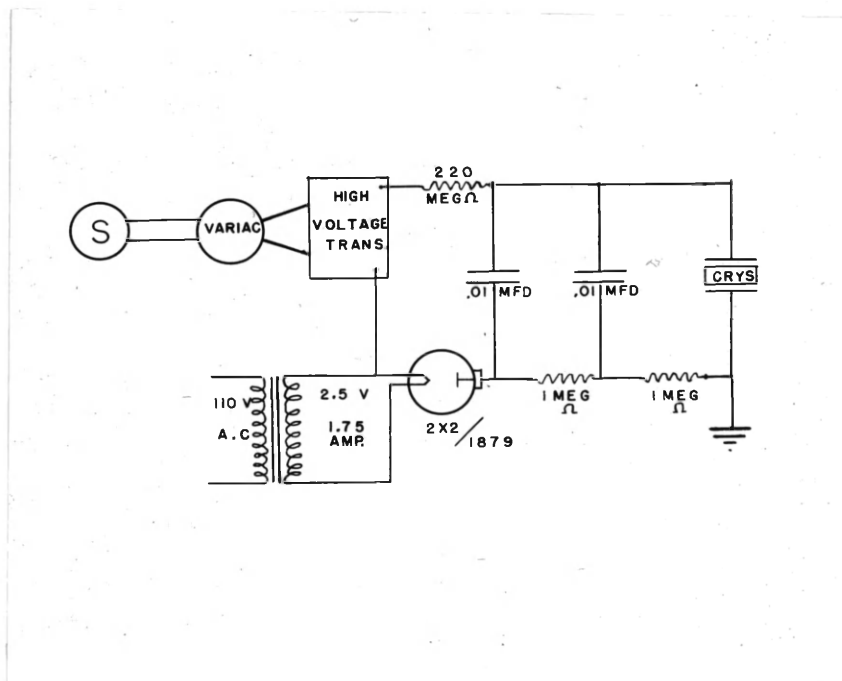


Figure 9. Circuit diagram of D. C. rectifier for the piezoelectric device.

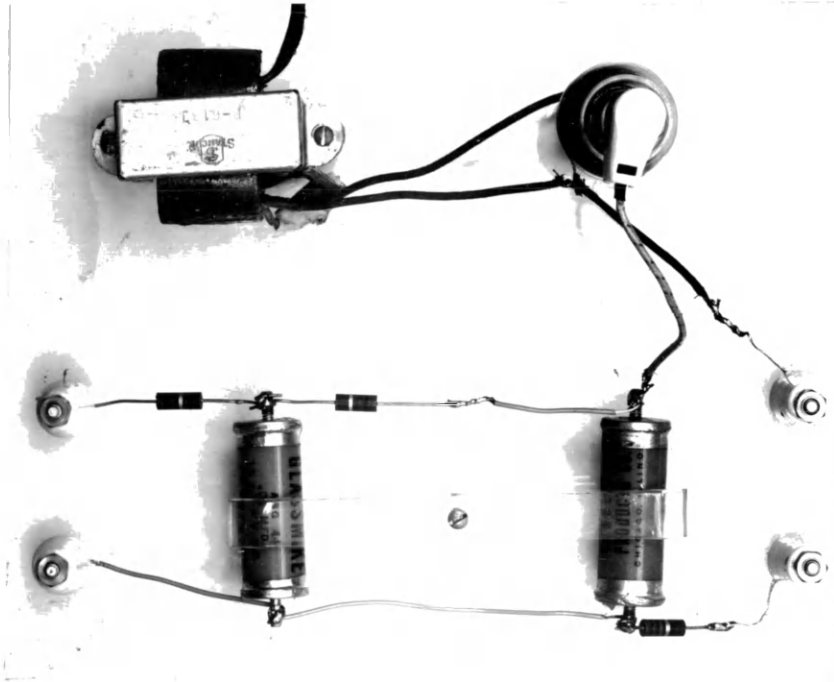


Figure 10. Top view of the rectifier. Variac and high voltage transformer are not shown.

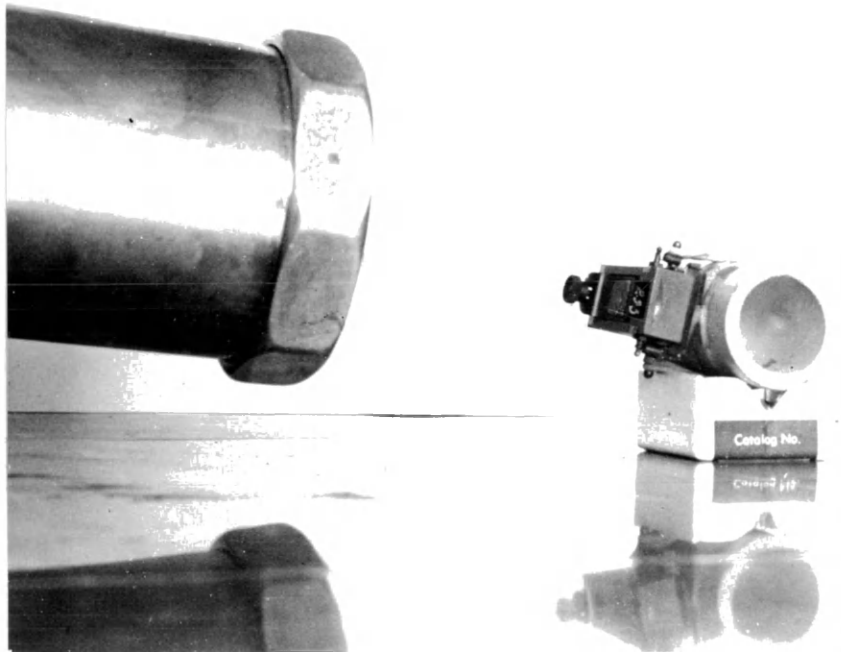


Figure 11. Measuring the change in length of the ceramic tube. At the left is the auto-collimator. The optical gage is held to the tube with a rubber band.

quiring a week or more, before firing them to ceramic maturity. An unfired tube appears in figure 6.

The tubes were fired in a platinum resistance furnace using a heating schedule of 3 degrees per minute; they were then slowly cooled. Several fired tubes are shown in figure 7. During firing a shrinkage of about 1/8-inch in the 1-inch diameter of the tube occurred. The finished tube is stable, rugged, and, because of the presence of the high molecular weight titanium, quite heavy.

A silver conducting ceramic was fired to both surfaces of the tubes except for a small area at the end of each tube which served to insulate the conducting surfaces from one another. A temperature of about 500 degrees centigrade is ample for firing the silver coating. Leads were then soldered to the silver. A silvered tube with soldered leads and attached to a plastic base is shown in figure 8.

An extremely simple D. C. rectifier was constructed to supply the energizing current. A plastic base was found to be more practical than a conventional metal chassis for the high voltage. The variac was used to regulate the high frequency A. C. entering the rectifier from the neon sign transformer and thus varied the D. C. output. Circuit diagram and completed rectifier are illustrated in Figures 9 and 10. In operation the tube is essentially a variable capacitor. The outer conducting surface is held at ground potential to protect measuring instruments adhering to it, while the exciting voltage is applied to the inner surface.

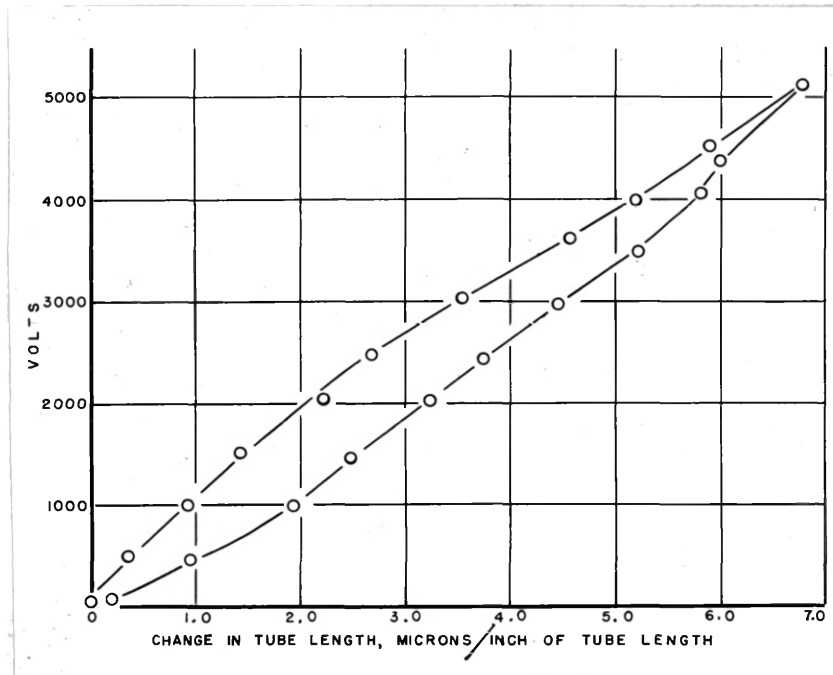


Figure 12. Typical hysteresis curve for one piezoelectric tube.

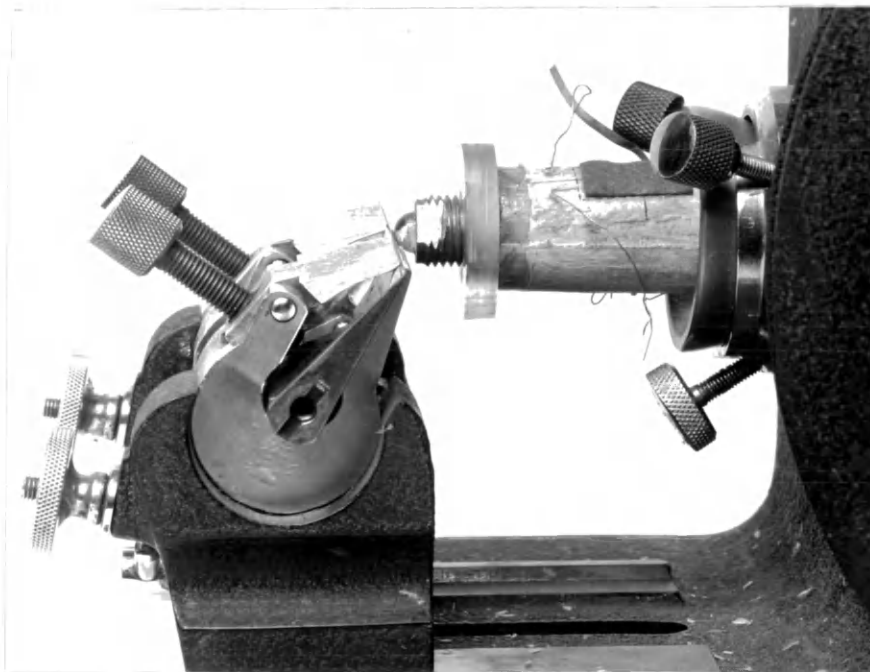


Figure 13. Assembled piezoelectric device in the microtome. Accessory equipment not shown.

Tuckerman strain gages, a type of optical lever, were used to determine the change in tube length under different voltages. An autocollimator was used to read the gages. The measuring method is shown in figure 11. Voltage plotted against tube length change appears in figure 12.

From the variation in the different runs represented in this graph it is obvious that the ceramic dielectric exhibits too much hysteresis to permit the use of the voltage input for determining the length changes. The optical levers used for these first measurements, moreover, do not permit operation of the microtome by a single individual, so they were replaced by two wire resistance elements attached to the outer surface of the tube. These consist of grids of one mil wire in close proximity to the tube but insulated from it by a sheet of paper and a film of Duco cement. When the tube lengthens the wires in this fine grid are attenuated and the cross-sectional diameter is decreased with a resulting increase in resistance while with tube shortening the changes have the opposite sign. These changes in resistance can be measured on a D. C. bridge with the proper type of ratio box but, for convenience, an A. C. continuously indicating bridge was used which read resistance changes in terms of dimensional change.*

It will be noted that for the tube represented in figure 12 each inch of tube length will give 6 microns of

*Sold by Baldwin Southwark as SR-4 indicating box.

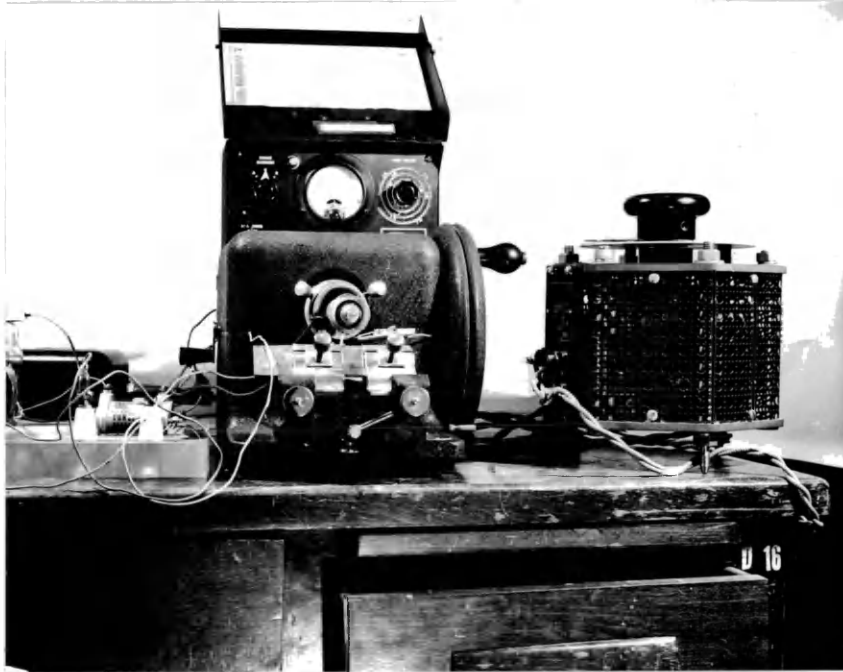


Figure 14. Assembled piezoelectric microtome.

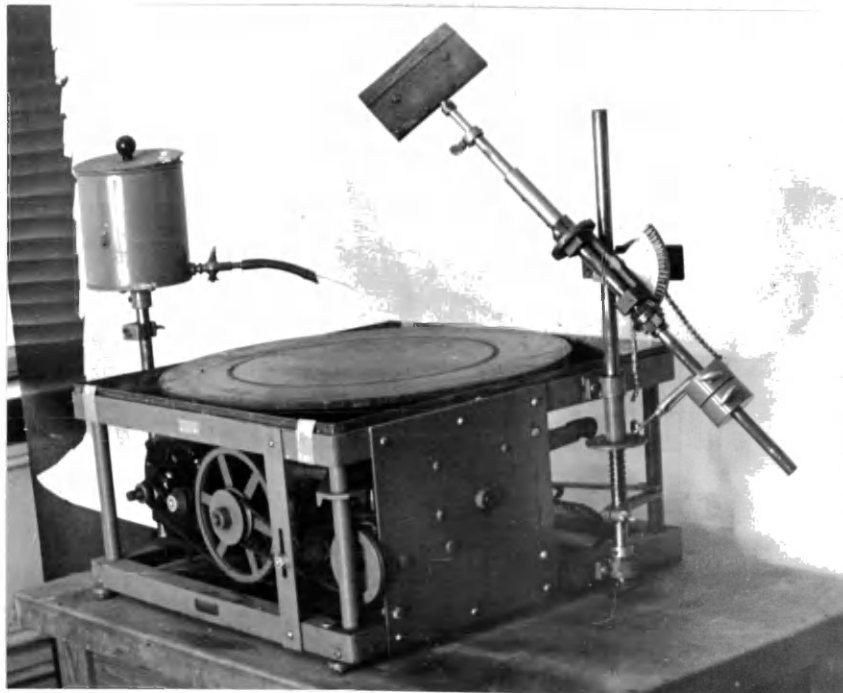


Figure 15. Fanz type knife sharpener.

change at 5000 volts. A maximum tube length of 3-inches is possible with the Spencer microtome but for most of the work done thus far a tube about 2-inches long has been found most convenient. The tube is fastened to a methyl methacrylate mount by means of the adhesive "Epon" and the assembly fits into the microtome by means of the modified head as shown in figure 13.

