

Chapter 13

MICROTOMY

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I. Introduction

It is generally said that the major steps in the knowledge of cell structure have resulted from progressive improvement of instruments for seeing cells, culminating in the electron microscope (Chap. 9, Vol. I, and Chap. 6, Vol. III). It is rarely appreciated, however, that the solution of many problems of cell and tissue structure have been almost equally dependent upon a series of improvements in methods of cutting slices thin enough to take full advantage of the increasing resolving power of microscopes. Thus, Leewen-

¹ This investigation was supported in part by Research Grant No. C1776(C3)M&G, National Cancer Institute, National Institutes of Health.

hoek, for his simple short-focus lens, was satisfied to shave by hand a section from a dried muscle. Early plant histology was worked out with the uncorrected compound microscope on material cut with a machine in which the twig or stem was mounted on a screw, which could be raised through a table, across which a razor was drawn by hand (the hand microtome). The full potentiality of the achromatic microscope, invented in 1809, only became realized after the middle of that century, when the hand microtome was improved by supporting the knife rigidly on a heavy block that could be pulled along polished ways to slice the tissue (the sliding microtome). Discoveries in cytology and embryology owe much to the convenience and precision of a later development, the rotary microtome, in which the turn of a flywheel automatically provides the cutting motion and advances the tissue to yield hundreds of successive slices that in thickness are nearly perfect replicates.

Instrumentation is but one phase of the developments that have led to modern microtomy. There has also been a parallel evolution of elaborate ancillary methods for preparing biological material for the cutting process. For the most part these latter do not involve physical techniques; but a minimum of knowledge of them is necessary if one is to understand all phases of the physical aspects of the process of preparing a specimen for microscopical study.

Higher plants are composed of tightly packed, boxlike cells, which are nearly plane geometrical figures bounded by solid walls of cellulose micellae. This woody structure is admirably suited for thin slicing, and from microscopic study of such sections plant anatomy was described on a cellular level long before it was suspected that animals had a similar structural basis. For, with rare exceptions such as cartilage, animal tissues are not solids suitable for cutting; instead they are essentially thick fluids or soft gels. Where a fibrous component (e.g., collagen or actomyosin) is present the tissue acquires a certain amount of tensile strength, but even if the fibrous components are in parallel orientation (tendon, muscle) or in a compact feltwork (dermis), the animal organ is naturally suited only to cutting thick, macroscopic sections. The butcher's slice of fresh meat is about the best that can be done. Obviously, therefore, sections thin enough for microscopic examination by transmitted light can be cut only if there is considerable alteration of the physical properties of animal material, like the change brought about by freezing or cooking meat. These familiar processes have exact analogues in techniques of freezing fresh tissue for microtomy and quick pathological diagnosis, and in the coagulation, or *fixation*, of tissues by heat or chemical treatment. The former converts an organ into a somewhat brittle solid, but a fixed organ is a firm mass that is not unlike plant material, and hence can be cut without much difficulty into slices well below a millimeter in thickness. The chief value of fixation in the early de-

velopment of animal histology, however, lay in the fact that the fixed organ was suitable for minute dissection or maceration, leading to dissociation of the structural units of the organ. Later it became the routine preliminary to embedding and cutting.

For most of the successful study of the cellular aspect of either vegetable or animal tissues it became necessary to cut much thinner sections, in the range of a few thousandths of a millimeter. Almost without exception, this accomplishment is impossible unless there is a further drastic change in physical properties by a process called *embedding*. Embedding media (Table I) are all substances which in solid form are suitable for this thin slicing. The tissue is saturated with a solvent of the medium, immersed in a liquid form of the medium, and after a suitable time for *infiltration* the medium is allowed to solidify about and within the specimen. In principle, infiltration must be considered to be a penetration of the fluid medium into all the spaces of the fixed tissue that are occupied by the solvent liquid; the latter under ideal conditions of fixation may correspond very closely with the aqueous phases of the living tissue. Commonly the latter is from one-half to nine-tenths of the tissue volume. It is not surprising, therefore, that the physical properties of the correctly infiltrated tissue are very similar to those of the embedding medium. It is obvious, nevertheless, that the solid, uninfiltrated regions, if there are any such, will resemble the medium least; but, since these in any case will be the naturally more solid regions and hence better suited for slicing, this lack of infiltration need not necessarily constitute an obstacle to cutting a uniform section. It is common experience, however, that with many embedding media the denser parts of the tissue appear to be significantly harder than the surrounding medium. For example, a paraffin section of testis will nearly always show at the surfaces some heads of spermatozoa that have been torn out of the main cleanly cut mass. Polymethacrylate, by contrast, is a medium in which such distortion is minimized; presumably this is due to the fact that this lucitelike plastic is closer to the hardness of the proteinaceous masses commonly encountered in fixed tissue, (but see Section II,2, a, below).

Somewhat to the surprise of orthodox microtomists, accustomed to considering $5\ \mu$ to be nearly the lower limit of thickness, it has proved possible in response to the demands of electron microscopy to extend this limit by approximately two orders of magnitude. The full range of application of microtomy today covers 5 orders of magnitude of thickness. Expressed in microns (10^{-3} mm., or $10,000\ \text{\AA}$) these are as follows: (1) 100 ± 50 (sections of brain to show complete neurones); (2) 10–40 (for histology, embryology, and histochemistry) and 2–10 (for cytology and cytochemistry); (3) 0.5–2 (phase microscopy); and (4) and (5) $100\text{--}1000\ \text{\AA}$ ($0.01\text{--}0.1\ \mu$, for electron microscopy).

To an engineer or physicist it is of course obvious that cutting biological

sections is an impressive accomplishment in applied physics. It may seem strange, therefore, that relatively little is known of the forces and properties of matter involved in this process. Microtomy is mainly an empirical achievement, closely bound throughout its history to medicine, and, like the latter, it shows *traces* of only lately having emerged from the barber shop. [For example, some of the cutting today is done with safety razor blades (see Section IV, 1, a).] This is necessarily so, for one looks in vain for a single example in industry or experimental physics where slices are to be cut with precision in such a range of thickness.

This lack of physical theory in microtomy was not of vital concern to biologists so long as the upper, or micron, ranges were involved. There is a huge storehouse of published lore of this art (e.g., Lee, 1937); and reference to this suffices to solve practically any problem which arises. In the Ångstrom range (below 0.1 μ), however, most of this traditional technique has proved to be quite inadequate, even though superficially the apparatus and methods for the different thicknesses seem to be very much alike. Actually, for electron microscopy the properties of older embedding media were unsatisfactory; the knives were far from sharp enough and had unsuitable facet angles; the advance mechanism had to be changed; and the whole microtome had to be redesigned to minimize many factors which, negligible for thicker sections, now became potential sources of major error. Nevertheless, within the short span of 10 years, these difficulties have been wrestled with so successfully that sections in the lower range are now being cut routinely in many laboratories (see Chap. 6 in this book). It is doubtful, however, if any worker is satisfied that there is not room for decided improvement in every aspect of section cutting for the electron microscope. The present authors have given considerable attention to this question of improvement, and some time ago concluded that advances were in many instances being retarded by lack of physical theory and of physical data on most aspects of the process. The present chapter is in part the result of this search for a more rational approach to microtomy; it also includes a certain amount of practical advice on the process.

II. The Specimen and Embedding

1. GENERAL CONSIDERATIONS

Biological microtome specimens are usually proteinaceous masses, which, more often than not, have been dehydrated. Such masses are spongy networks of variable density, low elasticity, and little plasticity (relative to substances which have been found to be more applicable to cutting) and therefore have the properties of *stiffness* and *variable hardness*. Spongy spaces are prominent in some regions and almost absent in others. Such a dehydrated tissue will deform permanently on the application of pressure.

The yield point (the pressure at which bonds rupture) will vary throughout the mass; some more dense regions will yield only after they have been pushed through the softer regions. Such specimens are obviously not suited to the cutting of sections for preservation of structure. Cutting is the rupturing of molecular bonds which lie in the path of the advancing knife edge. A wave of compression precedes the edge exerting tensile stresses transversely. As the tensile stresses increase with the advancing knife, the molecules first undergo an elongation along the lines of tension, then rupture when molecular chains break, or slip over each other (plastic flow) as they tear out from the body of the material when the yield point is exceeded.

It is the purpose of the embedding medium to fill the spaces of the tissue thereby giving support to the more tenuous cellular components when the knife exerts pressure on them. To this end, it is desirable for the embedding medium to have a density approaching that of the more dense regions of the specimen, and have bond strength equal to that of protein in order to present as uniform cutting resistance to the knife as possible and thus minimize the relative displacement of structural elements of the specimen. The embedding should be *elastic* enough so that the deformations (other than bond rupture) which the specimen suffers during cutting can be recovered by the positive reextension of the embedding. It should be *plastic* enough (have a relatively low viscosity) so that there will be a steep pressure *gradient* ahead of the knife edge, that is, so that the compressive wave and the associated stresses on the specimen will remain essentially local. If it were hard (dense with high bond strength), elastic, and had an extremely *high viscosity* (like glass at room temperature), the pressure gradient ahead of the knife edge would be small and the point in the specimen which would yield first would not necessarily be directly in line with or near the edge. In other words, the object would be *brittle* and would fracture along a surface which might or might not be the plane of travel of the knife edge. On the other hand, if the viscosity is too low, the embedding will flow so easily that the total cutting properties will be essentially those of the unembedded tissue.

We will now briefly discuss the different classes of embedding media which have been used in microtomy with reference to how well they fulfill the above requirements.