The voltage response of the tube is practically instantaneous and the tube can be lengthened or shortened while observing the changes on the A. C. bridge. The piezoelectric response is negative so that in normal operation it is found most convenient to start with the full potential across the tube wall and to decrease the voltage to advance the specimen. The microtome in operating condition is shown in figure 14.

While such a microtome has been found to give a high degree of precision and control to the cutting of thin sections it does raise the question of the value of using it with the Spencer microtome. In view of the defects in this instrument described by Hillier and Gettner (23), it does not seem possible, in using it, to attain the highest performance from the piezoelectric advancing unit. However, Cocks and Schwartz (27) have recently designed a microtome for thin sectioning which eliminates all bearings and ways. This instrument consists of a set of heavy flexure plates for providing vertical motion of the specimen. At present thermal expansion is used for advancing the specimen but it

is believed that the piezoelectric principle could be applied with best results to this microtome.

KNIVES

The knife for the ultramicrotome requires much more careful preparation than the edge used for cutting the thicker sections used in light microscopy. Unfortunately, there is as yet no practical way of defining or specifying a satisfactory edge for knives. The author has made a survey of purchasers and testers of large quantities of knives, among them Sears Roebuck and Company, Montgomery Ward and the National Bureau of Standards. All of these depend largely on specifying steels of known quality. One of the large mail order houses tests its knives by measuring the depth to which a knife will cut a rubber strip while under a given load. Such a test does not really simulate a microtome knife in action.

Richards (28) has described two methods for determining knife sharpness by means of the microscope. These methods are useful in checking the improvement in an edge during sharpening but they are not sufficiently critical to use for checking the suitability of a knife for cutting thin sections. As a result most microtomists have been reduced to the empirical test of using an apparently suitable knife and then judging whether the resulting sections are satisfactory.

It is known, of course, that among the more important elements in determining the suitability of a knife for ultra-

microtomy is sharpness, although this alone does not determine the knife's adequacy. Because of the importance of sharpness, however, detailed descriptions are presented here of some methods of microtome knife preparation. Almost all of the laboratories working with thin sections have found the Fans type of knife sharpener an important aid. This instrument, figure 15, consists of a turntable, a means for sweeping the knife across the turntable and turning it over after a definite number of sweeps, and a means for dropping a liquid on the abrasive field. However, the manner as well as the extent to which this device is used varies with the laboratory. There is variation in the abrasive employed and in the material used for the turntable plate. Hillier and Pease and Baker are known to use the glass plate supplied with the microtome knife sharpener and prefer the conventional knife polishing and sharpening agents such as alumina and rouge. In the work to be described later in this paper, it has been found to be more expeditious to use diamond dust as an abrasive and to replace the glass plate with one of bakelite. With the glass plate the abrasive fails to become embedded in the surface and is continually swept either over or off the plate. With bakelite, however, the plate becomes charged and can be used over and over again without the addition of more diamond dust. The automatic knife turning action is usually discarded because it is almost impossible to adjust it so that the knife makes a gentle descent on the plate each time.

Both Pease and Baker and Hillier find it unnecessary to strop their knives after the machine sharpening. On the other hand, in the work described in this paper all knives were given a stropping, just before use, on a flat leather strop charged with diamond dust. Without this final treatment inferior results were obtained.

Quite recently Latta and Hartmann (29) reported the use of a glass knife in thin sectioning for electron microscopy. A careful evaluation of glass knives was instituted. The results indicated that the glass knife was a good solution only for those without the time or facilities for sharpening steel. The proportion of good knives produced was much less than the inventors achieved. Furthermore, glass knives were found to be much less satisfactory than steel in the number of sections produced. The ability to shift from one part to another of a steel blade is a great convenience. The static electric charges that are commonly found developed on steel knives are often considered a nuisance. However, with glass knives the sections show little tendency to stick to the surface of the blades and recovery of straying specimen sections becomes a constant problem.

If we can assume that a worker will tend to publish only good illustrations of his results, we can judge knife quality to some extent by the micrographs presented by microtomists. Sections presented by Hillier and his coworkers (17, 23) and Pease and Baker (16, 30) are badly scored by knife marks while those of Newman et al (12) are practically

free of them.* The difference in embedding techniques must be considered in such a comparison since the first two were prepared in collodion-paraffin and the last in methacrylate.

Knife sharpening by whatever method, however, is arduous and time consuming. The two factors causing the deterioration of knives are corrosion of the edge due to exposure to air and the failure of the edge during the cutting of the specimen. Metals and other substances, more corrosion resistant or harder than steel, when used in knives might greatly reduce the sharpening problem.

Faberge (31) has reported the successful use of a knife of beryllium copper. This is a relatively hard copper alloy, used for some special purpose tools, but having only about half the hardness of tool steel. It is, however, very corrosion resistant and has a fine grain which permits sharpening to an excellent edge.

A knife of beryllium copper having the conventional American Optical Company design was produced in the laboratory shop. It was found to be much too soft to cut methacrylate embeddings, the edge failing after one or two cuts. Such a knife is suitable for cutting soft tissues in paraffin or wax but will not be satisfactory for hard plant materials embedded in resins. A similar knife of phosphor bronze gave the same results. Both of these experiments led to the con-

*Additional evidence can be found in references 13, 14 and 15 which were prepared essentially as the material in reference 12.

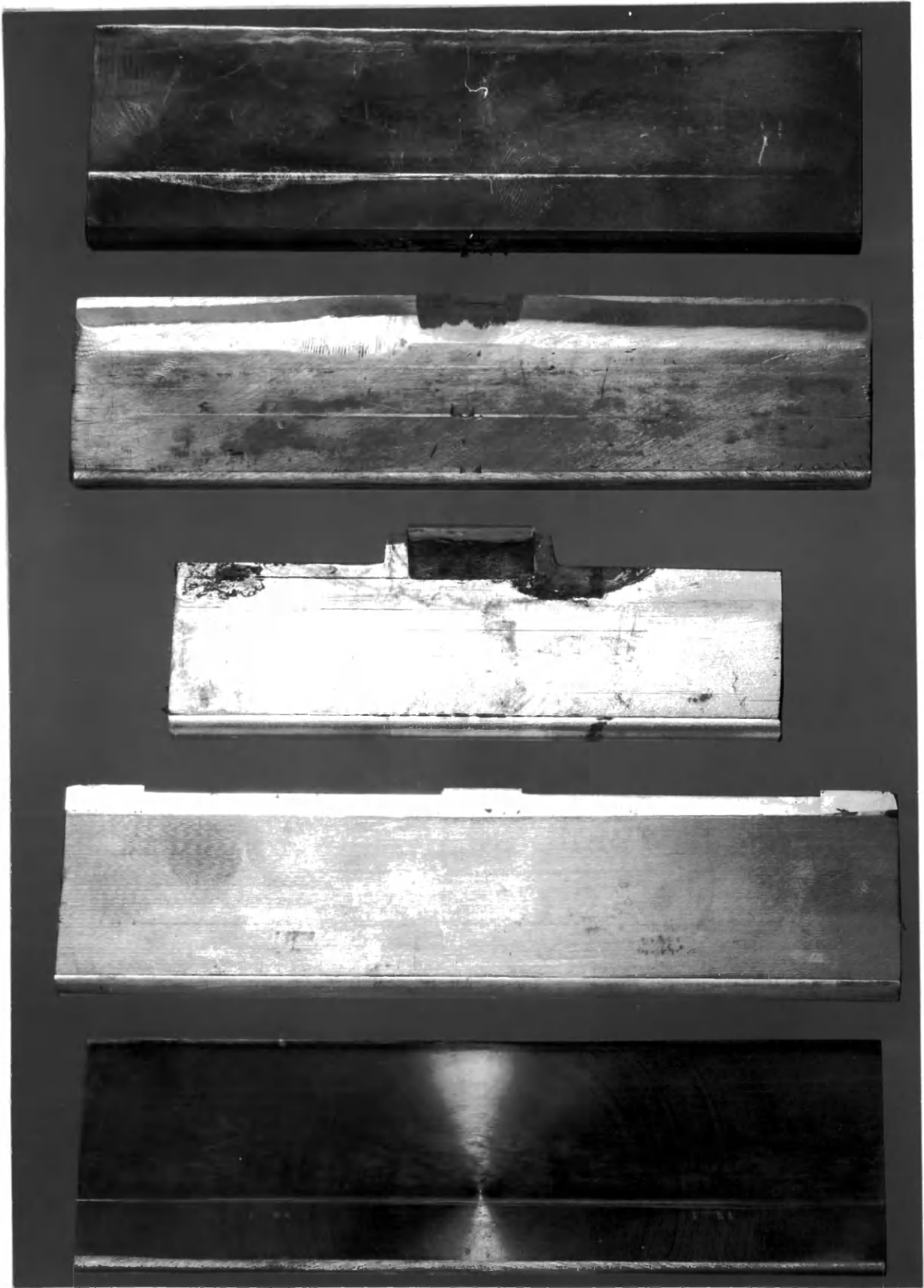


Figure 16. Experimental knives: (from top) beryllium copper, red sapphire insert, white sapphire insert, tungsten carbide insert, phosphor bronze.

clusion that the knife must be contrived from a substance with at least the hardness of steel. Dr. R. D. Stiehler suggested the use of Carbolloy in an experimental knife. Carbolloy is the trade name for a cemented tungsten carbide product (over 90 per cent tungsten carbide) used in metal turning tools and similar applications. It does not oxidize at ordinary temperatures and is about 30 per cent harder than tool steel.

Tungsten carbide inserts were placed in a monel replica of a microtome knife as shown in figure 16. The knife was sharpened by methods found successful with steel blades, but it appeared impossible to produce a usable edge on the tungsten carbide. Since this is a cemented material the particles of tungsten carbide chipped out of the matrix at the edge rendering the knife useless for ultramicrotomy.

Non-metallic knife blades of some industrial synthetics were also attempted. The most readily available of these is synthetic sapphire and several blades with sapphire inserts were completed. These are shown in figure 16. A knife capable of cutting sections was finally fabricated from a wafer cut from a sapphire boule, parallel to the C-axis of the boule. However, this blade showed little improvement over the steel knife. It chipped continually at the edge, presumably along the rhombic axes and required considerably more care in resharpening than the steel knives.

The results of all of our investigations so far lead us to conclude that we have arrived at a very satisfactory knife sharpening method which, while conservative is also

efficient. It is essentially as follows:

A badly nicked knife is also placed on the mechanical knife sharpener and sharpened on a bakelite plate charged with diamond dust of 25 micron particle size for 4 to 8 hours. This is followed by sharpening on a similar plate charged with 12 micron dust for 2 to 4 hours. During these first two steps the automatic knife turn over mechanism can be applied. Water is used as the abrasion lubricant in all steps.

The knife is then worked on a plate charged with 1 to 5 micron particle size diamond dust and finally on a plate containing 0 to 2 micron dust. For these last two operations the automatic knife turn over is disconnected and the knife is reversed manually every 15 minutes. When in satisfactory condition the bevel near the edge will have a high polish; the edge will have practically no nicks when viewed by direct illumination at 100 diameters nor any "stars" when examined by reflected light as described by Richards (28). Such a knife when directed transversely against a short animal hair, held at one end, is capable of entering it transversely and then cutting it longitudinally.

Knives, of course, vary in the quality of the steel from which they are made. Five knives varying in age from 2 to 30 years have all been used in this work. Though there is little to choose between them from performance considerations there is some variation in hardness as indicated by table 1.

Table 1
Vickers hardness of microtome knives

Knife	Edge	Side	Base
1	894	473	464
2	1003	870	847
3	870	782 (weld)	548
4	847	803	743
5	1003	847	673

It would appear that the variations in hardness at the knife edge which appear in table 1 are not significant in evaluating a knife for use in ultramicrotomy. The knives, in general, show considerable decrease in hardness toward the base. Continued use of such knives might result in reaching base metal which would fail to give satisfactory performance because it was too soft.

One knife purchased about 3 years ago failed to take a suitable edge. Microscopic examination revealed that holes were actually forming in the thin part of the bevel just behind the edge. Crystalline aggregates of the base metal were falling out of the porous structure. The knife was replaced and the substitute has functioned satisfactorily, but the incident indicates that knife sharpening techniques cannot be considered apart from the variable of knife quality.

It should be mentioned finally that two knives sharpened by the American Optical Company were supplied to Dr. Bang's group at Johns Hopkins Hospital. These knives were found to cut sections of fair quality but the use of

commercially sharpened knives does not appear practical. Knife edges apparently age or deteriorate even when unused. Their life may be only a matter of days so that the time lost in shipping would be prohibitive, even if cost were not considered.

HANDLING OF SECTIONS

Newman, Borysko and Swerdlow (12) had concluded that the maximum size of the specimen to be embedded should not exceed 8 cubic millimeters. The improved embedding technique previously described makes possible the processing of larger specimens. It was noted, however, that the number of bubbles in the embedding mass increased and that impregnation was poorer as the size of the specimen increased. For most materials it is well not to exceed the 8 cubic millimeter size.

This small specimen size is responsible for some mortality of sections. The electron microscope specimen screens are 1/8-inch diameter circles of Number 200 wire mesh cloth. Because of the stops and other considerations in the specimen stage it is impossible to scan more than the center 25 screen openings. To place the screen on the all but invisible section so that it is properly oriented requires the aid of a microscope and an infallibly steady hand. Despite considerable experience in handling sections a certain number were lost when the screens were misaligned over the sections. Once a screen has contacted a section embedded in a collodion film it is usually impossible to separate them again.

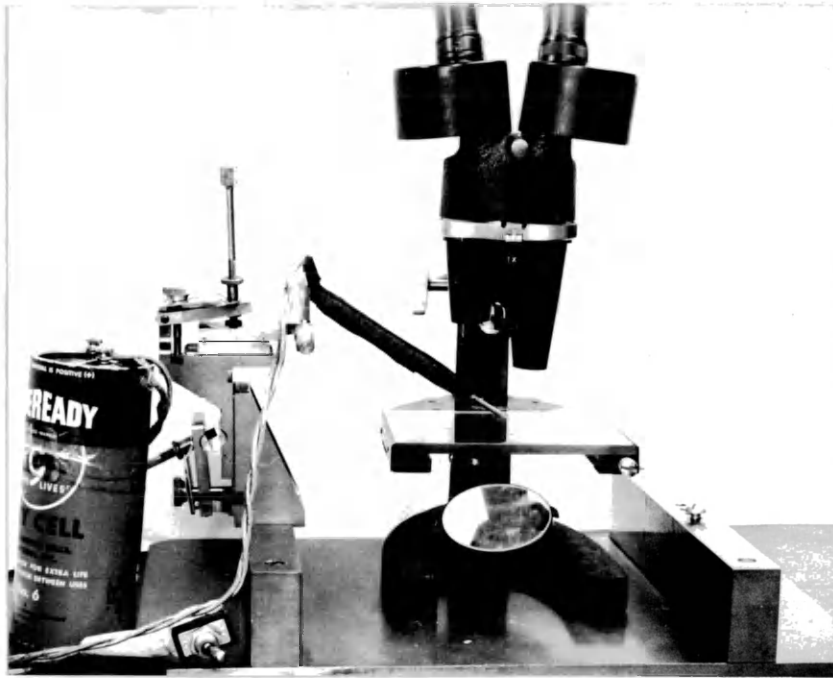


Figure 17. Micromanipulator assembly for placing electron microscope specimen screens on sections.



Figure 18. Dispenser for partially polymerized methacrylate. The bulb forces the liquid out through the capillary.

It seemed obvious that a micromanipulator would be of considerable aid in controlling the placing of the specimens. The main difficulty here was in designing a tool that would hold the screen and still permit the section to be seen during its positioning. The stainless steel screens supplied by the Radio Corporation of America are slightly magnetic and this property was used to advantage in constructing the micromanipulator tool.

A soft iron core, 6-inches long and 1/8-inch diameter, was wound with several hundred turns of magnet wire. A V-cut was made at one end of the core; the other end was held, by means of a set screw, in a simple pivot to the micromanipulator. The whole assembly appears in figure 17. A single dry cell is attached, through the reversing switch, to the magnet windings.

The screens were picked up by the energized magnet and centered over the V-cut. The micromanipulator placed the screens on the sections which can be seen fairly well through the wire mesh and the V. The micromanipulator was then drawn away from the section. In most cases the screen will adhere more tenaciously to the section than to the magnet and will be left on the former. However, if a gentle pull failed to release the screen, the polarity on the magnet was reversed and the screen fell away from the tool.

By the use of this instrument sections with extremely small areas can be mounted. The extreme emphasis placed on serial sections by some workers seems rather questionable in view of the difficulty in orienting the mounting screens.

Even with the instrument described here the screen wires could not easily be placed on the same area in even two adjacent sections. A change in orientation as great as 0.005-inch would result in considerable difference in specimen areas revealed.

IMPROVEMENTS IN EMBEDDING TECHNIQUE

The conventional embedding materials, paraffin, wax and collodion, have been commonly used also by electron microtomists. Carnauba wax alone (31) and double embeddings of collodion and high melting point paraffins (11, 23) have been the media in much of the recent work. Newman et al (12) found that the softer members of the methacrylate series gave excellent results. In their early work a polymer of pure n-butyl methacrylate was employed but this was soon replaced by a copolymer of 80 per cent of n-butyl- and 20 per cent of methyl methacrylate which had superior cutting properties.

Experience with methacrylate embedding revealed a rather serious problem connected with its use. When the specimen was placed in the catalyzed monomer and polymerized in an oven at 40 to 45 degrees centigrade the embedded specimen would often show serious damage due to the heat evolved in the exothermic polymerization reaction.

It was realized that decreasing the amount of catalyst and using even lower polymerization temperatures would result in a less rapid evolution of heat. Such experiments, however, showed that conditions which increased the polymerization time to 24 hours or more would cause frequent production of insoluble methacrylate polymers. The conditions under which such insoluble resins are likely to be produced

are somewhat difficult to determine. The organic materials and tissues placed in the embedding liquid are very apt to contain small quantities of polymerization inhibitors which constitute an uncontrolled variable. The insolubility is presumed due to the formation of high molecular weight resins which are insoluble in the conventional solvents.

Newman (32) in using n-butyl methacrylate in leather microscopy had discovered that partially polymerizing the monomer to a thick syrup before embedding the specimen resulted in a matrix relatively free of bubbles. Profiting from this previous experimental work a method of embedding based on partial polymerization was developed and used for the projects to be described later. The mixture of monomers (2 parts of methyl methacrylate and 4 parts of n-butyl methacrylate) are placed in a modified test tube. The bottom of this test tube is merely drawn into a short capillary as shown in figure 18. From 0.5 to 1.0 percent of a catalyst, 2, 4-dichlorobenzoyl peroxide, was dissolved in the monomer mixture. The tube was corked and warmed in a water bath at 50 to 55 degrees centigrade with agitation every 5 or 10 minutes. This agitation was necessary in order to produce a homogeneous liquid since the high molecular weight substance will tend to settle and form a gel near the bottom of the tube if it is undisturbed. After about two hours the liquid will have attained the viscosity of corn syrup.

The liquid in this condition would not flow freely into the Number 00 gelatin capsules used as embedding cells.

The tip of the tube was broken and the methacrylate forced out of the test tube in a thin stream by means of a rubber bulb as shown in figure 18.

The capsule, containing the syrup and a specimen, was placed in an oven at 40 degrees centigrade and was completely polymerized in 2 or 3 hours.

Some 500 embeddings have been prepared by this method and there has been no sign of heat damage in any section cut from these embeddings.

THICKNESS OF SECTIONS

It is difficult to determine accurately the thickness of ultrathin sections. The techniques of interferometry (28) applicable to thicker sections cannot be used. The thin sections do not flatten very well and errors induced by folds and bulges might result in a measured thickness several times the true value. Equally important is the fact that only usable portions of sections should be considered. In almost all papers containing electron micrographs of sections, some of the sections are clearly fragments or alternate thick and thin areas or both. This can be ascertained by examining the micrographs in the published reports.

Although instrumentation is pointed toward producing sections of a few tenths of a micron the published work usually avoids making direct claims to any values as small as these. Problems described in the discussion on microtomes set forth some of the reasons.

However, the questions of how to determine section thickness and of how to evaluate the results of ultramicrotomy in terms of section thickness are raised again and again. As a guide in instrumentation studies, some consideration of this point would appear justified. An attempt was therefore made to measure section thickness by the obvious relationship

$$t = \frac{m}{ad}$$

where t is the thickness, m the mass, a the area and d the density of the section.

The density of a methacrylate block was determined by hydrostatic weighing. Then sections were cut from the block by the thermal expansion technique. Selection of thin sections was made on the basis of the interference colors produced by the sections when floated on a liquid surface (50 per cent dioxane-50 per cent water). Some of the sections were checked for thinness by examination in the electron microscope, others were weighed. The cross sectional area was obtained by measurements with a calibrated ocular and by direct measurement of the methacrylate block with a screw micrometer.

Table 2

Thickness of sections

Density of block was 1.085 grams/cubic centimeter. Area of block was 0.1534 square centimeters

Section	Weight, milligrams	Thickness, microns
1	0.0057	0.3
2	.0085	.5
3	.0097	.6
4	.0097	.6
5	.0084	.5
6	.0129	.8
7	.0088	.5
8	.0093	.6

Weighings were made on an assay type balance using a reading telescope and finely divided index scale. Readings were estimated to 0.1 division.

The first group of four thin sections were weighed as a group, then intercompared in all possible combinations and reweighed as a group. A least square adjustment of these observations was made which gave the values for sections 1 through 4 accurate to within 10 per cent.

Sections 5 through 8 were weighed by single weighings considered good to 0.001 milligrams or better.

A second group of nine sections were weighed with values ranging from .0065 milligrams (0.4 microns) to 0.0112 milligrams (0.7 microns) with a mean value of 0.008 milligrams (0.5 microns).

Values of twenty-five sections from a third group ranged from 0.0051 milligrams (0.3 microns) to 0.0093 milligrams (0.6 microns) with a mean value of 0.007 milligrams (0.4 microns).

The thickness of these sections is much greater than is usually claimed for sections appearing in published reports. The reason for the successful sectioning work of Bretschneider (20) becomes obvious.

The area of the sections used in these determinations is much larger than those of sections cut routinely. Sections with large areas may be cut as thin as those with small areas but only with increasing difficulty. It is possible therefore

that, despite the criteria of selection these sections are not as thin as those appearing in the micrographs. If specimens had been included in the embeddings the determination of their usefulness by examination in the electron microscope would have been more valid. However, the evidence indicates that the thickness of the sections given here is of the same order of magnitude as those used in applications of ultra-microtomy.

SOME APPLICATIONS TO BOTANICAL MATERIALS

ELECTRON MICROSCOPY OF STARCH

Introduction. -- In his monumental work on starch, Reichert (33) gives a resume of the literature that had appeared before 1913 on the structure and form of the starch grain. Considerable detail is presented of developments in starch grain study from the original observations of Leeuwenhoek to the comparatively recent concept of the grain as a spherocrystal. Kerr's (34) summary in the 1950 edition of his standard text finds most of the ancient controversies still raging. However, he points out that it is noteworthy that recent workers have outlined the same general concept of structure.

These concepts deal with the macromolecular components of the starch granule. Since these macromolecules are relatively short chains of glucose residues we cannot hope to study them directly within the limitations of practical electron microscopy. These hypothetical structures, however, are based on conjectures and speculations from observations of treated grains. Alsberg (35), for example, considers the question of the presence of enveloping membrane and of the striations or lamina in his analysis.

Useful observations have been made with the light microscope, but the electron microscope should be able to

give superior resolution to the finer details of structure. The only recorded work on the electron microscopy of the starch grain is an abstract (36) of a paper presented by Southwick at the 1946 meeting of the Electron Microscope Society of America. The author has attempted to communicate with Miss Southwick in the hope of obtaining further information but without success. Since the abstract lacks details it was necessary to depend on the memories of several people who attended the meeting for additional data.

The work apparently consisted of observations on starch dispersals. The micrographs were rather poor due to the thickness of the grains. An attempt was made to relate the starch grain structure to the plastid.

With such little background it is impossible to correlate these new observations with those of Southwick. The work to be described is intended to present the structure of the starch grain as revealed by the electron microscope and to indicate some of the problems in microtechnique encountered in the study.

Materials and methods. -- Wafers of tissue were cut from the centers of commercial root tubers of Ipomoea batatas, Lam. and from commercial stem tubers of Solanum tuberosum L.

Some wafers were immediately fixed in Bouin's (Sass' modification), others in Navashin's fluid and still others in medium chrom-acetic or 2 percent formalin. Johansen's (37) formulae for these fixing solutions were used throughout. The

formalin was buffered by means of a tartrate buffer to reduce the acidity.

In addition, commercial starches obtained from the Organic Chemistry Section of the National Bureau of Standards were used. These included sweet potato (I. batatas), corn (Zea mays L.), and white potato (S. tuberosum) starches. This group was not fixed before embedding.

The tubers after fixation were washed in water and dehydrated through an ethyl alcohol series to absolute alcohol, then into 50 per cent absolute alcohol-50 per cent monomer mixture* and finally into pure monomer mixture. Embedding was accomplished as described in the section on improvements in embedding technique.

The capsules were then capped and polymerized at 40 degrees centigrade for 4 hours. At the end of that time the methacrylate embedding matrix was hard. The gelatin capsules were removed from the embeddings by soaking them in warm water. A brass pipe plug shown in figure 3 was used for mounting the embedded tissue in the microtome. Both the thermal expansion device and the new piezoelectric advancing mechanism, previously described were used for sectioning the tissues.

The sections were removed from the knife with a camels hair brush, picked from the brush with a needle and deposited on the surface of a 50 per cent solution of dioxane in water

*This monomer mixture was 80 per cent n-butyl methacrylate and 20 per cent methyl methacrylate as previously described.

as recommended by Pease and Baker (38). Sections placed on this liquid immediately flatten. The container with the sections was allowed to sink in a considerably larger volume of distilled water. This precaution was necessary because of the degradation products associated with even the chemically pure grades of dioxane. In some of the later steps the undiluted dioxane produces films which appear as artifacts and contamination in the final preparation.

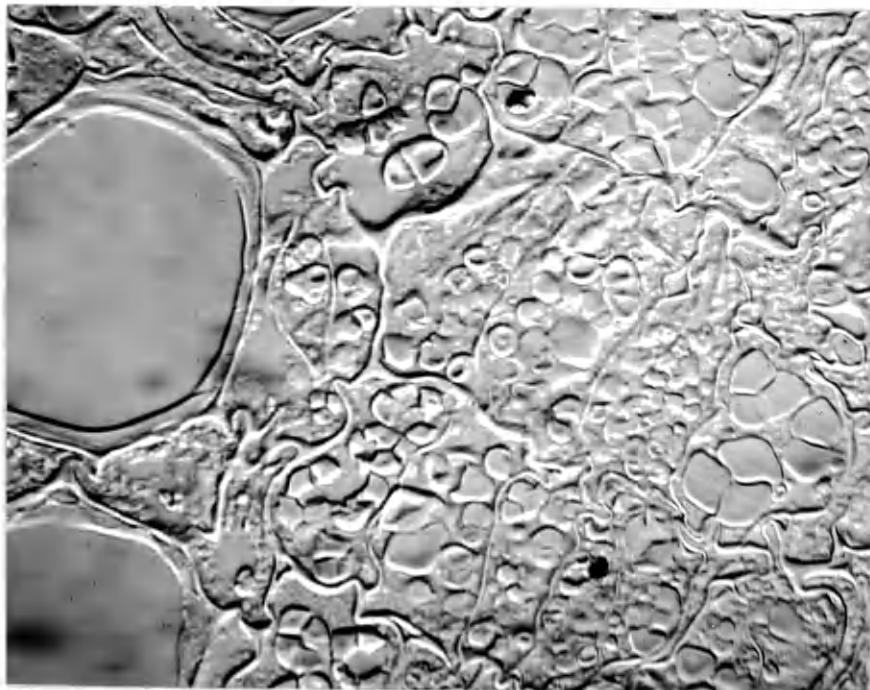
Needles were used to manipulate the sections to the surfaces of clean microscope slides where they were allowed to dry. The matrix of methacrylate was then removed by immersing the slide in amyl acetate. While still moist with the amyl acetate the slide was flooded with a 10 per cent solution of U. S. P. collodion in amyl acetate. It was then tilted to spill off the excess collodion and allowed to dry in a vertical position.

The section at this stage was found embedded in a thin film of collodion on the slide. This film was slightly wedge-shaped since the end of the slide at the bottom would accumulate somewhat more collodion as the excess drained from the top.

Films, with the embedded sections, were then stripped from the slides. This was accomplished by submerging the slide at the thin edge of the film. The film would usually leave the slide as the surface tension membrane of the water contacted it. As the slide was pushed below the surface the film floated off until it floats free. Figure 19 shows a



Figure 19. Stripping collodion film from the microscope slide. Note the specimen screens on the film.



100 μ

Figure 20. Double-diaphragm micrograph of a section of sweet potato tuber fixed in Navashin's.

step in this process. Film forming and stripping is performed most expeditiously at low relative humidity. It is extremely difficult to accomplish at much more than 50 per cent relative humidity and most of these films were poured and stripped at about 30 per cent relative humidity.

The floating film was then picked up on a glass plate and placed on the stage of a dissecting microscope. Electron microscope screens were placed on the sections by means of the micromanipulator (figure 17) described in the discussion of the handling of sections.

Finally, the sections and film were again floated on a large volume of water. One narrow end of a microscope slide was touched to an end of the film and the film pushed under the surface and then out in a scooping motion, so that the film again rested on the glass surface. After drying, the screens and sections could be easily separated from the empty film by gently manipulating with forceps.

Many of the sections produced by this technique adhered to the slide when the film was stripped and could not be used. To avoid this difficulty sections were allowed to dry on slides which were already coated with a thin collodion film. The matrix was then removed from the sections by immersing the slides in chloroform which dissolved the methacrylate but not the collodion. The chloroform was then allowed to evaporate from the slide. No difficulty was encountered in stripping these films and sections.

The sections were examined in an RCA-EMU (50 kv) electron microscope. Most of the micrographs were taken

using a biased gun as a source of electrons.

A few of the sections were shadowed with chromium as described by Williams and Wyckoff (39) to increase the resolution and contrast.