Paraffin and paraffinlike substances have been among the most successful embedding media. The "bond strength" of a paraffin is less than that of a protein. Paraffins are about $\frac{2}{3}$ as dense as dry protein. It can be readily observed that crystalline paraffin is slightly more elastic than a dry "amorphous" protein, such as alcohol-dehydrated muscle. The viscosity of paraffin is very dependent on molecular weight and temperature, and it is through control of viscosity that it is adapted to the role of an embedding

medium. In general, a high viscosity (relative to the viscosity of the simple paraffins) is desired and this is achieved by increasing the molecular weight or lowering the cutting temperature. The higher the molecular weight, the higher the melting point of the paraffin, but high temperatures are often deleterious to biological specimens. For this reason, in order to cut thin paraffin sections (less than 5μ), the microtomist must resort to a judicious combination of high-melting-point paraffin and low cutting temperature. Paraffin is a polycrystalline substance, and its viscous properties are distributed unequally between the inter- and intracrystalline bonds, the interior of the crystal being the more viscous (Jeffries, 1917). As a result, during cutting, the intercrystalline bonds tend to flow more readily, and crystals tend to slip past one another. This nonrecoverable deformation can be easily detected. The two surfaces of a cut approach, in smoothness, the regularity of the cutting edge itself. Thus the cut face of a paraffin block and the underside of a cut paraffin section will appear quite smooth and shiny. However, the upper surface of the section (which was smooth before the section was cut) appears dull and scatters considerably more light. This is due to the protruding ends of crystals which have slipped and become rearranged during the cut. This change in reflectivity is a very sensitive test, and in thicker sections usually only represents a few per cent of nonrecoverable deformation (as measured on a section floating on water a few degrees below the melting point of the paraffin). Paraffin sections of 3μ thickness and less are found to show rapidly increasing nonrecoverable deformation, even with the sharpest knives, and this can be attributed to the low density, viscosity, and elasticity of the paraffin (see Fig. 1). Carnauba wax, a denser, more viscous paraffinlike substance, which is about as inelastic as paraffin, does not show this behavior until the Ångstrom range (0.1μ) is reached. It is, however, almost useless for cutting biological material. When 5% Clarite X is added to paraffin (Wehrle, 1942), the crystal size of the latter is reduced (rapid quenching in cold water also reduces crystal size) and the intercrystalline viscosity is increased, lowering the cutting range by a factor of two. Reduction in crystal size results in a more uniform yield point and therefore less relative deformation within the section. Multiple mixtures of paraffins and other waxes have been advocated (Lee, 1937; Waterman, 1939) but have not been found by the authors to lower the cutting range of paraffin-embedded tissues appreciably. The addition of a trace of rubber (Johnston, 1903) does not seem to increase the elasticity of paraffin noticeably.

The paraffin embedding procedure removes fats and other lipid substances from tissues. When it becomes desirable to study the lipids in sections, nonpolar media must be avoided. Relatively recently, the use of Carbowax (polyethylene glycol) which has cutting properties similar to

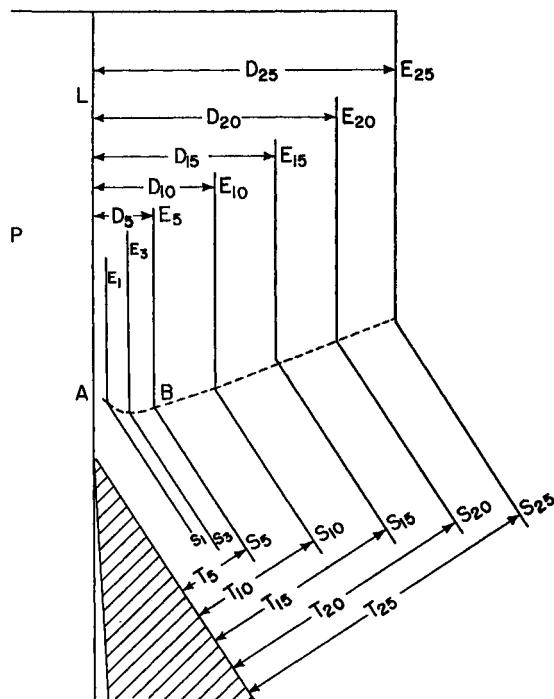


FIG. 1. Compression vs. section thickness. This is a replot of the data of Dempster (1943) showing compression in a physical picture. D_{10} , desired thickness of a $10\text{-}\mu$ section; E_{10} , edge of tissue block for a $10\text{-}\mu$ section; L , line of travel of the knife edge; P , paraffin block; S_{10} , outer surface of section; T_{10} , resulting section thickness. All section thicknesses contain both recoverable and nonrecoverable deformation. The increase in slope from B to A represents increasing nonrecoverable deformation.

paraffin has come into vogue for the embedding of tissues in which lipids are preserved (Firminger, 1950). The Carbowaxes are available in varying molecular weights, and their cutting properties vary with molecular weight and temperature as do those of the paraffins. In general, they are less elastic than paraffins, and somewhat denser and, therefore, harder. The ratio of inter- to intracrystalline viscosities is lower and therefore crystal slip is a bigger problem. Deformation is mainly nonrecoverable. The addition of 50% glyceryl monostearate (Clyman, 1955) increases the intercrystalline viscosity appreciably, and results in cutting properties closely approaching those of paraffin.

The cutting properties of ice, ice-glycerine "glasses," and frozen gelatin embedding (Lee, 1937) are quite similar to those of the different paraffins and Carbowaxes. Less elasticity and intercrystalline viscosity make the sectioning of these materials even more difficult; however, the specimen in

this case is usually a *hydrated* proteinaceous mass and is much more elastic in its own right than the embedding medium and so a reasonable recovery occurs on warming after cutting. Except when the minimum processing and speed of the frozen section technique is at a premium, the Carbowax embedding is to be preferred for lipid-containing materials.

We will now consider the amorphous embedding media. Next to paraffin, collodion (celloidin) has enjoyed the greatest popularity over the years. The greater density, slightly greater "bond strength," and total lack of the crystal slip problem together with a higher viscosity made it possible to cut large fairly thin sections where paraffin was very inadequate. (Although the elasticity of collodion is less than that of paraffin, the yielding of paraffin at the intercrystalline planes as the section is bent from the block, even with thick sections, results in more permanent deformation than in collodion.) As the 1 μ range is approached, the inelasticity of collodion becomes the determining factor in sectioning, and extreme, nonrecoverable deformation puts it at a disadvantage with respect to paraffin.

Before passing on, it should be noted that in the amorphous class of embedding media fall gelatin and lead-gum embedding (Lee, 1937). The cutting properties of these media limit the microtomist to the 10 to 15 μ range and have been mainly superseded by Carbowax and frozen section techniques.

Double embedding with collodion and paraffin (Kultschitzky, 1887; Pease and Baker, 1948) represents an attempt to combine the more desirable properties of both collodion and paraffin, taking advantage of the elasticity of the paraffin, and the higher yield point and viscosity of the collodion. As the percentage of collodion is increased, the size of the paraffin crystals is reduced due to the increased number of mycells of collodion per unit volume and therefore the reduction of the maximum paraffin crystal size. This combination permitted an improvement in sectioning of one order of magnitude, and provided the means for the first really widespread investigation of sectioned tissue in the electron microscope. The compression at even 0.1 μ , however, is very disturbing.

Newman, Borysko, and Swerdlow (1949a,b) introduced the use of *n*-butyl polymethacrylate as an embedding matrix for thin sectioning for the electron microscope. This substance, which is formed by the *in situ* polymerization of *n*-butyl methacrylate with the aid of a dissolved catalyst and moderate heating, is an amorphous glassy solid made up of long polymethacrylate molecules. Its viscosity and elasticity at room temperature, as well as its density and "bond strength," leave little to be desired in comparison with paraffin-collodion embedding. It supports all but the most dense protein structures well.

It is generally believed that, at this date, it is the knife which prevents

the micrometrist from *consistently* entering the 100 Ångstrom range, rather than the characteristics of the embedding matrix or the mechanical limitations of many of the various ultramicrotomes that will be described further on. There is no doubt, however, that the properties of *n*-butyl methacrylate, or of any other embedding medium, must necessarily become inadequate for sectioning below the 100 Ångstrom range where the total thickness approaches atomic dimensions. Cutting necessarily involves a permanently deformed layer at the surface of the section, and eventually a range is reached where the section must consist of nothing but deformed layer.

2. MEDIA FOR THE ÅNGSTROM RANGES

The physical properties of an embedding material that concern the electron microscopist are those that facilitate cutting the biological material to very small thicknesses, i.e., the range 0.1 to 0.01 μ . To select an embedding material the physics of image production should be understood (see also Section VI). The image of a tissue specimen in the electron microscope is a picture of the variations of density and thickness in the specimen. There is no material that is truly transparent to the electron beam; every substance whether tissue or embedding, is a real object and scatters electrons out of the beam according to its density and thickness. In order for the tissue structures to produce any contrast at all, if the embedding material is left in (Hillier and Gettner, 1950b), it is essential that the supporting material be of different density than the tissue; in practice embedding materials have been chosen to have the lowest possible density. In Table I the densities of various tissue substances and embedding materials are compared.

In an attempt to reduce the background density, the supporting material has at times been dissolved out. If the sectioned material is allowed to air dry, the microstructures of the tissue will collapse under the surface tension of the receding solvent (Hillier and Gettner, 1950b). The embedding material may be dissolved out and the solute substituted with liquid CO₂ under pressure, which is then heated until it passes through its critical point becoming a gas, at which time the pressure is gradually released (Anderson, 1951). Even the application of this critical point method, in which no phase boundary passes through the specimen, does not completely prevent the collapse of fine structures (Anderson, 1951). (See also Chap. 5 in this volume.)

The reduction of background density by a sublimation of the embedding material had been introduced by Claude and Fullam (1946) with their use of the eutectic, Camphor-naphthalene. After the section is cut, the Camphor-naphthalene is sublimed *in vacuo* (but leaving the tissue structures unsupported). Differential sublimation *in vacuo* of the embedding matrix of paraffin-collodion sections by the electron beam was proposed as one of

TABLE I
Densities of Embedding Materials and Various Biological Substances

Embedding material	Density	Tissue substance	Density
Camphor	1.0	Gelatin, anhydrous	1.27
Naphthalene	1.15	Silk	1.56
Paraffin	0.9	Amino acids, crystalline	1.1-1.8
Celluloid	1.4		
Spermaceti wax	0.90-0.95	Tallow	0.9-1.0
Beeswax	0.96	Oleic Acid	0.85
Stearic acid	0.85	Nucleic acids, anhydrous	1.5-1.8
Carnauba wax	1.0		
Carbowax	1.2	Starch	1.5
<i>n</i> -Butyl polymethacrylate	1.05	Cellulose	1.3-1.4
		Sucrose	1.59
		Bone	1.7-2.0
		Rock salt	2.3

the assets of this embedding combination (Hillier and Cannon, 1951). The partial sublimation of *n*-butyl polymethacrylate also occurs during exposure to the electron beam allowing a mass thickness reduction of as much as 50% (Williams and Kallman, 1955) and thereby greatly enhancing the contrast of the tissue structures which show little or no loss (Morgan, 1956).