Observations and discussion. -- The application of ultramicrotomy to the starch grain involves a considerable problem in microtechnique. This problem primarily involves fixation. Wafers of the tubers placed in Bouin's were visibly disrupted after 12 hours in this fixing solution. The tissues swelled and cracks of considerable depth appeared all over the surface. A second sample of tissue was then placed in a different batch of the fixing solution with the same result. The two batches of Bouin's had a pH of 1.89 and 1.90 respectively. Pieces of the tubers were teased out on microscope slides and examined. They showed quantities of swollen and gelatinized starch granules. This is at least indicative that the tissue was damaged by the development of internal pressure due to the acid induced swelling of the starch contents. The Bouin's fixed material was discarded.

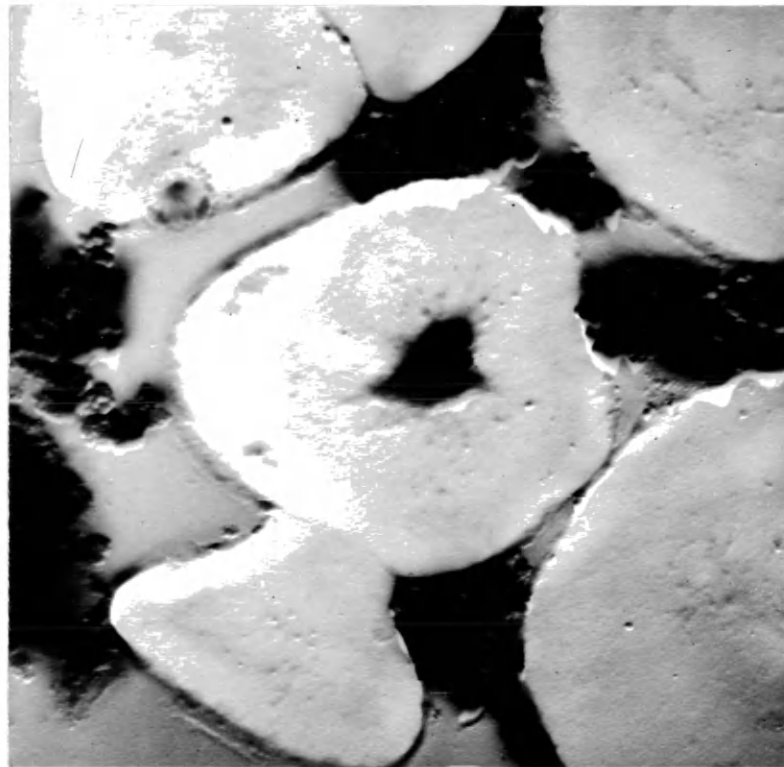
The remainder of the tissues showed no gross distortion from the other fixing solutions.

Some sections cut at 5 microns from the embedded tissues were examined in a microscope equipped with a double



1 μ

Figure 21. Electron micrograph of a section through a sweet potato starch grain. Navashin's fixation, chromium shadowed.



1 μ

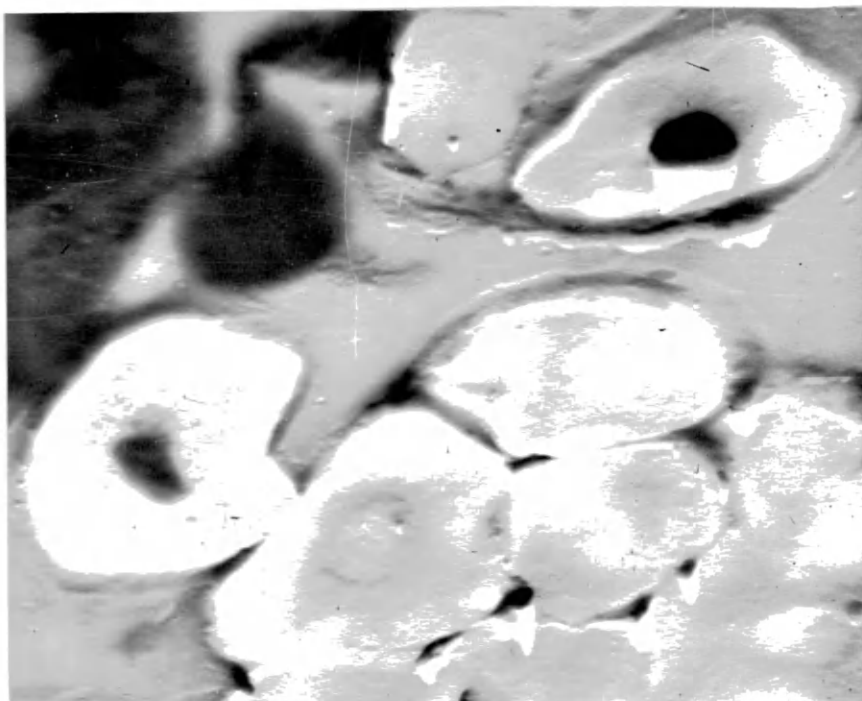
Figure 22. Electron micrograph of a section through a sweet potato starch grain. Navashin's fixation, chromium shadowed.

diaphragm* in order to correlate light microscope with electron microscope observations.

In figure 20 is a section of sweet potato tuber (I. batatas) fixed in Navashin's fluid. Many of the starch grains visible in this field show definitely differentiated centers. These centers appear to have a higher index of refraction than the peripheral areas. This phenomenon was also observed in the tissues fixed in medium chrom-acetic and in formalin. It was visible in starch grains which were not fixed before embedding.

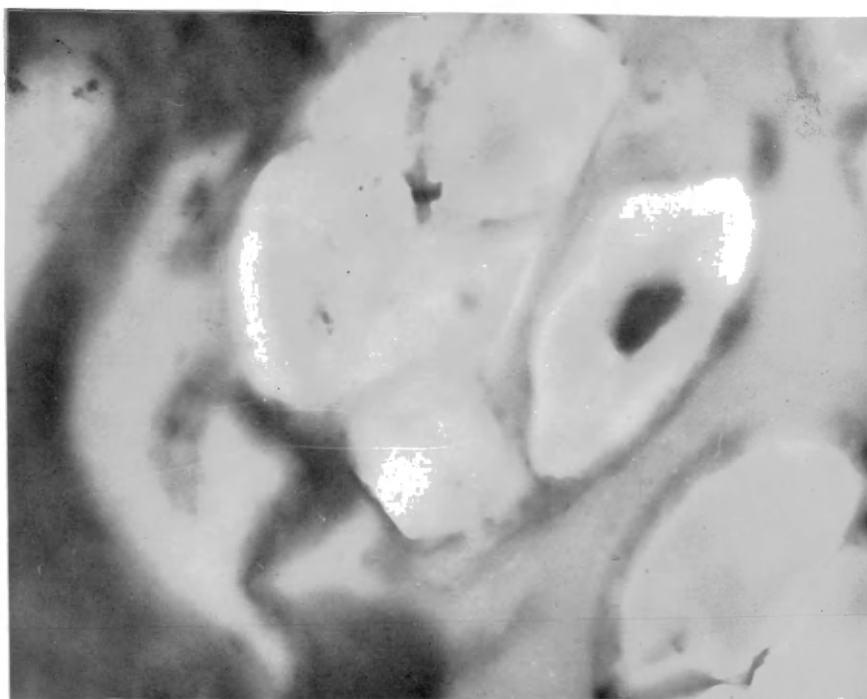
When thin sections of tubers fixed in Navashin's and embedded in a thin collodion membrane are examined in the electron microscope these differentiated centers appear as dark rather sharply delimited bodies. The starch grains in figures 21 and 22 show them clearly. Both the large and small grain sections display them to some extent in the light and electron micrographs. Some of the grains observed did not seem to possess the dark center but even assuming that the structure is common to all grains, many sections would be through planes which did not contact the center.

* This double diaphragm technique was first described by Saylor (40) and provides a simple and efficient method for examining unstained tissues. The tissues are differentiated by variation in the index of refraction of the component structures. Though widely used in index of refraction measurements it is practically unknown in other applications. Since Dr. Saylor anticipates publishing a discussion of its uses it is not deemed necessary to discuss them further here.



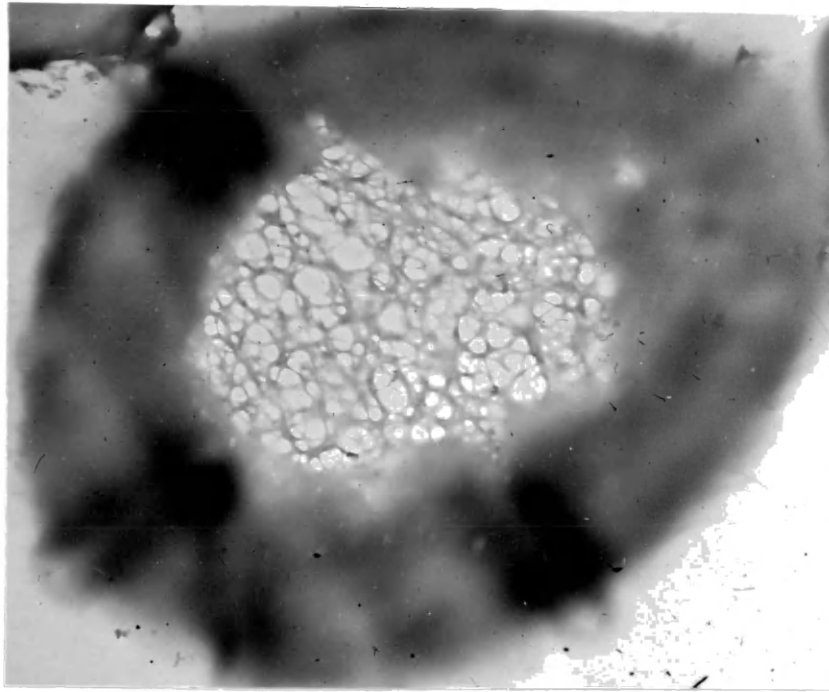
1 μ

Figure 23. Electron micrograph of a section through sweet potato starch grains. Navashin's fixation, chromium shadowed.



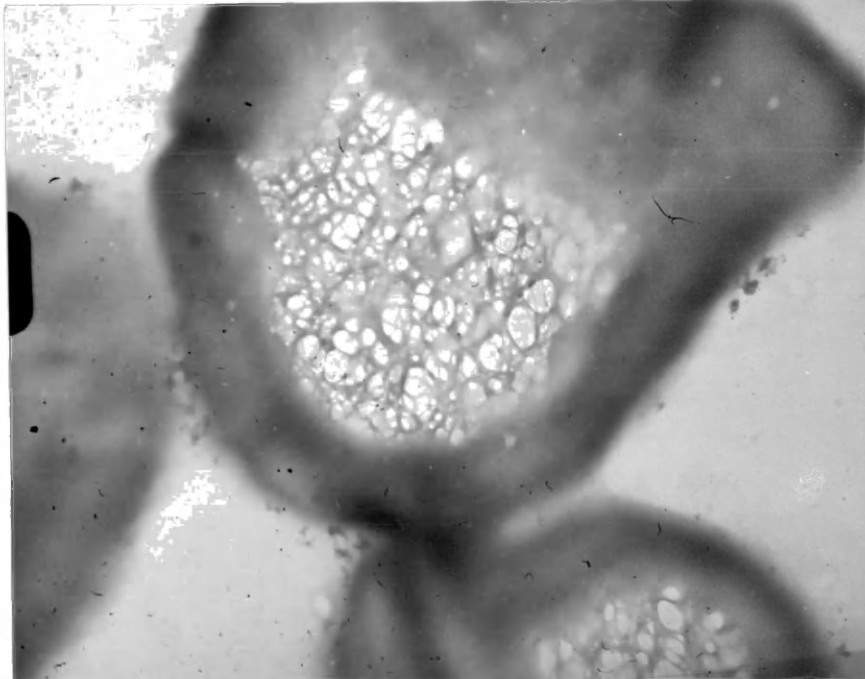
1 μ

Figure 24. Electron micrograph of part of the section in the preceding figure before shadowing.



10 μ

Figure 25. Electron micrograph of a section through a sweet potato starch grain. The section is lying on top of the collodion film and is desiccated.



10 μ

Figure 26. Electron micrograph of a section through a sweet potato starch grain. The section is lying on top of the collodion film and is desiccated.

Chromium shadowing* of the sections permits some additional information to be derived from the sections. In figures 23 and 24 are shadowed and unshadowed fields covering overlapping areas. Despite the assertion of Black et al (41) that shadowing contributes little to the visibility of fine structure in sections, the shadowed section seems the more desirable for study. Sections in figures 21 and 23 show that the dark centers usually do cast considerable shadows proving that they are higher than the remainder of the grain. In some step of preparation after sectioning, the center area of the Navashin fixed material swells and absorbs the solvents and supporting collodion so that a hump of more or less magnitude exists in the final section. It should be noted that in these sections the protoplasmic contents are also quite opaque and cast significant shadows. When the sections are placed on top of the collodion film, the matrix removed and the solvent allowed to dry, the starch structure is unsupported and has the appearance shown in figures 25 and 26. The center area assumes the "brush heap", reticulated structure characteristic of many dried gels.

* Chromium shadowing is a well established technique for obtaining contrast in electron microscopy. Its use on sections was first reported by Newman et al (12) and has, admittedly, justifiable criticisms. However, it does increase the resolution of some structures and for certain purposes there is no available substitute. The technique involves the projection of metal from a fixed point (electrode) over a preparation in a high vacuum. Projecting points accumulate the metal which travels in straight lines while low-lying areas are "shadowed" or protected from the "rays" of metal.

In a preparation of reasonably homogeneous thickness, such as these sections, the opacity of a limited area can be due only to an accumulation of heavy metals. The image formation in the electron microscope is by differential scattering of electrons rather than by differential absorption, as is usually the case in the light microscope. The heavy metals such as molybdenum, tungsten and chromium scatter electrons much more efficiently than the carbon, hydrogen, oxygen and nitrogen of which most living matter is composed. The selective absorption of salts of heavy metals have been used in electron microtechniques of rather limited usefulness (42).

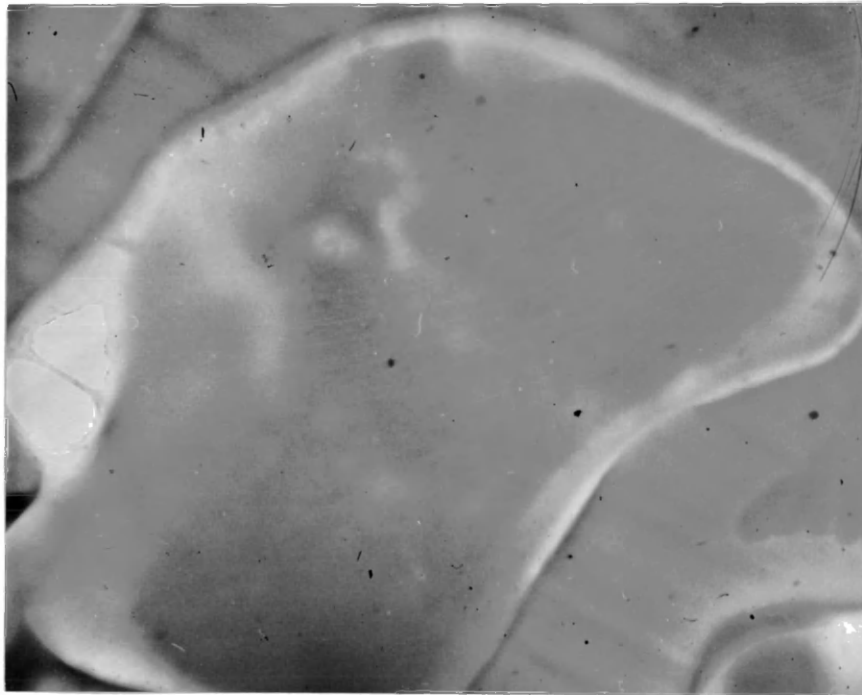
The Navashin's fluid used as a fixative contains chromic acid. When sections fixed in other fluids were examined in the electron microscope the opaque central areas were never observed. This was true even of the medium chromacetic which because of its chromium content could be expected to yield similar artifacts. An initial pH of 1.2 was found common to both of the solutions. However, after several hours the Navashin's fixing solution turned a greenish-brown indicative of the reduction of the chromium, probably by the formalin. As the color change took place the pH rose to about 2.7.