In consideration of the above prerequisites, the electron microscopist, or molecular cytologist, has diverged from the practices of the histologist in choosing the embedding material; paraffin by itself cannot be cut much below the 1 μ range (see Section II, 1). It is not known if camphor-naphthalene can be cut in the Ångstrom range; the thinnest that Claude and Fullam (1946) reported was 0.3 μ , although one microtome was reported capable of advancing in unit steps of 0.1 μ (Claude, 1948). Polyethelene glycol (Carbowax) sections have been produced between 0.2 and 0.1 μ (Richards *et al.*, 1942). Carnauba wax has been cut to thicknesses of less than 0.05 μ (Fabergé, 1949). A mixture of beeswax and resin has been sectioned at 0.05 μ (Baker and Warren, 1952). Although paraffin-collodion double embedding, (Pease and Baker, 1948), enjoyed several years of popularity, it has rarely been cut successfully below 0.10 μ and not at all below 0.05 μ due to the large permanent compression which becomes manifest at the lower thickness. Polymethacrylate, introduced by Newman *et al.* (1949a,b) has been sectioned as low as 0.007 μ (70 Å) (Sjöstrand, 1953a).

a. n-Butyl Polymethacrylate

The physical properties of the polymer of *n*-butyl methacrylate are greatly different from those of paraffin. It is a clear thermo-setting plastic with a brittle point (the temperature below which the material will shatter

rather than shear in a standardized procedure) near 16°C and a softening point near 33°C (Neher, 1949). It is a linear molecule adding end to end in polymerization, which gives it an elasticity of a few per cent.

Elasticity (deformation which disappears when the deforming force is removed) is an important characteristic of the polymethacrylate. When a section is cut, it folds and compresses slightly, but on being floated on the trough liquid (see Section III, 2, e), having no facet sliding frictional forces to overcome, it re-extends itself to its original dimensions, especially if the trough liquid contains a reagent in which the embedding is partially soluble, such as acetone. This re-extension of the section can occur regardless of the cutting angle of the knife. Even with a glass knife with a 75° facet angle and 80° to 85° cutting angle it is possible to cut sections of polymethacrylate without excessive permanent deformation. While the advantages of elasticity are apparent after the polymethacrylate section is cut, the quick recovery of this polymer after compression can disadvantageously affect its sectioning (see Sections III, 2, a, and f, for descriptions of some of these difficulties).

Upon first contact, the polymethacrylate in the neighborhood of the knife resists being cut and compresses slightly. When the transverse tensile stress has reached the yield point of the polymer, molecular chains are pulled apart and bonds are broken. The elongation of the molecular chains before reaching the yield point is a function of the temperature at which the polymethacrylate is cut in relation to the second order transition point, or softening point, characteristic of the polymer. For *n*-butyl polymethacrylate the second order transition point is 33°C with a possible spread of 5 to 10°.

The second order transition point is a temperature at which many properties of the polymer change due to the gain (as temperature is increased) or loss (as temperature is decreased) of the secondary degrees of freedom of the molecules, such as rotations and vibrations (van Amerongen, 1950). Such physical properties as specific volume, specific heat, compressibility, thermal conductivity, tensile strength, etc., exhibit radical and discontinuous changes. The maximum elongation before rupture occurs is a maximum and decreases as the temperature is either raised or lowered from this point. Viscous flow is exhibited above this point as some of the secondary bonds are unfrozen. The second order transition point is lowered and spread out by the addition of plasticizers and is raised by cross-linkages between the molecular chains. It is raised by compression and lowered by tension.

As the temperature of the polymethacrylate is lowered below the second order transition point, tensile strength increases but maximum elongation before rupture decreases and a "brittle point" is reached; for *n*-butyl polymethacrylate this point is 16°C. The "brittle point" is *that tempera-*

ture at which the rate of deformation of the polymer equals the rate of application of an impacting force, below which it fractures rather than deform in any other manner (see Section II,1). Since the value of "brittleness" so determined is dependent on the rate and method of application of the force and the dimensions of the piece under test (commercial values are obtained from the application of successive hammer blows), it has little bearing on the cutting characteristics except if it is determined separately for the cutting speed of the ultra microtome and the thickness of the sections cut. We have found that hardness measurements on polymethacrylate blocks at various temperatures through this region show no indication of the "commercial brittle point." Good sectioning will be possible, however, only between the "sectioning brittle point" and the softening point.

The range of hardness of polymethacrylate in which good sectioning can be performed is very limited. If the polymer is "soft" (easily indented with thumbnail pressure), it will be difficult to cut with anything but the sharpest of presently available knives. A "soft" block implies a nearness to the second order transition point or a greatly spread out point extending into room temperature. Such a block exhibits unusual compressibility (its deformation-before-rupture is extreme) and during sectioning will readily be compressed behind the knife resulting in no section (skipping or missing). Sections will be cut only after 2, 3, or more passes of the knife, the sections being proportionally thicker than desired. With a sharper knife, no sections are skipped, but thickness may change one to several times during the cutting of a single section. In a soft polymethacrylate block the structural components of the biological tissue may have greater tensile strength and yield point than the embedding, and may therefore be torn out of either the section or the block during cutting (Williams and Kallman, 1955). Such tearing (scratches or knife marks) has often been observed in the embedding matrix of the section when the knife edge is knicked or chipped, or when a hard particle is picked up by the knife and carried through the section (Hillier and Gettner, 1950b). A glance at Table II shows that OsO_4 fixed and dehydrated tissue is 10 to 50 times "harder" than *n*-butyl polymethacrylate. The sectioning problem is resolved, therefore, into cutting a hard fibrillar network with spaces filled in by a much softer embedding medium.

On the other hand, if the polymethacrylate block is too "hard" (not impressionable by thumbnail pressure), other phenomena arise which are equally discouraging. Striations (due to "chatter") will appear, as either the polymethacrylate block or the knife edge or even the whole advancing structural member is set in vibration (Porter and Blum, 1953). The knife edge, which is not infinitely hard and tough, will be dulled and chipped by

TABLE II
Representative Hardness Values of Some Materials

Material	Knoop hardness value	Other hardness value
Diamond	5500-6950 ^a	
Synthetic white sapphire	1850-2000 ^b	
Corundum (air contaminant)	1620-1680 ^a	
Commercial razor blade	840 ^b	Rockwell C 65 ^b
Jung microtome knife	840 ^b	
Spencer microtome knife	740 ^b	Rockwell C 61 ^b
C-Cr-Steel microtome knife	829	Vicker 800 ^c
Beryllium bronze microtome knife	419	Brinell 380 ^d
Plate glass	484 ^e	
Pyrex glass	457 ^e	
Crystal quartz	710-790 ^a	
Fused quartz	846 ^e	
Osmium, metal	360	Vicker 350 ^f
Aluminum 99% (2S-O), annealed	30 ^e	Brinell 23 ^f
Bryzoa, Devonian fossil	60 ^e	
NaCl, crystalline	28 ^e	
Sucrose, crystalline	60 ^e	
Sucrose, caramelized	2 ^e	
Carnauba wax	8 ^e	
Carbowax 6000	7 ^e	
Paraffin 62°C m.p.	1.0 ^e	
Methyl polymethacrylate	22 ^e	
n-Butyl polymethacrylate	0.2-3.0 ^e	
Gelatin, anhydrous	25 ^e	
Gelatin, anhydrous OsO ₄ fixed	31 ^e	
Dried split pea, seed coat	18 ^e	
Dried split pea, seed coat OsO ₄ fixed	22 ^e	
Liver, Carnoy fixed, in paraffin 62	4.4 ^e	
Paraffin 62°C m.p. of same block	1.0 ^e	
Ovary, OsO ₄ fixed, in n-butyl polymethacrylate	6 ^e	
Polymethacrylate of same block	0.3 ^e	

Knoop hardness values for various knife materials, lapping surfaces, tissue and embedding substances. The Knoop hardness scale (Robertson and van Meter, 1951) was chosen because it can include all solid materials on one single scale.

^a From Sharp and Badger (1953).

^b By Mr. D. F. Sklar, Torsion Balance Co., Clifton, New Jersey.

^c From Ekholm *et al.* (1955).

^d From Fabergé (1949).

^e These values are averages of 10 measurements made by the authors through the courtesy of Mr. E. H. Enberg, Torsion Balance Co.

^f From Everhart *et al.* (1943).

"hard" blocks. The sharper the knife the more delicate the edge. We have found that after as few as 25 sections 0.02 μ thick have been cut with a Spencer steel knife, the edge has become sufficiently damaged for skipping to begin.

It has been noticed among several electron microscopists that the hardness of *n*-butyl polymethacrylate varies with the lot number of the manufacturer, and from different manufacturers. Using a standard embedding procedure, quantities from several lot numbers can be polymerized and those lots not producing a final polymethacrylate capsule with the most suitable cutting characteristics may then be discarded. A more uniform and reproducible hardness is achieved if air is kept from contacting the monomer during polymerization. Even the air pocket encapsulated by the top half of a gelatin capsule may be enough to inhibit complete polymerization. One simple means to insure total exclusion of air is to close a capsule by the insertion of the bottom of another capsule sealing the two by first wetting the latter with water and making sure that some monomer overflows. Another distressing aspect of polymethacrylate is its tendency to increase in hardness with age, as though the polymerization process were never quite complete. We have found that blocks that were too soft for good sectioning at one time had attained the proper hardness a half year later. Also that blocks with good cutting characteristics became too hard to section at a later date. Many ultra-microtomists prefer to take steps designed to alter the cutting characteristics of a given batch. The addition of 5 to 25% methyl methacrylate (a shorter length molecule producing a polymer with higher second order transition and "commercial brittle points") to *n*-butyl methacrylate for hardening the tissue block is in common practice. Details of embedding procedures may be found in Newman *et al.* (1949a,b) and Porter and Blum (1953).

b. Artifacts of Polymerization

Although the polymethacrylates approach the ideal embedding medium, they all suffer from a very undesirable feature of the actual polymerization process; among electron microscopists this is called polymerization damage or "explosion." It may be related to what has been called in polymer chemistry "popcorn polymerization." Explosion may be described as a swelling of the methacrylate embedding plastic during its polymerization process. The magnitude of explosion may be from a slight enlargement (less than 50% linear increase) to a gross rupture (many times enlargement) of a small region. In extent it may be confined to a small area or may generally pervade the tissue. Figure 2 shows some typical cases of most explosion patterns normally encountered. It has often proved difficult to differentiate this artifact from the distortions which are usually attributed