Mechanisms for the selective absorption of chromium at the grain centers are somewhat difficult to establish. It might be assumed that an accumulation of reducing sugar at these loci was responsible for immobilizing the metal in insoluble form. The addition of reducing sugars to the solu-

tions, however, does not result in the accumulation of chromium metal about the sugar.

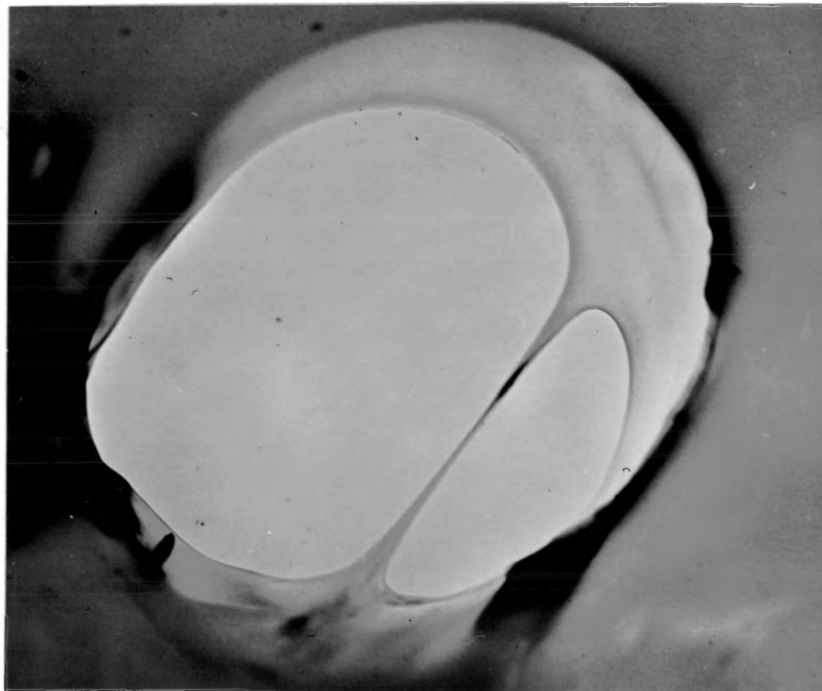
If the presence of protein is responsible, the action of the fixatives on pure protein should give a clue. Both of the solutions cause the accumulation of metallic chromium around particles of egg albumen, gelatin and collagen. It might be conjectured, of course, that the chromium ions can only penetrate the periphery of the grain in the reduced form, or alternately, that the formalin renders the envelope permeable to the chromium ions. Either of these hypotheses would account for the specific action of the Navashin's solution, the latter supported by the somewhat obscure reaction of aldehydes with starch. The gel structure, noted previously, would be more indicative also of protein structure than of a crystalloidal sugar.

Regardless of the nature of the reaction, the dark center is significant in evaluating certain assumptions that have been made on the structure of the starch grain. The opaque center in these grains has a definitely delimited area. There is no gradient of opacity from the center outwards. This is strongly indicative of a central area which differs sharply in its reactivity with chromic acid from that of the grain periphery. This center probably differs at the molecular level since it can not be differentiated even at the highest magnifications in untreated starch grains. Also it does not vary significantly in its electron scattering ability from the envelope surrounding it.



10 μ

Figure 27. Electron micrograph of an unfixed section through a sweet potato starch grain.



10 μ

Figure 28. Electron micrograph of an unfixed section through a corn starch grain which has been damaged by electron bombardment.

The untreated starch grain shown in figure 27 does not differentiate the center. This situation is similar to that encountered in cross sections of certain animal fibers in which the intercellular substance has about the same electron scattering power as the cortical cells and thus obscures their outline. In the latter case differentiation was obtained through the use of selective enzymes.

Kerr (34) has criticized the general picture of structure presented by recent workers because it rests largely on the concept of chemical homogeneity of the carbohydrate constituents. The observations made in this study are good evidence that in the region of the hilum, the organic center or nucleus around which the granule has grown, this homogeneity does not exist. Teller (43) has studied the development of starch granules in young growing potatoes. He finds first a thin amorphous haze which is blued by very dilute iodine. Around this a coating forms. This binds the interior into the completed granule and protects it against solution. The accompanying electron micrographs indicate that this starch nucleus* is still present in the mature grain.

None of the electron micrographs show any evidence of the outer membrane noted by earlier investigators. This is in agreement with Alsberg's (35) conclusion, based on optical and chemical evidence, that the membrane is an artifact produced during gelatinization. There does not seem to be much justification for relating the non-chromium absorbing periphery to the gelatinization membrane.

Striations or lamellae are not present in any of the grains studied, but according to Meyer (44) these disappear when water is removed from the lattice of the grain. After exposure to the exceedingly high vacuum in the electron microscope the grain would not be expected to reveal these structures. However, if these lamellae are due to variations in density due to fluctuations in physiological activity, as has often been suggested (33), it is difficult to understand why they would not have sufficient difference in electron opacity to be visible. In passing, it has also been impossible thus far to see by electron microscopy the lamellated structure in cross sections of cotton. The two phenomena may be related.

The sections used for these observations have been found to be extremely sensitive to exposure in the electron beam. In figure 28 an unfixed starch grain is seen shrinking after exposure. The starch exhibits, under this treatment, the high degree of elasticity common to most polymeric materials. Chromium shadowing and "curing" the sections in a low intensity beam increased their stability.

INVESTIGATION OF THE NATURE OF PLASMODESMATA

Introduction. -- The classical theory of cellular continuity by means of protoplasmic connections through the cell wall has been supported by dozens of investigations and reports. Sharp (45) distinguishes between large protoplasmic connections and strands of exceedingly small diameter. These latter, requiring special methods for demonstration, are called by him plasmodesms.

Modern proponents (46, 47) of the protoplasmic continuity concept hold that these intercellular strands are ubiquitous. The term plasmodesmata has replaced the earlier designation and the concept has been extended to incorporate nucleodesmata and plastodesmata (48). Eames and MacDaniels (49), among others, believe that the failure to find them in some cases is due to their excessive fineness.

Jungers (50) has questioned the view that plasmodesmata in cells with thickened walls are protoplasmic in nature. Their resistance to reagents and their arrangement in growing walls suggested that they were constituents of the wall itself. He was not able to demonstrate this, however. In studying living tissues Chambers (51) finds little direct evidence of protoplasmic bridges and believes that in many cases the reported connections are either fixation artifacts or fibrous structure in the intercellular substance.

The favored materials for observing plasmodesmata are some hard, thick walled endosperms of the type known as "horny". According to Johansen (37) such endosperm is cellular from the first mitotic division. The individual cells, therefore, must be subjected to tremendous pressures from the developing seed which would tend to destroy any delicate connections. Protoplasmic strands could rise secondarily but their formation through these thick walls is difficult to imagine.

Callan and his coworkers (52) examined the nuclear membrane in the electron microscope after removing it from the cell. They found numerous small holes but the identification of these as nucleodesmata is somewhat suspect. It is difficult to dissect out such a fragile film and then dry it without inducing stresses and breaks. There is no other report of electron microscope studies of cellular connections. The oft repeated claim that the fineness of the strands is the main detriment to their study would indicate that the electron microscope would be a considerable aid in detecting these structures. The general techniques, previously described, were therefore applied to some of these tough endosperms with the hope of determining the real nature of plasmodesmata.

Materials and methods. -- Endosperm of the American persimmon (Diospyros virginiana L.) the commercial date (Phoenix sp.) and the white oak (Quercus alba L.) were fixed

in 70 per cent ethyl alcohol. The endosperm tissue was taken from mature ripe seeds only. In the case of the persimmon the product of several trees and of two seasons were sampled.

The tissues were dehydrated in an ethyl alcohol series and placed in a monomer mixture of 80 per cent n-butyl methacrylate and 20 per cent methyl methacrylate for several days in order to insure thorough impregnation. The pieces of endosperm were then embedded by placing them in partially polymerized methacrylate and completing the polymerization in an oven at 40 degrees centigrade.

Sections were cut from the embeddings for electron microscopy using the thermal expansion device and the piezoelectric tube. In addition, the microtome was used to cut sections at 3 and 5 microns in the usual way for light microscopy.

The conventional stains for demonstrating plasmodemata were used. These included the iodine-potassium iodide solution followed by sulfuric acid used by Scott (47), as well as the haematoxylin procedure recommended by Johansen (37). It was also found that excellent results in differentiating the walls of the horny endosperm cells was obtained with the Herzberg stain. There are several versions of this reagent but the most recent (53) is simple to formulate and gives the proper staining reactions. A saturated solution of C. P. zinc chloride in distilled water is first made. Fused sticks of zinc chloride in 50 gram, unbroken, sealed bottles are best for this purpose. Then 0.25 grams of iodine

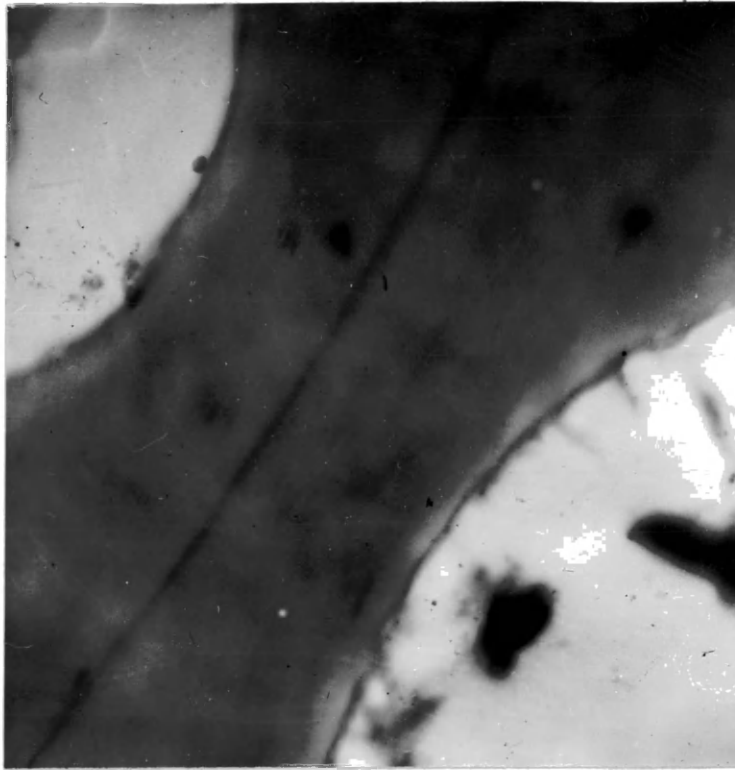
and 5.25 grams of C. P. potassium iodide are dissolved in 12.5 cubic centimeters of distilled water. Twenty-five cubic centimeters of the first solution is mixed with the second solution. The liquid is then poured into a narrow cylinder and allowed to stand until clear (12 to 24 hours). After decanting the supernatant into an amber-colored, glass-stoppered bottle a small piece of iodine is added to the solution. A small amount of sulfuric acid sometimes improves the action of the stain for certain purposes.

A microscope equipped with a double diaphragm or with phase contrast equipment aided in observing some of the structures.

Observation and discussion. -- Despite the hardness of these endosperms they cut with surprising ease when embedded in the methacrylate co-polymer. Both thin sections for electron microscopy and thicker sections (3 to 5 microns) flattened easily when placed on the 50 per cent dioxane solution.

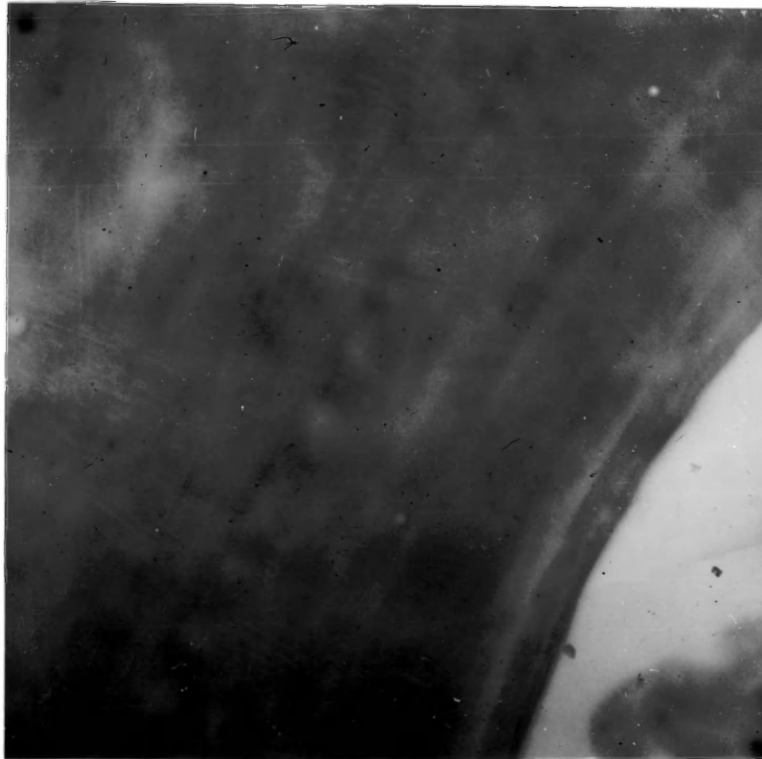
Endosperm of the white oak (*Q. alba*) is not a horny endosperm. The tissue has relatively thin walls and each cell has a large central area well supplied with starch. Because of the thin cell wall and the dark staining starch this was found much less desirable material for study than the horny endosperms and the investigation was largely confined to these latter.

The endosperm of persimmon (*D. virginiana*) was examined in the electron microscope at about 3500 and 5000 diam-



10 μ

Figure 29. Electron micrograph of a section through the wall of an endosperm cell from Diospyros. Note the middle lamella and the amorphous structure of the wall.



10 μ

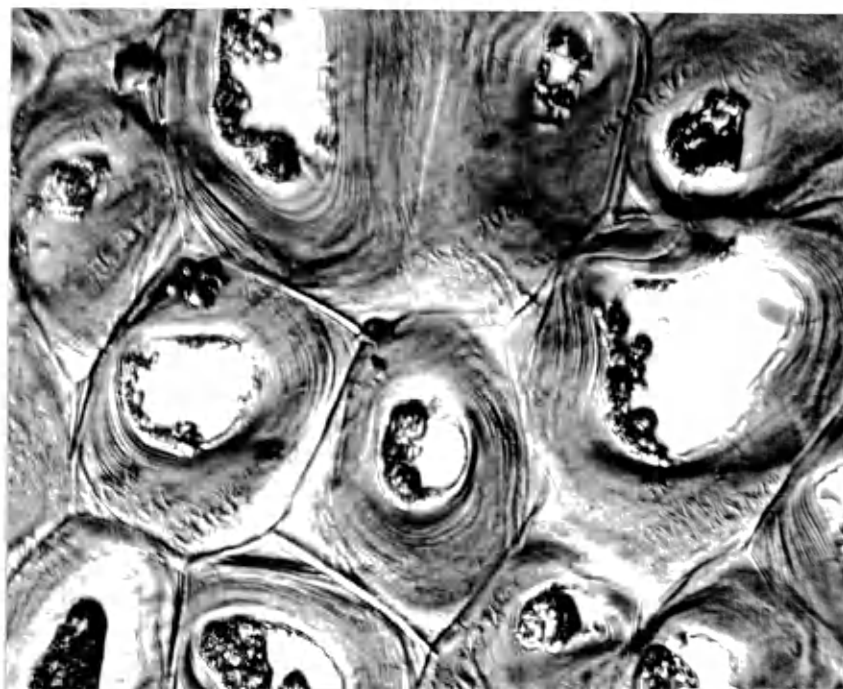
Figure 30. Electron micrograph of a section through the wall of an endosperm cell from Diospyros. Note the annular structure of the wall.

eters. In the 112 sections from the endosperm of 7 persimmon seeds there was no identifiable evidence of transverse protoplasmic connections. The fortuitous sectioning of a plasmodesma along its length would occur only occasionally. However, a goodly number of the sections of cell wall would be expected to contain circular, elliptical or conical sections of the plasmodesmata from cuts through non-parallel planes. These were not found and the wall seemed largely amorphous as can be seen in figure 29.