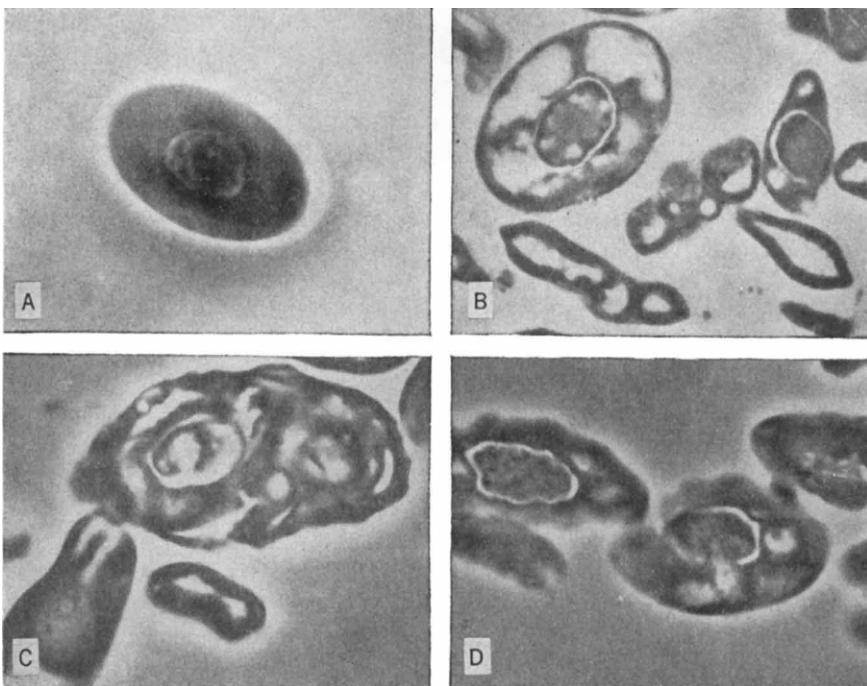


FIG. 2. Artifacts of polymerization (explosion) shown in amphibian erythrocytes (*Rana pipiens*) which have been fixed in OsO₄ and embedded in *n*-butyl methacrylate. A, Erythrocyte in *n*-butyl methacrylate before polymerization. Magnification: $\times 1260$. B, C, and D, Erythrocytes in *n*-butyl methacrylate after polymerization (1.0- μ sections). Magnification: $\times 1260$. B, Gross explosion occurring in interior of cytoplasm. C, One erythrocyte showing large over-all swelling with local areas of gross explosion, and an adjacent cell showing very slight general swelling. D, Gross explosion simultaneously involving both nucleus and cytoplasm.

to poor fixation. The manner or exact process by which explosion takes place is not known. The authors have seen gross swelling take place in an *n*-butyl polymethacrylate block when immersed in its monomer, a property also common to methyl polymethacrylate (Rohm and Haas Co., 1953). At another time a block of previously polymerized *n*-butyl methacrylate with "unexploded" tissue was placed in monomer and repolymerized, with the result that extreme explosion ensued. *n*-Butyl polymethacrylate will swell 40% and more (one-dimensional measurement) when placed in a 50% acetone-water solution, and will return to its original size when dried. It has been noticed that the lower molecular weight methacrylates (methyl methacrylate) are inclined to explosion patterns of greater magnitude than those of high molecular weight (lauryl methacrylate). Osmium-fixed tissue itself catalyzes the polymerization of methacrylate. A capsule containing

osmic-acid-fixed tissue immersed in *n*-butyl methacrylate with inhibitor removed, will polymerize at room temperature (25°C) much sooner than methacrylate without tissue.

The above evidence suggests that explosion is the result of a spotty or uneven polymerization process during which regions already polymerized swell upon the invasion of monomer or less polymerized methacrylate from adjacent regions (see also Borysko, 1955a). Such points of advanced polymerization may be the osmic-acid-fixed tissue, e.g., the nuclear membrane, as well as other membranous structures, commonly exhibits a wide separation (gross rupture) from the surrounding cytoplasm (see Fig. 2C). Working with this hypothesis, the authors have loaded the methacrylate with catalyst, adding as much as 10%, to establish many more centers for polymerization to begin, but no marked improvement has been observed. Other factors which have been observed to have no effect on explosion, either in increasing or decreasing it are: The presence of alcohol up to several per cent; the presence of a trace amount of water; the presence or absence of the inhibitor (hydroquinone); the presence or absence of the plasticizer (dibutyl phthalate) which is normally a constituent of the catalyst (Luperco CDB); the temperature of polymerization from 0 to 100°C; the length of time of polymerization; polymerizing under vacuum; polymerizing with ultraviolet light and no catalyst.

It has become evident that some tissues exhibit explosion more than others. In particular the amphibian erythrocyte shows explosion more consistently and to a greater extent than other tissues. Explosion is in fact so marked that a 1 μ section may be studied under oil immersion in the phase microscope and the extent of explosion in cytoplasm and nucleus observed and compared with fixed erythrocytes placed in the monomer. Using these cells as test objects (see Fig. 2), the authors have performed a series of chemical tests to discover if one specific chemical group is more responsible for explosion than others. These involved blocking or removing certain reactive groups of the tissue protein, such as: benzoylation in pyridine; acetylation with acetic anhydride; acylation with 2,4-dinitrofluorobenzene; methylation in methanol-HCl; iodination in acid and base; extraction with hot trichloroacetic acid and with normal HCl at 60°C. Lipid extractions were also performed with hot acetone, hot acetone-ether, chloroform, and carbon tetrachloride. None of the above chemical treatments eliminated explosion.

It has recently been claimed that the following procedure reduces explosion (Borysko, 1955a). The tissue after being dehydrated in alcohol or acetone and passed through several changes of *n*-butyl methacrylate monomer, is put into a very viscous pre-polymerized methacrylate with catalyst and is stored at 0°C for 24 hr. for good infiltration. It is then com-

pletely polymerized in an oven at 60 to 80°C. This final stage of polymerization is rapid and may take as little as 2 hr. Pre-polymerized methacrylate is prepared by processing *n*-butyl methacrylate in the usual way; catalyst is added to the inhibitor-free monomer and the mixture stirred frequently at 80°C until a viscosity similar to *very thick molasses* is reached. This may be stored at 0°C for several days (Borysko, 1955b). The authors have found this method to show remarkable improvement with respect to polymerization damage in some materials; however, it does not eliminate it completely or consistently.

III. Microtomes

1. STANDARD MICROTOMES FOR THE MICRON RANGES

The most widely studied biological tissues are sectioned at the middle range thicknesses (1 to 40 μ) on the rotary microtome (motion is transferred to the mechanism from a hand-operated flywheel. See Fig. 3). The specimen block (usually paraffin-embedded tissue) is carried in a vertically reciprocating motion and simultaneously advanced toward a rigidly mounted knife. The advance is obtained by the engagement of a ratchet and pawl

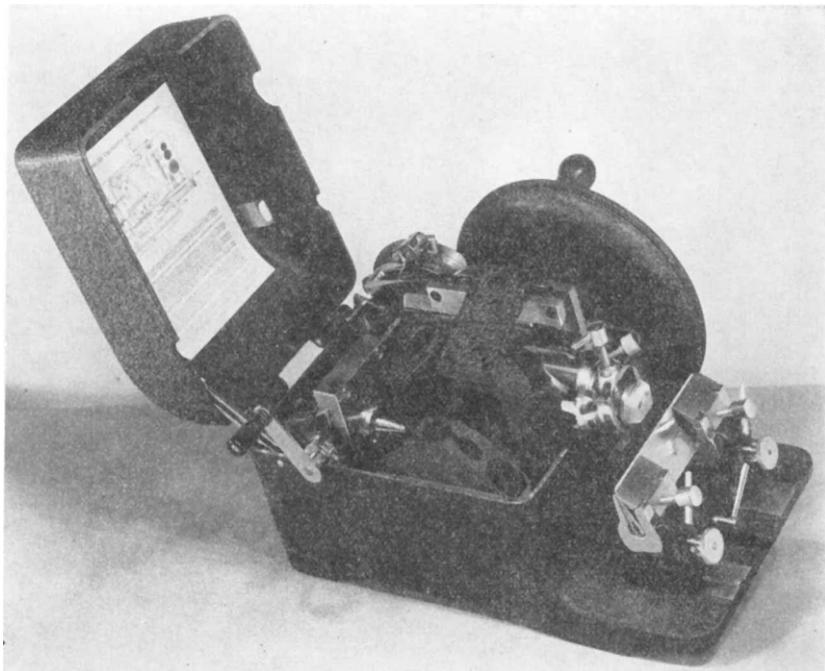


FIG. 3. American Optical Spencer rotary microtome 820. (Courtesy of American Optical Co., Instrument Division, Buffalo, New York.)

which turns a precision screw directly connected with the specimen vise thereby pushing (advancing) the specimen toward the knife at a time when the tissue block is above the knife. Greater dependability is obtained with the Spencer model 820 microtome in which a wedge or inclined plane attached to the specimen vise arm is acted upon by a screw-drawn travelling cone. The angle of inclination of the plane is such that the distance of specimen advance is one-half the distance along which the cone travels per section, thereby also reducing the error of the screw by one-half. Greater details of this type of microtome and its use can be found in Lee (1937) and Richards (1949).

At the upper end of the thickness span, 50 to 150 μ , serial sections of brain, whole embryos, and woody botanical specimens, often embedded in collodion, are cut on a sliding microtome (see Fig. 4). In this type of instrument the specimen is advanced upward by the same type of advance mechanism as used in the rotary microtomes. The tissue block is cut by a horizontal slanting movement of a hand-drawn knife which is mounted in a carriage running in ways, or which slides on glass-bearing plates. The freezing microtome, in which unembedded frozen tissues are cut, is similar in design and operation to the sliding microtome.

With rotary microtomes the direction of motion of the specimen relative to the knife edge is perpendicular and therefore the rate of motion of the surface of the section across the facet surface is equal to the rate of advance of the specimen into the knife (a chopping action). When specimen mate-

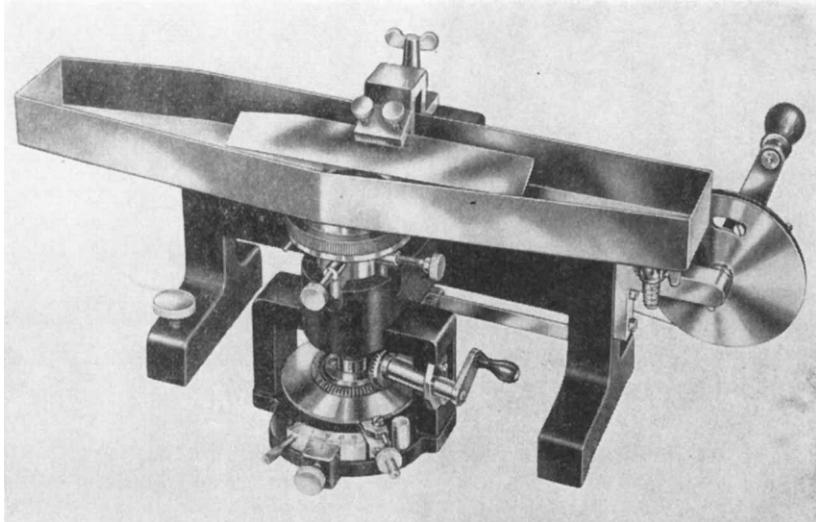


FIG. 4. Jung universal sliding microtome model ML IV. (Courtesy of William J. Hacker and Co., New York.)

rials, which have a relatively high viscosity and are not self lubricating, i.e., collodion (in contrast to paraffin which has a lower viscosity and self lubricating properties), are cut by a chopping action, static friction becomes excessive and specimen sections are produced with a relatively large proportion of permanent deformation. When these materials are cut by a slicing action (the slanting knife edge of the sliding microtome), in which the section slides over the facet surface with a velocity considerably greater than the rate of cutting, *static friction is largely replaced by a much decreased sliding friction*, and the thickness of the permanently deformed layer is greatly reduced. In the case of collodion, the thickness of an alcohol film between section and facet increases with higher sliding velocities, further decreasing sliding friction.²

2. ULTRAMICROTOMES FOR THE ÅNGSTROM RANGES

In the relatively new ultra-thin region (0.1 down to 0.01 μ), tissues are sectioned for cytological study in the electron microscope. The ultramicrotomes and techniques being used are still in the development stage; some of the mechanical principles involved are not essentially different from those employed in instruments which cut in the higher ranges, but considerably more attention has been focused on the limits of accuracy and the elimination of errors which are inherent in mechanical systems in the attempt to obtain reproducibility at molecular dimensions.

a. Early Ultramicrotomes

Probably the earliest attempt to prepare biological tissues in sectioned form for the electron microscope was made by von Ardenne (1939). A tilting specimen holder was made as an attachment to a standard Minot rotary microtome, by which wedge-shaped sections were cut (the trimmed face of the tissue block was covered with a supporting film of paraffin) with the idea that somewhere in the wedge shape the sections would have an ideal thickness for the electron microscope. Wedge-shaped sections were also cut on a Minot microtome modified to advance in unit steps of 0.25 μ (Richards *et al.*, 1942). The embedding (Carbowax 4000) was dissolved out with water and the section was then dried by a freeze-drying procedure. Sectioning by hand (Richards and Anderson, 1942) has been resorted to in ultramicrotomy as it was more than 100 years earlier for histological sectioning. The tissue, hard and soft insect cuticle, was inserted into a slit in a carrot and shaved or worn away with glancing strokes of a razor blade.

² The function of the slant of the knife has often been attributed to some ill-defined advantage of the more acute cutting angle that the specimen "sees" when cut by a slanting knife (Lee, 1937; Richards, 1949). In reality, the specimen is bent through the same real angle, (facet plus clearance angles) whether cut with a slanting or square-set knife.

The next attempts at thin sectioning were made by O'Brien and McKinley (1943) and Fullam and Gessler (1946) with high-speed microtomes. The theory was advanced that with higher cutting speeds (3.3×10^6 cm/sec) the distribution of strains within the tissue block preceding the knife becomes more localized, and that this thin stress plane readily overcomes the forces resisting separation of the section. Claude, after working with the high-speed microtome (Claude and Fullam, 1946) designed an all-rotary, slow-speed, double-screw ultramicrotome (Claude, 1948) in which the advance mechanism consisted of two concentric screws of slightly different pitch, the screw of greater pitch being the nut for the screw of lesser pitch, both screws being mutually coupled by spur gears to revolve in opposite directions. A razor blade was used as a knife and a trough was built into the razor blade holder. The tissue block was mounted eccentrically on the vise rotor and therefore this was the first of the slow-speed ultramicrotomes using a "single-pass" principle (in which the tissue block passes the cutting site only on the cutting stroke) as well as a trough and continuous motion in all moving parts. The sections were fully extended on the surface of the trough liquid and were collected by immersing a specimen-supporting grid in the liquid and picking them up.

Thin sectioning came into favor rapidly after Pease and Baker (1948) adapted the popular Spencer model 820 rotary microtome to advance at a much reduced rate. This "solution," a reduction of the angle between the inclined plane and the feed screw axis by the attachment of a wedge, contained the essence of simplicity and made use of an instrument already popular to every biologist. These workers also employed the familiar paraffin-collodion double embedding, dissolved out the embedding material, and thus demonstrated for the first time in thin section the presence of some of the membranous structures of the cell.

Hillier and Gettner (1950a,b,c) next showed that by reducing static and sliding friction and, by employing a constant-speed motor to turn the Pease and Baker modified Spencer microtome, greater reproducibility in section thickness was obtained. At the same time Gettner and Hillier (1950; also Hillier and Gettner, 1950c) rediscovered the trough, which soon became a standard addition to all ultramicrotomes. [Cocks and Schwartz (1952) showed that a cantilever spring system would provide frictionless reciprocating motion. Similarly Kuroha *et al.* (1953) showed that the elastic deformation of a pair of steel spring plates can give a mechanical advance free from static or sliding friction.]

At about this same time Newman *et al.* (1949a,b) introduced the thermal advance principle for obtaining the small incremental movements needed for thin sectioning. A brass chamber, used as a specimen holder, was mounted in the Spencer rotary microtome and was chilled by the expansion

of CO₂ in the chamber which caused an initial contraction of the metal. As the temperature and therefore the length of the brass chamber increased and approached room temperature, the microtome was operated at zero mechanical advance (ratchet advance not engaged), cutting sections whose thickness was determined by the rate of thermal expansion and the time lapse between consecutive sections. These workers also introduced the plastic *n*-butyl polymethacrylate (see Section II,2,a, as an embedding material. The elastic properties which gave it an initial success, however, threatened its use by the resulting problem raised by the tendency for the return stroke of the specimen to pick up the section. This "pick-up" phenomenon is not peculiar to *n*-butyl polymethacrylate. It often occurs with paraffin in the higher ranges, and it is especially noticeable with doubly embedded tissue blocks when the trailing end of a section is torn from the block with the resulting fragments overhanging the knife edge. These are "picked up" by the returning tissue block of a double-pass reciprocating instrument such as the rotary microtome. The methacrylate polymer, in addition to being more difficult to cut, shows slight elastic deformation during cutting which causes the tissue block to rub against the knife on the return stroke of a "double-pass" microtome increasing the probability of "pick-up."

These early attempts to obtain thin sections soon revealed a new molecular cytology of tremendous scope. Nuclear membranes came into evidence as definite delineated structures whether real or artifact. Mitochondrial membranes, evidences of ergastoplasmic fine structure, and internal fibrillar structure of cilia, are only a few that excited the imagination and spurred the curiosity to discover more structures and their possible relation to cellular function. (See Chap. 6 in this volume.)

b. Single-Pass Ultramicrotomes

When the "pick-up" problem was appreciated, many workers applied their efforts to the design of "single-pass" ultramicrotomes; mechanisms in which the tissue block passes the site of sectioning only during the cutting stroke, returning via another path. These instruments were of two kinds, those obtaining specimen advance by employing a principle of mechanical advantage, and those using the thermal expansion of a metal.

Gettner and Ornstein (1951) introduced the second single-pass, slow-speed ultramicrotome in the first successful attempt (Ornstein *et al.*, 1951) to eliminate the pick-up phenomenon which is especially severe with polymethacrylate. The reciprocating mechanism of a Spencer 820 microtome was replaced by a rotating inclined plane (see Fig. 5). Motion was transferred from the constant-speed, motor-driven flywheel through a pair of spiral gears to a horizontal shaft. A rotor with an embedded glass plate, the

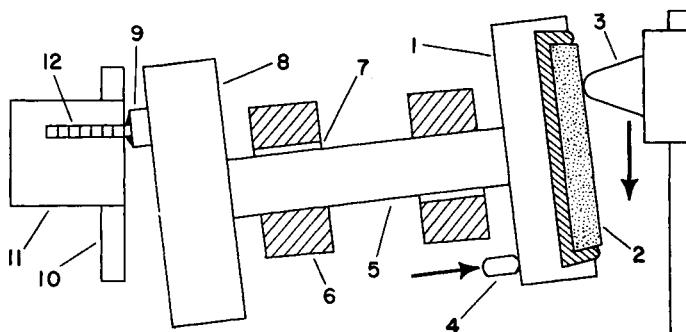


FIG. 5. Rotary inclined plane ultramicrotome. 1, Inclined rotor; 2, embedded glass plate; 3, travelling cone; 4, spring-activated lever; 5, main shaft; 6, bearings; 7, bearing clearance; 8, vise rotor; 9, specimen; 10, knife; 11, trough; 12, ribbon of serial sections.

rotating inclined plane, was mounted at one end of the shaft and pressed against the travelling cone of the Spencer microtome, the plane of the glass plate being displaced from the line of motion of the travelling cone by an angle of $\arctan \frac{1}{100}$. The tissue block was mounted in a rotating vise at the other end of the shaft. As the tissue block passed the knife in its downward motion, a section was cut, but when the tissue block passed on its upward movement, it was removed from the site of cutting by the diameter of its circle of rotation and by an angular backward displacement of $\arctan \frac{1}{100}$. A spring-activated lever was placed to press against the rear edge of the rotating inclined plane diametrically opposite the travelling cone to annul all sideward movement due to clearance in the bearings of the main shaft and to provide extra loading so that the force needed to cut the tissue block was only a small part of the force needed to turn the shaft (see Section III, 2, f).

The principle of the rotating inclined plane was later incorporated into an all-rotary ultramicrotome (Gettner and Ornstein, 1953) (see Fig. 6). The main shaft with its two rotors and spring-actuated lever were placed on a rotatable base plate which allowed the angle between the rotating inclined plane and the plane of movement of the travelling cone to be set at will. The precision screw drawing the travelling cone was driven at a constant velocity being connected with the rotating motion of the main shaft through a set of reduction gears. Since all motions were interconnected and driven from a constant-speed motor, a change in section thickness was obtained by a change of the angle between the inclined plane and the travelling cone, section thickness being proportional to the tangent of that angle. A gear change was provided to increase the lead screw velocity by a factor of 10, permitting consecutive sections of 0.1 and 1.0 μ thickness to be cut,

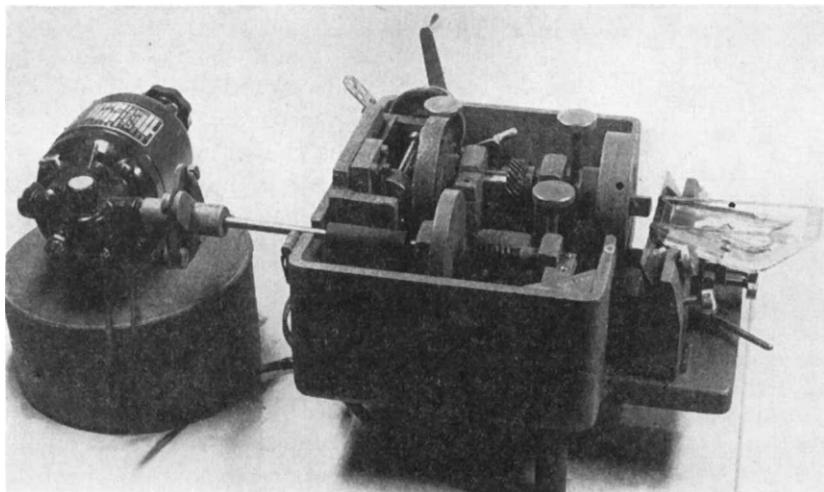


FIG. 6. All-rotary ultramicrotome

thereby making possible a section to section comparison by electron and phase microscopes (Ornstein and Pollister, 1952).