Plasmodesmata in stained sections, 3 to 5 microns thick, are easily visible in an optical system containing a 4 millimeter objective and a 10 power ocular. Therefore they must exceed 0.1 micron in width. Numerous electron micrographs at magnifications which would show these structures as 2 or 3 millimeters wide, fail to reveal them. If the fineness of the plasmodesmata were the only obstacle to their visualization it would be overcome by the superior resolving power of the electron microscope.

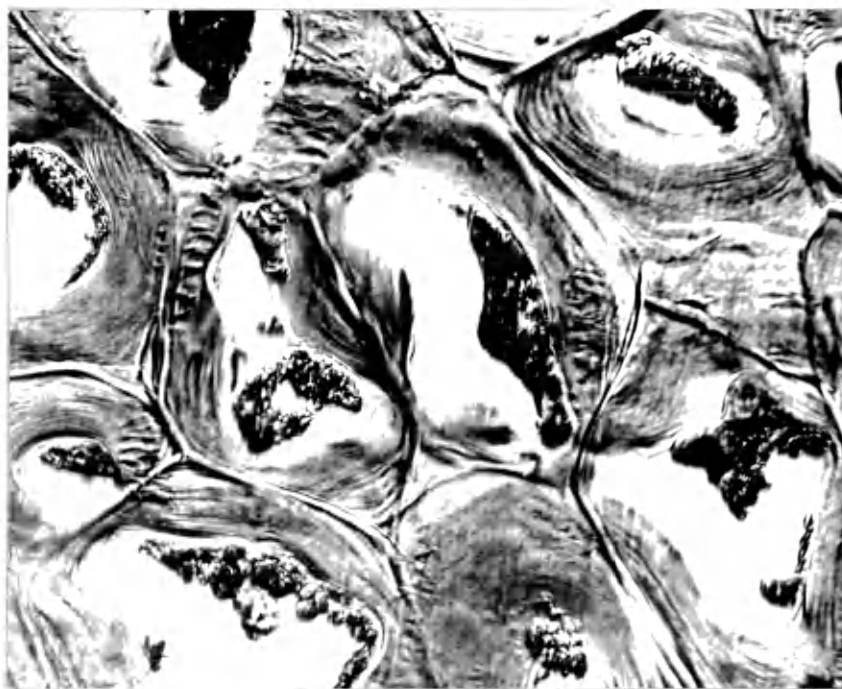
Though no evidences of plasmodesmata were noted, an annular structure could be observed in the walls of some of the horny endosperm cells. These appeared as faint, alternating layers of more and less dense material. They were not visible in all cells but in some preparations were seen rather frequently. The annular structure is shown in the electron micrograph in figure 30.

Some of the 3 and 5 micron sections were treated with the Hertzberg stain and examined in the light microscope



100 μ

Figure 31. Section through the endosperm of Diospyros. Stained with Herzberg's and photographed with the aid of a double-diaphragm.



100 μ

Figure 32. Section through the endosperm of Diospyros. Stained with Herzberg's and photographed with the aid of a double-diaphragm.

equipped with a double diaphragm. The cell walls turn a light brown color in the stain, indicative of lignin or pectin. In some of the cells the walls reveal hints of an annular structure. If a few drops of a 30 per cent solution of sulfuric acid are drawn under the cover slip, these annular lines become more distinct. They also appear in more cells than they do with the Herzberg stain alone. The accentuation of these annular structures with sulfuric acid increases with time until after about fifteen minutes the maximum effect is attained. Only sections 5 microns or less in thickness permit this ringed structure to be observed satisfactorily. Several different areas of such sections are shown in figures 31 and 32. Although both the double diaphragm and phase contrast objectives increase the resolution of the layers they can also be detected in ordinary transmitted light.

The annular structure is only generally concentric. In the immediate region of the protoplast the rings are sometimes truly annular but, in general, they exhibit considerable variation. Some cells show compression of the entire cell wall at certain points so that the rings appear normal to the intercellular layer. At other part of the tissue the peripheral layers are actually not rings, but change direction and appear to cross the middle lamella into an adjoining cell.

Endosperm tissues of the date (Phoenix) present a somewhat different picture. When stained with the Herzberg



Figure 33. Section through the endosperm of Phoenix.
Stained with I-KI.

100 μ



Figure 34. Section through the endosperm of Phoenix.
Stained with I-KI.

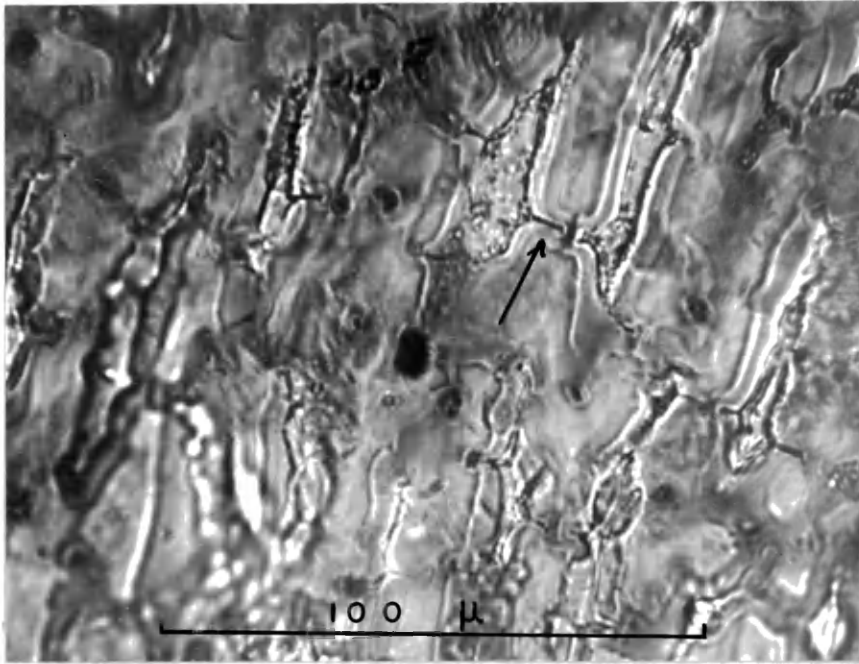


Figure 35. Section through the endosperm of Phoenix stained and swollen with I-KI and sulfuric acid. Arrow indicates the plasmodesma-like artifact.

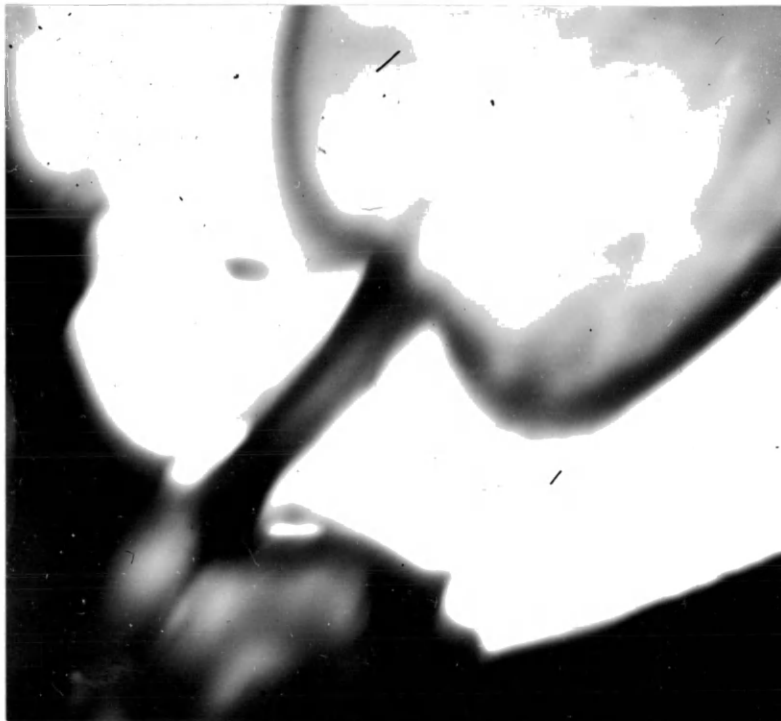


Figure 36. Electron micrograph of a section through the endosperm of Phoenix. This is the partition between two cells.

stain the walls become a dilute blue, indicative of some celluloses. The protoplasts in longitudinal section appear to touch the middle lamella in adjacent cells at numerous points (figure 33). The relatively large protoplasmic connections penetrate the cell wall and appear to be separated from similar projections in the next cell only by the lamella. In focusing up and down this appears as an unbroken, refractive line between the two protoplasmic connections. Numerous protoplasmic projections are cut in cross section and can be seen as approximately circular tubes (figure 34).

Unlike the persimmon endosperm the date endosperm is extremely unstable in acid solution and in the Herzberg stain. The walls swell, diminishing the size of the protoplast. The protoplasmic connections shrink to mere threads. In this condition the protoplasmic projections approximate the delicate strands called plasmodesmata. Since sulfuric acid is commonly used in differentiating plasmodesmata this severe swelling must be kept in mind in interpreting tissue structures. While the swollen material is difficult to photograph satisfactorily it can be seen in figure 35.

Observations in the electron microscope showed no indication of plasmodesmata in the thickened parts of the walls. The drawing of Eames and MacDaniels (49) shows the large protoplasmic projections ending in the thickened wall. Thin linear strands then connect with the protoplasmic projections in the next cell. Figure 33 shows that this is not the true picture. The only visible barrier to the proto-

plasmic projections is the pectinous middle lamella. No plasmodesmata through this thin film were visible, even in the electron micrograph in figure 36.

According to Meyer and Anderson (54) the middle lamella is largely or entirely composed of colloidal pectic materials. The primary walls at the tip of these protoplasmic connections are, if present, very thin. Such a membrane could conceivably pass dissolved carbohydrate food materials from cell to cell of the date endosperm.

The thickness of the middle lamella in the persimmon endosperm appears to have the same average value as the plasmodesmata. This was true in the commercial preparations examined, in Johansen's (37) picture of plasmodesmata in persimmon and in sections cut during this work. In the micrographs shown in figure 31 the lamella is plainly visible. Evidently the fineness of plasmodesmata is not the reason for their absence.

These fine threads through the cell wall would be extremely inefficient as a mode of transport in any case. The rate of flow will obviously be less under a given pressure as the radius of the tube is made smaller. If it is assumed that flow is due to diffusion pressure, we can use Poiseuille's (55) relationship to determine the order of magnitude of this flow. This is expressed as

$$v = \frac{\pi r^4 \Delta p t}{8 \eta l}$$

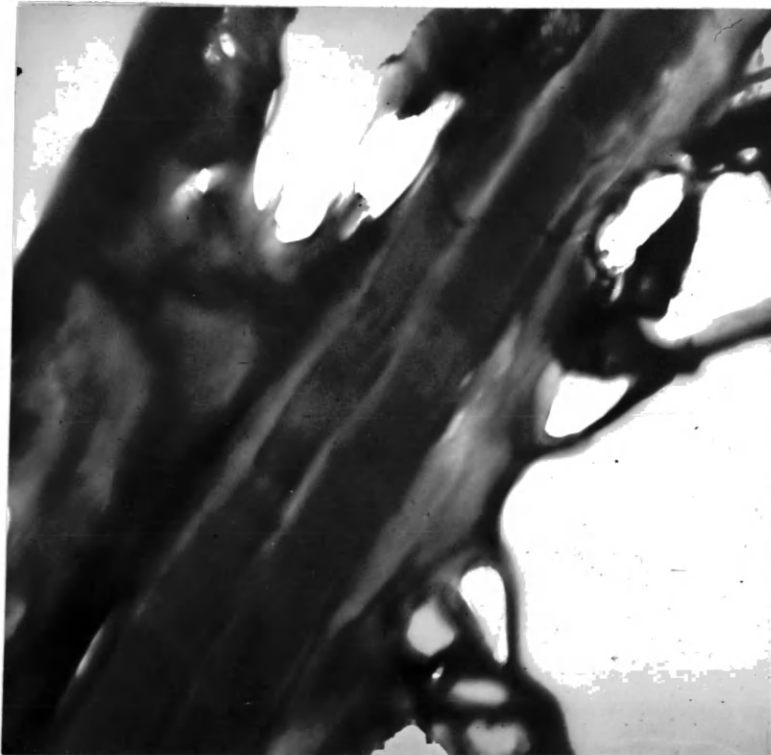
for a liquid with a coefficient of viscosity η , flowing with a uniform velocity at a rate of v cubic centimeters in t seconds, through a narrow tube of radius r centimeters and length l centimeters under a driving pressure of p dynes per square centimeters. If conservative estimates are made for some of the variables it becomes obvious that the flow is small. For example it would take almost a million tubes (0.25 microns radius) to move one cubic centimeter of a liquid with the viscosity of glycerine, 20 micron under a driving force of 30 atmospheres in one day. If the lower range of pressure, say 4 atmospheres is assumed, it would take more than five million plasmodesmata to perform the same transport. Since plasmodesmata are probably much smaller than the assigned value they would have to be present in unbelievable numbers to be effective. The most powerful factor in this relationship being the radius.

It is difficult to believe, in the light of observations described here, that plasmodesmata in the classical sense do exist in these walls. However, the mechanism which causes these cell walls to preferentially absorb stains is still obscure.

GYMNOSPERM ANATOMY BY ELECTRON MICROSCOPY

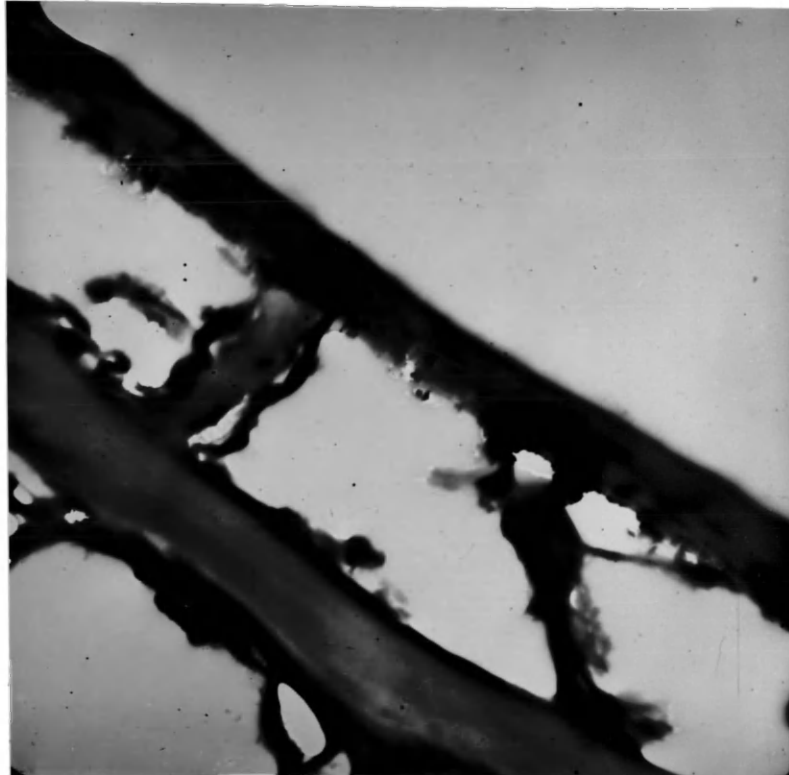
Introduction. -- The range of magnification of the electron microscope overlaps that of the light microscope. At the same magnifications the electron microscope will give superior resolution to many structures and therefore justify its use at magnifications in the light microscope range. It is thus possible to make the transition more easily from the familiar light microscope image to the greater enlargements possible with electrons. The same section cannot be examined advantageously in the two instruments since thin sections do not form adequate images in the light microscope. Likewise, at low magnifications the electron microscope image is considerably distorted in most commercial instruments. At higher magnifications resolution falls off sharply with most sectioned material. Experience indicates that rarely, if ever, is it desirable to examine sectioned material at magnifications greater than 5000 diameters.