The Porter and Blum (1953) side-motion cantilever microtome has proved to be the most popular of thin-sectioning microtomes. It is the third of a series of microtomes the first of which consisted of an aluminum rod mounted at one end in a massive brass block and the other in an eccentric hole of a hand-rotated disk, a circular motion of the latter end being obtained (Porter, 1952). A collet-type specimen holder, to clamp the cylindrical polymethacrylate specimen block which was polymerized in a gelatin capsule, was screwed into the free end of the aluminum rod. When operating, an electric lamp was placed directly above the aluminum rod thus obtaining a thermal type specimen advance.

In the second ultramicrotome of this series (Porter and Blum, 1953), the aluminum rod was mounted at one end in a gimbal consisting of two sets of pivots, one set horizontal and the other set vertical. A lateral motion was obtained in the specimen end of the aluminum rod by causing it to follow the trajectory of a parallelogram (see Fig. 7). During the cutting stroke the aluminum arm is drawn vertically downward, passing the knife at its half-way point; near the bottom of this stroke the end of the parallelogram plate produces a sideward thrust to the aluminum rod causing its upward return stroke to be displaced from the downward cutting stroke; near the top of the return stroke the upper end of the parallelogram plate produces a reverse sideward thrust causing the aluminum rod to return to a point vertically above the cutting site. An electric lamp was used to produce a thermal expansion in the aluminum rod.

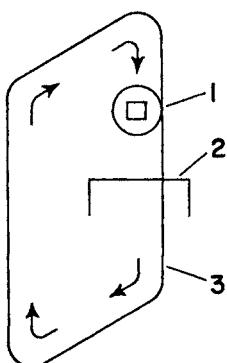


FIG. 7. Specimen trajectory of the Porter and Blum (1953) ultramicrotome. 1, Cantilever arm with specimen; 2, knife edge; 3, parallelogram plate.

The final model of this series (Porter and Blum, 1953) is little changed from the second model except that the thermal expansion of the aluminum rod is replaced by a purely mechanical movement. The horizontal pivots of the gimbal have been mounted in a yoke which in turn is mounted to the supporting frame by another pair of horizontal pivots slightly vertically displaced from the horizontal pair of the gimbal (see Fig. 8). These two pairs of pivots act as a lever with great mechanical advantage. When the yoke, which joins the gimbal to a nut on a precision lead screw, is drawn backward, the cantilever arm moves forward. The incremental advance is the yoke nut movement along the lead screw axis reduced by the ratio of the distance between the two pairs of horizontal pivots to the distance from the pivots to the lead screw.

Another ultramicrotome using a lateral displacement of the specimen to achieve a single-pass sys-

tem is that of Weinreb and Harman (1954). A rotary displacement specimen holder consisting of two disks held together by a central tapered pin, one disk being clamped into the vise of any standard reciprocating microtome with a reduced advance mechanism such as the Spencer rotary or the Cambridge rocking microtomes, and the other containing an eccentrically mounted chuck to hold the specimen block. On the downward movement of the specimen block, the chuck is positioned in line with the cutting site (see Fig. 9). The chuck disk is then rotated so that on the upward stroke the specimen block is laterally displaced from the cutting site. While Weinreb and Harman have used a glass knife, it is suggested that, by a slight angular reorientation of the disk rotation axis so that a small backward displacement of the specimen block occurs in addition to its circularly lateral displacement, a steel knife may be used.

A third mechanical principle used on at least two ultramicrotomes is that of backfeeding in conjunction with pure reciprocating motion. Grey and Bieseile (1953) adapted a double-ratchet gear to the lead screw of a Spencer model 815 microtome, which on the down stroke will advance the lead screw by a predetermined number of ratchet teeth, and which on the return stroke will retract the lead screw by a number of ratchet teeth less than the advance motion. The section thickness is determined by the difference between the advancement and retraction of the lead screw.

In a novel adaptation of magneto-striction, Haanstra (1955a) has produced a backfeeding cantilever ultramicrotome. A coil is wound about a

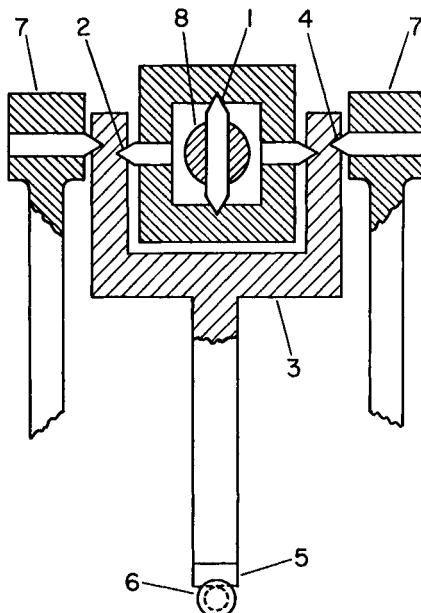


FIG. 8. Gimbal and yoke mounting of the Porter and Blum (1953) ultramicrotome. 1, Vertical gimbal pivots; 2, horizontal gimbal pivots; 3, yoke; 4, yoke suspension pivots; 5, yoke nut; 6, precision lead screw; 7, frame; 8, specimen-carrying cantilever arm.

rigidly anchored nickel bar and a rod holding a specimen vise is attached to the nickel bar by a leaf spring (see Fig. 10). On the down stroke no current flows in the coil and the nickel bar assumes its maximum length. On the upward stroke current flows through the coil and by the phenomenon of magneto-striction the nickel bar retracts thereby also retracting or back-feeding the specimen from the cutting site. Since the current in the coil builds up to a maximum virtually instantaneously, the retraction and relaxation of the nickel bar are also nearly instantaneous. Reciprocating motion is obtained by a solenoid raising the specimen arm on the return stroke. The force of gravity activates the downward stroke; cutting speed is controlled by the viscosity of oil or liquid in a dash pot. Specimen advance is by thermal expansion of the nickel bar due to hysteresis losses as its coil is continuously turned on and off, the section thickness being proportional to the ampere turns of the coil and to the time lapse between consecutive sections.

The earliest attempt to apply thermal expansion in a single-pass ultramicrotome was that of Hillier (1951b) which culminated in an instrument in which an electrically heated thermal rod applied an advancing force ec-

centrically to a specimen vise rotor (Hillier and Chapman, 1953). The system was spring loaded axially to oppose the forward motion of the heated rod. The couple, produced by the off axis forward thrust of the thermal rod and the backward thrust of the spring, annulled the bearing clearance play of the shaft (see Fig. 11).

Another thermally advanced ultramicrotome is that of Sjöstrand (1953a; see also Chap. 6 of this book) which also uses a rotating specimen head. A wheel rotating within a large precision bearing holds one central and two eccentric shafts at the other end of which is mounted a supporting bar. The central and one eccentric shaft are rigidly mounted on both the rotating wheel and the supporting bar, while the other eccentric shaft, or thermal rod, is rigidly mounted to the supporting bar but longitudinally free within the rotating wheel. The thermal rod is constructed of two steel rods mounted end to end, one of which is heated by an electric coil wound around it and insulated from the other parts by plexiglass, and the other carrying the specimen block in a chuck-type vise after passing

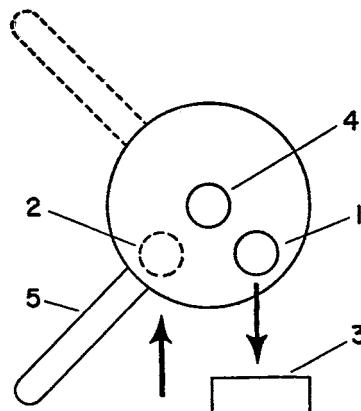


FIG. 9. Rotary displacement specimen holder of Weinreb and Harman (1954)
1, Specimen position on cutting stroke; 2, specimen position on return stroke; 3
knife; 4, tapered pin; 5, actuating lever.

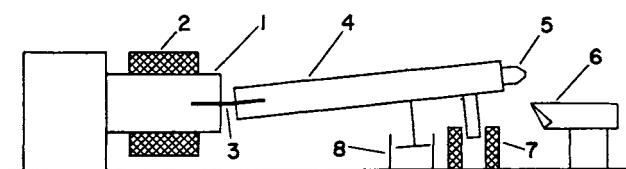


FIG. 10. Magneto-striction ultramicrotome of Haanstra (1955a). 1, Magneto-
striction nickel bar; 2, electro-magnetic coil; 3, leaf spring; 4, cantilever arm; 5,
specimen; 6, knife and trough; 7, solenoid; 8, dash-pot.

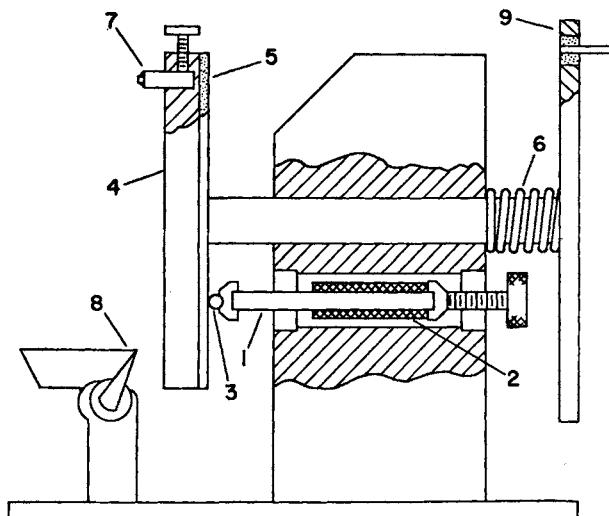


FIG. 11. Thermally advanced rotary ultramicrotome of Hillier and Chapman (1953). 1, Thermal rod; 2, electric heating coil; 3, ball-bearing thrust; 4, specimen vise rotor; 5, glass thrust plate; 6, spring; 7, specimen; 8, knife and trough; 9, motor drive coupling rotor.

through the rotating wheel and bearing. A constant-speed motor drives the rotating wheel by means of a belt. In addition to a horizontal torque produced by the pull of the belt on the rotating wheel thereby tending to annul bearing clearance play, the wheel is greatly loaded by the tight fit of its bearing.