An additional advantage of the ultramicrotomy techniques is their adaptability to difficult materials. Woody tissues, horny endosperms and silica-containing plants, like the horsetails, can be easily sectioned. This is counterbalanced somewhat by the inability to produce ribbons and the necessity for limiting the specimen to extremely small



10 μ

Figure 37. Electron micrograph of a section through the foliar epidermis of Picea. Navashin's fixation.



10 μ

Figure 38. Electron micrograph of a section through the foliar epidermis of Cedrus. Navashin's fixation.

dimensions.

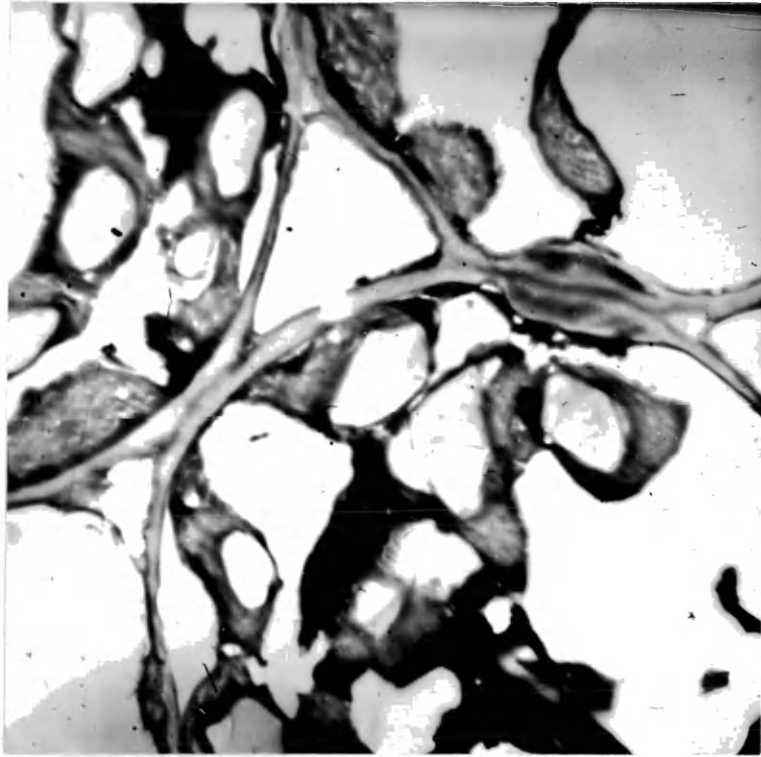
Wyckoff (56) recently showed the seriousness of knife artifacts in wood sections being observed at high magnifications. It will require further refinement of technique before the macromolecular structure of such botanical materials can be made visible. However, the grosser aspects of such tissues can be studied in greater detail by ultramicrotomy than by usual methods as indicated by the following study of some gymnosperm tissues.

Materials and methods. --The needles and young wood from Cedrus deodara (Roxb.)Loud., Pinus virginiana Mill., Picea glauca var. densata Bailey and Taxus baccata L. were sectioned by the methods previously described and examined in the electron microscope.

Fixation of all tissues was by Carnoy's fluid.

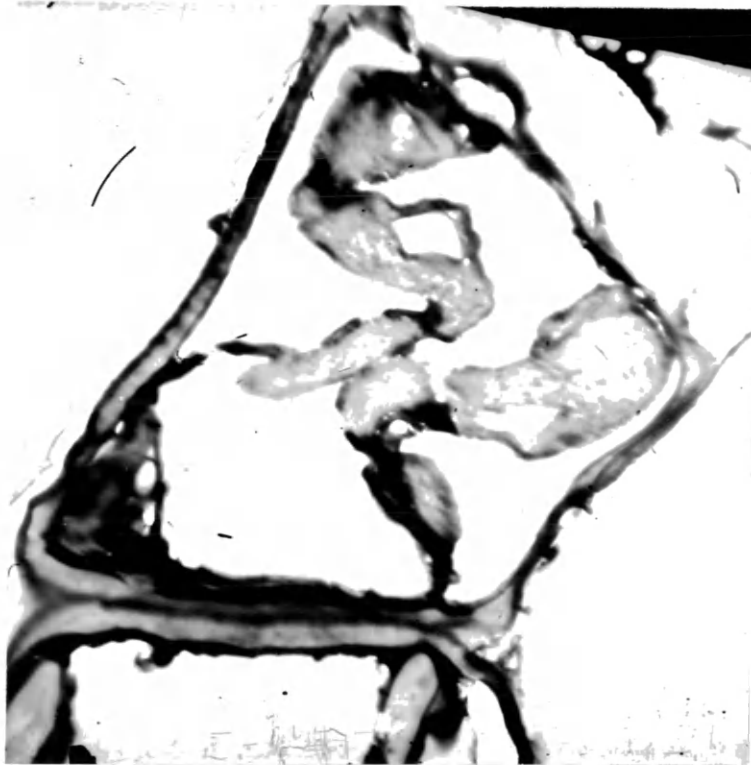
Observations and discussion. -- Carnoy's fixation was used because of its rapid and complete penetration which, in turn, appeared to aid the penetration of the methacrylic embedding mass.

Epidermal cells of both needles and young wood were often seen in sections. The walls of all of these cells are greatly thickened and only remains of the protoplast can be seen. In Picea the well developed hypodermis can be seen but in Cedrus the mesophyl appears to begin immediately beneath the single layer of epidermis. In figure 37 is a longitudinal section through the epidermal cells of P. glauca and in



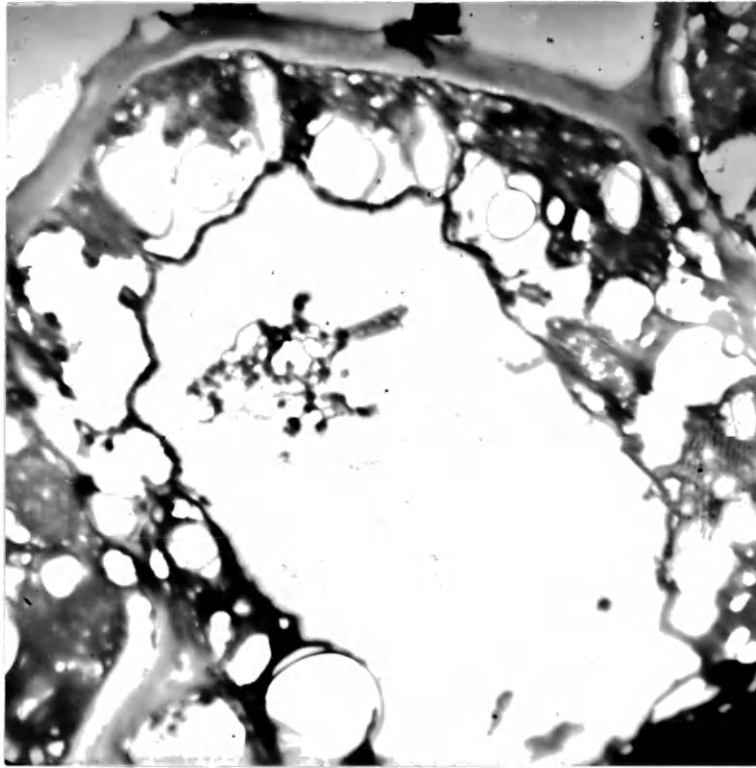
10 μ

Figure 39. Electron micrograph of a section through the stem mesophyll of Taxus. Navashin's fixation.



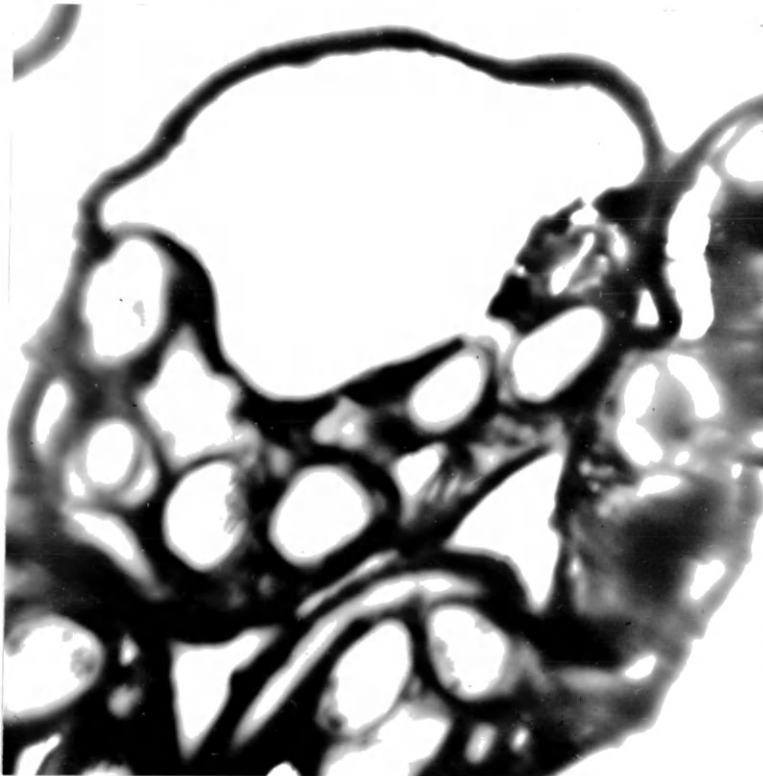
10 μ

Figure 40. Electron micrograph of a section through the foliar mesophyll of Cedrus. Navashin's fixation.



10 μ

Figure 41. Electron micrograph of a section through the foliar mesophyll of Cedrus. Navashin's fixation.



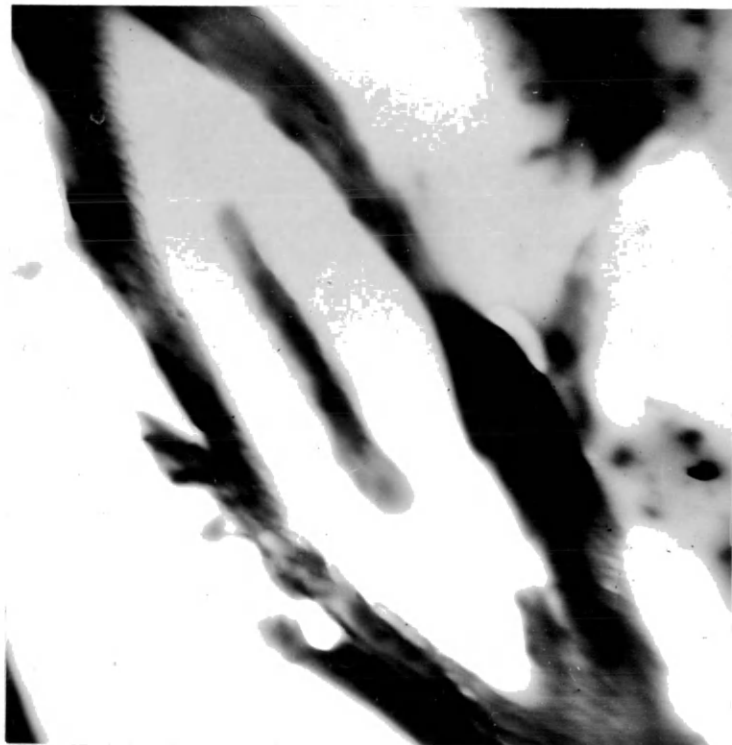
10 μ

Figure 42. Electron micrograph of a section through the foliar mesophyll of Cedrus. Navashin's fixation.



10 μ

Figure 43. Electron micrograph of a section through a foliar bordered pit of Pinus. Navashin's fixation.



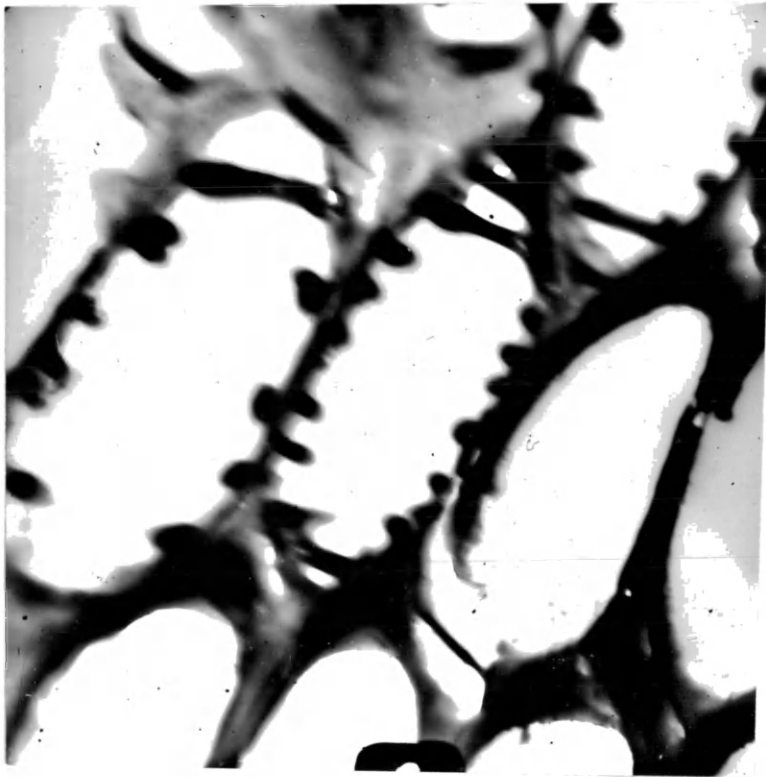
10 μ

Figure 44. Electron micrograph of a section through a bordered pit from the wood of Pinus. Navashin's fixation.



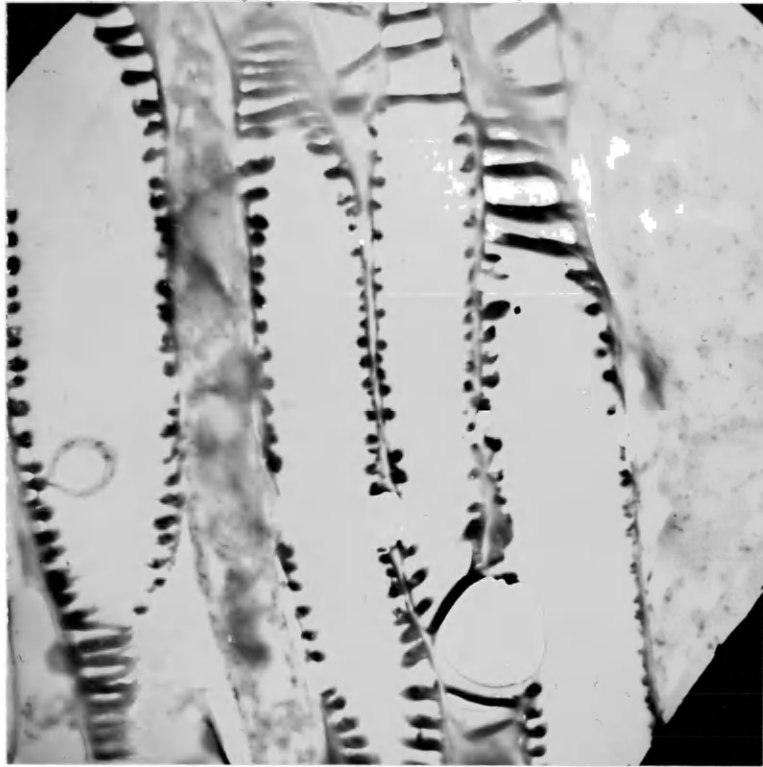
10 μ

Figure 45. Electron micrograph of a section through a bordered pit from the wood of Pinus. Carnoy's fixation.



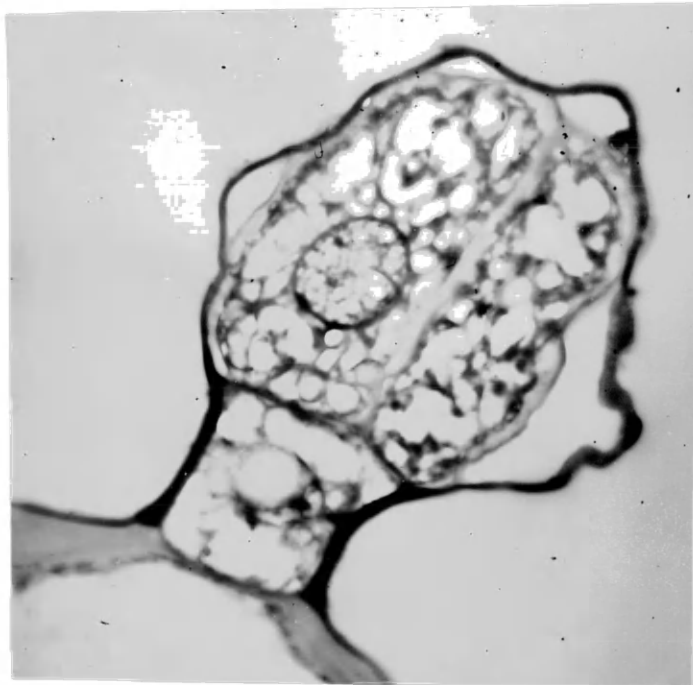
10 μ

Figure 46. Electron micrograph of a section through the vascular tissue of the stem of Taxus. Carnoy's fixation.



10 μ

Figure 47. Electron micrograph of a section through the young wood of Pinus. Navashin's fixation. Note the tylose forming on the left.



10 μ

Figure 48. Electron micrograph of a section through a foliar hair of Nepeta. Bouin's fixation.

figure 38 a section through the same cells in C. deodara.

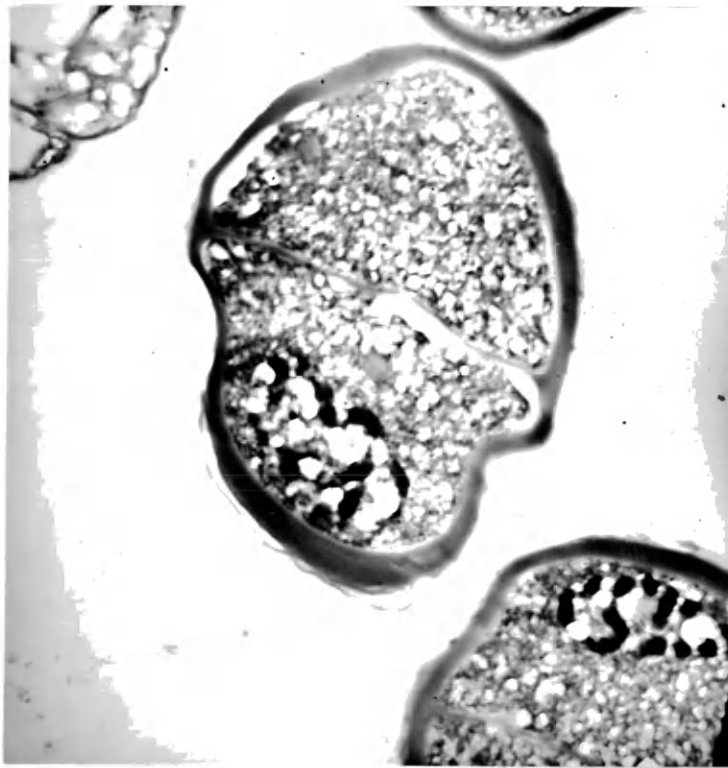
The leaf mesophyl in Taxus, Cedrus and Picea and the stem mesophyl in Taxus are characterized by great variability in cell shape. The protoplasm forms a layer around the periphery of the cell. A single or double layer of small starch grains are embedded in the cytoplasm. Plasmodesmata are not visible in the cell walls. Typical cells are shown in figures 39, 40, 41 and 42.

The endodermis could not be definitely differentiated in any of the sections examined.

In Pinus the parenchyma-like transfusion tissue of the needle bundle contained numerous bordered pits which distinguished it from the mesophyl. One of these leaf pits is shown in figure 43. The needle pits were smaller than those found in the young wood of the same plant. The pit membrane of the leaf pit appears to be lenticular while that of the wood pits is membranous. Frequent discontinuities are seen in the pit membranes of the wood (figures 44 and 45), evidence of a perforated condition. According to Jeffrey (52) the occurrence of foliar pits in the bundle transfusion tissue is much more marked in Pinus than in allied genera. They were not noted in the needles of Cedrus or Picea.

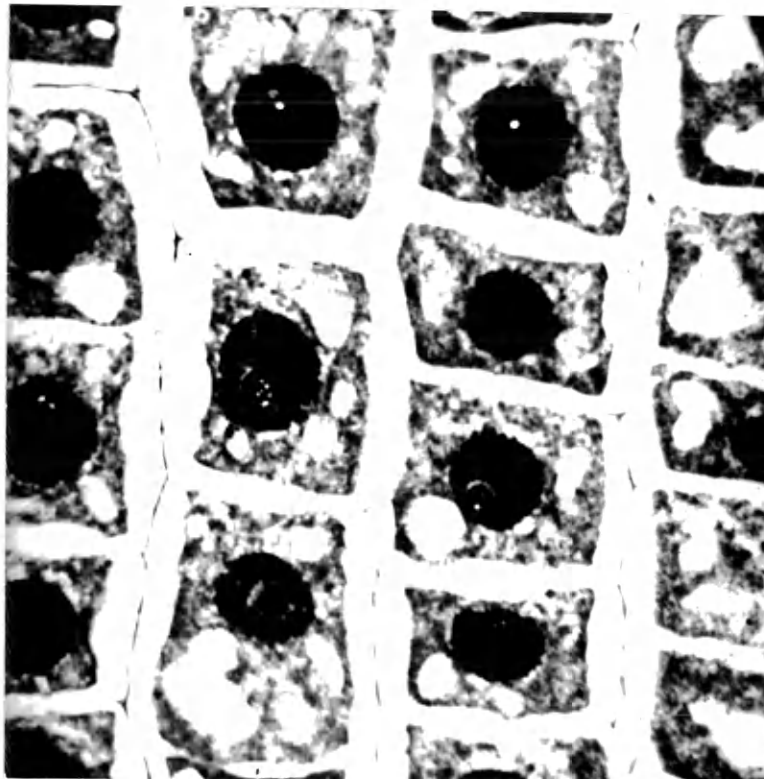
Primary xylem and adjacent metaxylem could be observed in some sections (figure 46). They were invariably devoid of protoplasts. Occasionally a tylose could be seen forming in the primary wood (figure 47).

It was not possible to make a comprehensive survey of a single section because of the small size of the viewing area. In addition a large part of the section would lie directly over the screen wires and could not be seen. The stiffer, more lignified cells did not tolerate the mechanical manipulations of film stripping and mounting in all cases and many broken cells could be seen.



10 μ

Figure 49. Electron micrograph of a section through developing microsporocytes of Lilium. Acetic-alcohol fixation.



10 μ

Figure 50. Electron micrograph of a section through the root tip of Allium. Fixation by hot water.

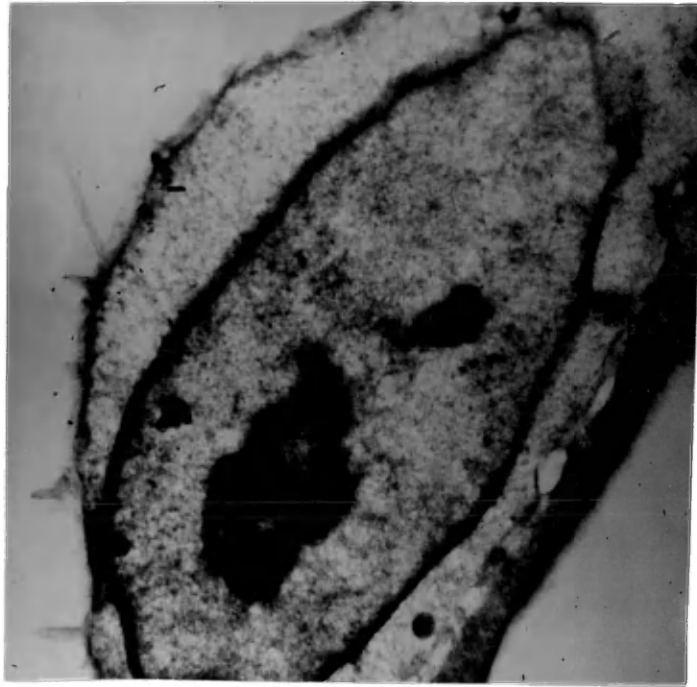
THE FIXATION IMAGE FOR ULTRAMICROTOMY

One of the major problems in ultramicrotomy is the preservation of the microstructure of the specimen. Considerable work is being carried out in different laboratories on the choice of preserving fluids for animal tissues. Though no reports have yet appeared which are devoted primarily to this problem, a general preference appears developing for fixation by osmic acid.

Fixation artifacts are a much more serious problem in electron than in light microscopy. The finer artifacts invisible in the light microscope become apparent in the electron microscope. In the thicker sections used in the light microscope these fine details are not resolved.

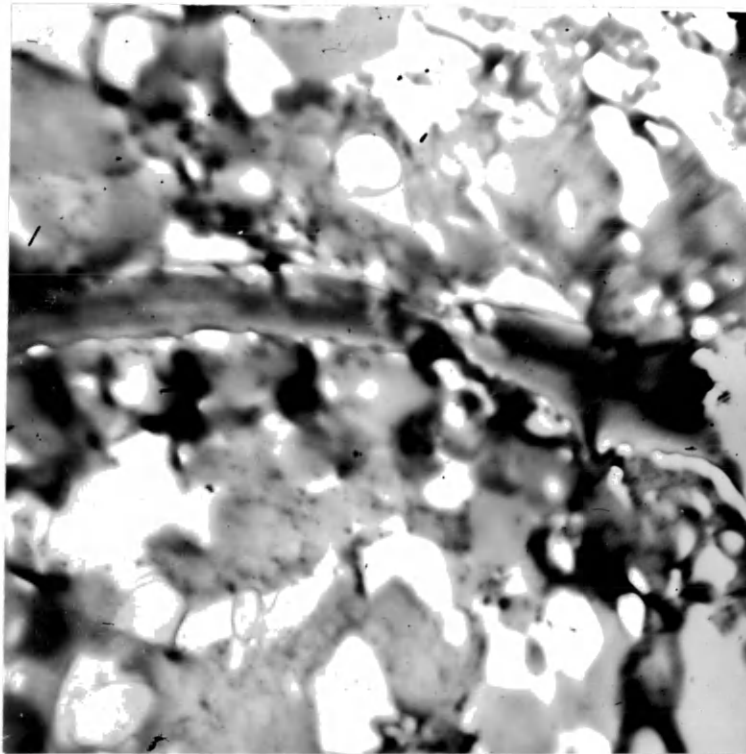
With most conventional fixatives the cell protoplasm is given a reticulated or coagulated structure. Bouin's fluid yields the rather coarse coagulum shown in figure 48. In addition to the coagulation some shrinkage occurs. Cell and nuclear membranes are distinguishable but disturbed.

Figure 49 shows Lilium superbum L. microsporocytes fixed in acetic-alcohol. This fixation gives a finer reticulum but some distortion results especially in the area of the chromosomes. It is obvious that such fixation is not suitable for studying the finer structure of the chromosomes



10 μ

Figure 51. Electron micrograph of a section through a callus culture of Rubus. Osmic fixation.



10 μ

Figure 52. Electron micrograph of a section through the leaf mesophyll of Taxus. Carnoy's fixation.

and spindle.

Immersion in hot water gives the fixation image in figure 50. The coagulum is less open than that obtained with Bouin's but this may be the result of compression from the large amount of shrinkage. The vacuoles appear well preserved.

The finest reticulum is achieved with 2 per cent osmic acid. There is little shrinkage and the membranes seem well preserved. The section in figure 51 shows osmic fixation. Though this fixative is excellent for many tissues the amount of osmium remaining in the section increases its opacity.

Carnoy's fluid yields a different type of fixation image. In figure 52 the amorphous cytoplasmic outlines are the result of treatment with Carnoy's.

The fixation image with the same fixative will vary slightly from tissue to tissue. However, in general they will resemble the examples given here. It is possible that none of the conventional fixing fluids will be the ultimate choice. At present osmic acid appears to give the most delicate fixation for plant as well as animal tissues.

SUMMARY

Because of the opacity of electrons to biological materials, sections for use in the electron microscope must be less than a micron in thickness. In recent years practical methods have been developed for producing such sections. These methods, however, are changing continuously and are being constantly improved.

During the course of the work described here some developments in instrumentation and technique were made in working with the methacrylate embedding method. A magnetostriction advancing mechanism was built and tested. This was discarded in favor of a piezoelectric device which gives better control of specimen advance than any instrument yet described. A simple micromanipulator tool was devised for applying electron microscope specimen screens to the sections. The problem of specimen damage through heat from the exothermic polymerization reaction was solved by partial polymerization of the methacrylate before introducing the tissue.

Determination of thickness was made on a number of sections by the weight-area-density relationship. Results indicated that the sections are somewhat thicker than presumed in published reports, but all were considerably under a micron.

Because knife sharpness is a critical factor in

ultramicrotomy, an investigation of knives of non-ferrous materials was made. Blades of beryllium copper, synthetic sapphire, phosphor bronze, tungsten carbide and glass were manufactured and tested. None of these had any advantage over steel knives in ease of sharpening, durability or quality of sections produced except the glass knife under certain conditions.

To test the applicability to botanical materials of the improved methacrylate technique several groups of plant structures were prepared and examined. Starch grains of Solanum tuberosum, Ipomoea batatas and Zea mays were examined for structural differentiation. With a chromium reagent a discrete center could be distinguished in the grains. The nature of this central area was investigated.

The endosperm of Diospyros virginiana, Quercus alba and Phoenix sp. were studied for evidences of plasmodesmata in the cell walls. The greater resolving power of the electron microscope did not succeed in making plasmodesmata visible.

Young wood and needles of various conifers were sectioned and the finer details of structure described. The most delicate fixation of cells was obtained with osmic acid.

These studies indicated that the methods of ultramicrotomy are useful in investigating the nature of those features which are at the limits of resolution of the light microscope.

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