Realizing the high cost of precision parts in an instrument, Hodge *et al.* (1954) have produced an ultramicrotome similar to Sjöstrand's but without the precision bearing. A rod heated by an electric coil is attached at one end to a rigid block by a thin steel wire clamped to the rod and rigid block by a pair of chucks (see Fig. 12). The other end of the rod passes through a rubber bearing in an eccentric hole in a rotating disk. Another chuck-type vise holds the specimen block at the free end of the heated rod. While the position of the specimen is always parallel to the knife edge, the relative circular motion of the heated rod within the eccentric hole of the rotating disk causes static friction resulting in tortional oscillations in the heated rod about its axis. Small elastic movements in the rubber bearing may also result from the developing static friction and may thus cause variations of section thickness if the knife edge is not oriented perpendicular to the axis of the heated rod. Modifications made in the *commercial* model have eliminated the rubber lined bearing and have provided a precision roller bearing for the rotating disk.

In all the above instruments, the tissue block is advanced toward a

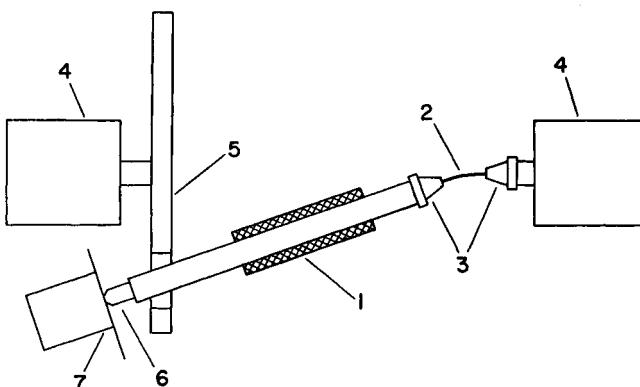


FIG. 12. Wire-coupled thermal ultramicrotome of Hodge *et al.* (1954). 1, Thermal rod with electric heating coil; 2, wire coupling; 3, chuck vises; 4, rigid frame; 5, rotating guide wheel; 6, specimen; 7, knife and trough.

stationary knife on the principle that if all motions are given to one mechanical member, reproducibility will be greater in addition to being mechanically simpler, than if more than one member are involved in motion. There is one ultramicrotome, however, in which the knife is advanced toward the specimen (Stryker, 1955). The knife is mounted on a carriage drawn through ways by a high-precision screw which is rotated by a ratchet and pawl through another worm and wheel (see Fig. 13). The specimen is mounted centrally on the surface of a drum which is rotated in precision roller bearings. A plan of improvement includes providing a second precision screw

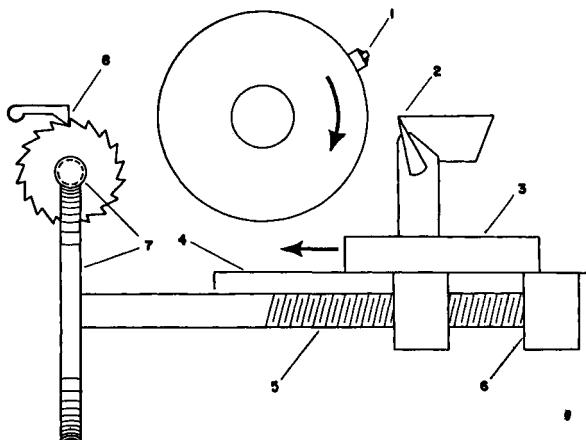


FIG. 13. Knife advancing ultramicrotome of Stryker (1955). 1, Specimen mounted on rotating drum; 2, knife and trough; 3, knife holder and carriage; 4, carriage ways; 5, precision screw; 6, screw thrust plate; 7, worm and wheel; 8, ratchet and pawl.

rotating with equal angular displacements and in a position parallel to the first screw. The carriage is to be mounted entirely on these two screws, similar to a recent double-screw ruling engine (Strong, 1951), instead of the present system of ways.

c. Mechanical vs. Thermal Advance Systems

The choice of one of two types of ultramicrotome (mechanical and thermal) is very often decided because experience with the other type has not been satisfactory. Convenience in the selection of a section thickness from an extensive thickness range is offered in almost all mechanical systems and as long as the instrument operates the sections produced will have a constant thickness plus or minus the error of the system in its surroundings. Thermal advance offers a motion which is smooth and is not subject to the irregularities of solid surfaces sliding on each other or of variations of the thicknesses of lubricating films. (Most thermal instrument designs, however, do not take full advantage of this.) On the other hand, thermal systems have limited periods of continuous operation. During these periods a relatively indefinite advance is obtained which is "smaller than" some maximum value.

d. Tissue Clamping Devices

The choice and design of a tissue block vise is as important for consideration as any other single member of an ultramicrotome because poor construction or manipulation of this part can cause faulty performance in an otherwise well-designed instrument. Most specimens to be sectioned will be embedded in materials that are soft, plastic, easily deformed, and subject to bending and vibration. By its vise the tissue block should be transformed into a *rigid extension* of the structural member onto which it is held.

Several vise systems are in current use, at least two chuck designs and one wax embedding (see Fig. 14). One chuck-type vise is a two-way split cylinder, similar to lathe chucks, which closes down on the cylindrical form of the polymethacrylate block. In another chuck-type vise the polymethacrylate block is pushed into a narrower diameter just allowing the tissue to protrude. One should expect with chuck-type vises in general some plastic flow and thereby extrusion of the polymethacrylate block under the applied force exerted on it by the vise. The (Carnauba) wax-embedded design, similar to procedures in histological technique, embeds a trimmed and oriented tissue block on four sides and cements the bottom to a Bakelite or laminated Nylon rod which in turn is secured to the advancing member of the ultramicrotome. The authors prefer the last technique because the rigid Carnauba wax flows into fingerlike intrusions sometimes several microns in depth into the polymethacrylate intimately holding the tissue

block without the application of pressure. Greater freedom of orientation of the specimen also results.

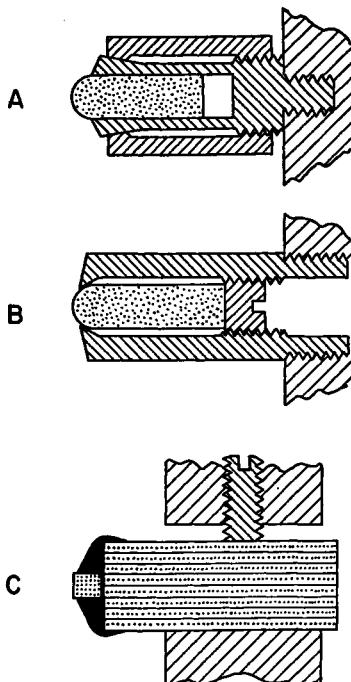


FIG. 14. Specimen mounting and clamping systems. A, Chuck-type vise with a number of jaws laterally clamping the polymethacrylate specimen block. B, Vise with conical bore longitudinally pressing the specimen block. C, Polymethacrylate specimen block embedded in Carnauba wax and cemented to a rigid Bakelite mounting block.

e. The Trough

In early thin-sectioning attempts, sectioning and section handling were copied from conventional histological technique. The sections were cut on a dry knife, picked up with a brush, and spread on the surface of warm water. The dry sections were extremely fragile and difficult to handle while serial sections were almost impossible to preserve (Hillier and Gettner, 1950c). This difficulty was overcome by the addition of a trough.

Troughs, or reservoirs, independently developed by Claude (1948) and Gettner and Hillier (1950), have been made in a number of ways (see Figs. 5 and 6). They have been cut from cardboard and attached to the steel or glass knives with molten paraffin. One design uses an aluminum, open-ended box where the steel knife acts as the end wall with a gasket seal and

with clamps passing under the knife. Malleable rubber has been formed into a large well and pressed to the sides of a glass knife.

The liquid of the trough serves three purposes; it releases the section from the knife facet, eliminating much compression due to the force necessary to move the section down the knife facet; it eliminates electrostatic charge and all accompanying effects; it spreads the section, eliminating folding, crimping, and handling in transport from a dry knife.

The liquid must, therefore, have certain definite characteristics. It must wet the knife facet to the very edge in order that the section will begin to separate from the knife as soon after cutting as possible. It must not wet the section, for if this happens the section will readily submerge into the liquid. It must have a low surface tension to creep between the section and the knife facet at all times; with a high surface tension the sections often push the liquid away from the knife edge with a resulting adherence to the knife facet. The surface tension must not be too low for then the liquid will creep over the knife edge and wet the back facet also wetting the tissue block. The liquid must not corrode the knife if a steel knife is used. One component of the trough liquid should be an agent miscible with the embedding material when in the liquid state. This is imbibed slightly by the section thus releasing the strains set up during sectioning.

Trough liquids which have proved most successful are surface-tension-reducing mixtures such as water solutions of acetone or ethyl alcohol. A 25% acetone mixture by volume (there has been some preference for mixtures varying from 10% to 40%) is a widely used trough liquid for polymethacrylate sectioning. The acetone is imbibed by the polymethacrylate facilitating a rapid relaxation of the compression that tends to occur during sectioning. If water alone is used as a trough liquid, the release of compressive strain proceeds at a very much slower rate, while if a high concentration of acetone is used, such as a 60% mixture, the methacrylate becomes soft and adhesive causing folds and crimps (which occur during sectioning) to be permanently molded in the section. Ethyl alcohol is used occasionally in concentrations of 20% to 60% with results similar to those obtained with acetone mixtures. Dioxane solutions have been used but the corrosive effect of its aqueous solution on a steel knife is rapid, and its effect on the tissue is uncertain. Soap and detergent solutions, oils of various descriptions, ethylene glycol, and glycerine solutions and silicone fluids all wet the section which immediately submerges. Plain distilled water has often been used with some success with glass knives probably because there is no sharp break, or edge, where the knife facet meets the body of the knife, and also perhaps because distilled water will easily wet a slightly dirty glass surface. If steel knives are used, special precautions must be taken to prevent corrosion of the knife edge; addition of 0.1% of sodium chrome glucasate

(Haering & Co., San Antonio, Texas) to the trough liquid has been found to accomplish this very well (Hillier and Gettner, 1950c).

The level of the liquid should be high enough to reach the cutting edge of the knife but not so high that wetting of the specimen block occurs. A slightly convex meniscus produces the best cutting characteristics almost entirely eliminating folding and crimping as the section is cut.

f. Microtome Problems

Chatter is a vibration of the tissue block toward and away from the knife and is evidenced by thick and thin striations of the section in a direction perpendicular to the movement of the section past the knife. Chatter striations are differentiated from knife marks or scratches because they blend into each other, whereas knife scratches are abrupt and often tear out small regions of the section. Chatter may arise in any of several ways the sources of some of which may be recognized by the periodicity of the striations.

When the tissue block receives the first impact from the knife, some member of the microtome must yield under the newly applied cutting force,—the tissue block, the knife, or the specimen arm. If the knife is dull, or if the tissue block is relatively soft and elastic, it can be compressed behind the knife and produce no section (see Section II,2,a). If the tissue block is long (in the direction of microtome advance), it can bend about its point of attachment. Bending will be greater with greater impact and cutting forces. These forces increase with increasing hardness and width of the specimen block (in the direction parallel to the knife edge), with higher cutting speeds, and with duller knives. *Such stresses, when suddenly released (as the yield point of the specimen block is exceeded and cutting begins), can give rise to vibrations in the less rigid or less massive member of the ultramicrotome.* Buckling and twisting can occur in the leaf springs of instruments which have employed these to be free from friction effects (Cocks and Schwartz, 1952). Oil films can change in their thickness, and even the micro-spring nature of two contacting surfaces (see Section III, 2,g) may yield especially if these surfaces are small in contacting area. While no measurements of cutting force have been published on polymethacrylate-embedded tissues, those recorded for paraffin tissue blocks are illustrative (Richards, 1949). Chatter from this source may be alleviated by reducing the width and length of the tissue block, thus reducing the force of impact and amplitude of vibration proportionally; the tissue block may be trimmed into the form of a truncated pyramid with a section width as small as 75μ (Sjöstrand, 1953a). The specimen arm may be loaded with a constant external force appreciably greater than the force of cutting, i.e., a spring-loaded lever applied in such a manner as to greatly increase the

force needed to advance the specimen vise (Gettner and Ornstein, 1951; see also Section III,2,b).

Chatter may arise from sources other than specimen-knife interaction. If the coupling from a motor to the ultramicrotome both through the mounting and through the power transmission does not thoroughly insulate motor noise, a chatter with the frequency of the motor r.p.m. will become evident. A more random periodicity than from motor noise will be produced if static friction or variations of sliding friction occur in any moving part of the ultramicrotome. If such static or sliding friction occurs in the specimen vise member, it will be more evident than if it occurs in one of the secondary mechanisms, such as any part of the advance mechanism or power transmission. In the latter case, minute variations and millisecond interruptions of constant velocity will be transmitted to the main structural member to produce variations of stress and flexure, or of critical oil film thicknesses. The nonlinear motion of spur gears, the bind and slip of inadequately lubricated spiral gears and worm and pinion gears, and the variation in friction of pivots can produce the same effects.

Missing or skipping of sections takes place when the tissue block passes the knife without any section being cut. When alternate missing and cutting occur, it is due to either a dull knife or a "soft" tissue block (see Section II,2,a). Recurrent short periods of continuous missing are indicative of a poorly functioning advance mechanism, such as an improperly aligned lead screw. When a long, extended period of continuous missing occurs, it is generally from some major factor, as, for instance, a sudden draft of air much colder or warmer than the air in the immediate vicinity of the instrument. Other minor deviations from good technique, such as a tissue block that is loosely held in the vise, or a knife that is not firmly secured, will give rise to both chatter and missing.

Very often with double-pass microtomes and not infrequently with single-pass microtomes the methacrylate tissue block is wet by the trough liquid. In double-pass instruments it occurs predominantly on the return stroke of the tissue block, but in single-pass microtomes the wetting takes place at the instant when the tissue block first makes contact with the knife edge. In the latter case, especially, this problem is due to a dull knife. The wetting takes place either because the trough liquid creeps over a rounded or chipped edge, or because the trailing end of the previous section was not cut but was torn from the tissue block producing fragments of methacrylate lying over the knife edge and giving opportunity for the trough liquid to creep over the edge by capillary attraction. If a section is cut, it will submerge into the trough liquid, but more usually the embedding material swells sufficiently that only a "sludge" is scraped off.

Another phenomenon, a corollary to the "pick-up" problem, may occur with a dull knife. An overhanging section fragment from the previously cut section may be *caught* or "picked-up" by the descending tissue block and the entire previously cut section drawn over the knife edge.

g. Reproducibility and Sources of Instrumental Error

Reproducibility of performance of a microtome, like any instrument, is limited by random fluctuations inherent in the design, for example, those due to stresses and strains within structural members. This random change may be due to several factors acting simultaneously, as, for instance, temperature fluctuations and flexure of structural members. In an instrument such as an ultramicrotome, where the designed events are movements as small as 200 Å and often less, reproducibility is largely a function of the ratio of the desired incremental movements to the total error, or random change, which occurs within the time lapse needed for the incremental movement.

One source of error from which no ultramicrotome is entirely free is thermal expansion and contraction of all structural members due to air currents passing through or about the instrument. Some of the more serious changes of this type occur as angular movements due to slight warping and twisting as well as simple length changes. It is obvious that if the parts of an instrument are short and massive and made from metals with low coefficients of thermal expansion and thermally insulated from the room air currents, that instrument will be least sensitive to temperature fluctuations. Computations indicate that in our microtome (Gettner and Ornstein, 1953) a change of temperature of 1°C per hour difference between the main shaft and the base will produce an error of $\pm 0.006 \mu$ per section, or $\pm 30\%$ in a 200-Å section, with a sectioning rate of 8 per minute. Cocks and Schwartz (1952) state that a change of more than $\pm 0.003^\circ\text{C}$ in a 25-cm arm between the production of two consecutive sections cannot be tolerated for a section thickness of 0.05 μ .

Another source of error common to all ultramicrotomes is the one due to changes of length and flexure of structural members which are caused by the stresses of forced motion. Hillier and Gettner (1950b) have reported that a certain time is required for the instrument to come to a state of equilibrium in motion (or dynamic equilibrium) before reproducibility of sectioning can be achieved. This source of error can be reduced by decreasing the number of moving parts and their contiguous surfaces. We have measured an over-all flexure of 4×10^{-6} inch/pound of force (2000 Å/kg) applied axially on the specimen vise rotor of our ultramicrotome.

In the world of everyday experience when one rigid piece rests on another, it does so at 3 points of contact. This is no longer true in the submicro-

scopic region, where a surface which appears flat by ordinary standards will appear as a field of many high points of varying heights (Bowden, 1945). When two such surfaces are in contact, the highest points will touch first but will be compressed into the body of the surface. The next highest points will next contact the opposing surface and will also be compressed, and so on, each high point being compressed until the sum of compressive forces equals the total force by which the two surfaces are pressed in contact. Thus the nature of contact of one such normally flat surface against another is similar to so many submicroscopic springs holding the surfaces apart. If, therefore, the external force holding the two surfaces together is varied, they will be separated or pressed together slightly and in the case of the ultramicrotome become a source of error (see Section III,2,f). If the number of high points or spring contacts between the two surfaces is greatly increased, as by increasing the area of surface contact in a bearing, thrust plate, or pivot point, the total effect of a variation of external force will be reduced proportionally.

In thermally advanced ultramicrotomes the lack of complete knowledge of the thermal gradients at every point of the heated rod is a source of error. Since the heating and cooling of a body follow a fourth power radiation law as well as second power conductivity and convection laws, it is difficult to predict how one may get a linear rate of expansion. (No author has claimed true linear thermal advance; Eden *et al.* (1950) show representative rates of expansion for precooled and for electrically heated thermal rods, and Haanstra (1955a) gives expansion rate data for his instrument.) The rate of expansion may be measured experimentally while the rod is in a stationary position, but when sections are being cut, the rod is in motion and the rate of thermal expansion may be quite different.

The force needed to overcome static friction can be a troublesome source of error (Hillier and Gettner, 1950b). When a part, such as the inclined plane and specimen vise casting of the Spencer 820 microtome, is pushed forward by the small incremental movement needed for thin sections, static friction can easily oppose the force of the travelling nut for one or perhaps two strokes, the frictional energy being absorbed in the stresses and strains of the structural members. The advancing force may then suddenly become larger than the static friction and the part may overshoot the desired rest point, thus cutting a section much thicker than desired after a period of no sections. Static friction may also produce error in the discontinuous movement of a lead screw turned by a ratchet and pawl.

In all ultramicrotomes in which one part may move against another part, sliding friction will vary both with the speed of motion and from point to point. Oil lubrication between these sliding surfaces reduces the sliding friction to $\frac{1}{20}$ to $\frac{1}{50}$ of its value in the dry state, thus reducing but not

eliminating this source of error. Oil films, however, introduce another variable, that of their thickness. It was observed in accord with the theory that if two contacting surfaces are relatively accelerated, their mutual oil film increases in thickness, while if the surfaces are decelerated, the oil film thickness reduces (Hillier and Gettner, 1950b). The use of oil films, therefore, necessitate constant speeds in all lubricated moving parts of an ultramicrotome. In the ultramicrotome of Gettner and Ornstein (1953) the lead screw is also turned at a constant velocity thus producing constancy of oil film thicknesses between the travelling nut and its way, the travelling nut and the lead screw, and finally between the lead screw and its thrust bearing.

Many thermally and mechanically advanced ultramicrotomes are driven by constant-speed motors; as a result the interval of time between sections is accurately controlled for thermal instruments and the oil films are kept at constant equilibrium thicknesses in mechanical instruments. The vibration of a motor, however, may easily be transmitted to the microtome to produce knocks and otherwise introduce large discontinuous stresses on the sensitive structural members of the instrument. In the case of Hillier's early thermal ultramicrotome (Hillier, 1951b) the motor and microtome were mounted on two separate stands, the microtome on a cement pillar and the motor on a table nearby, in an attempt to prevent the transmission of vibrations and motor noise to the instrument. Sjöstrand (1953b) has also mounted a Hillier and Gettner (1950b) microtome on a separate cement pillar, driving the instrument through a plastic belt. In the Gettner and Ornstein (1953) microtome the motor is dynamically balanced, with a lead plate sandwiched between foam rubber on which the motor is mounted (this construction is also employed in the Hillier and Chapman microtome, 1955). The shaft from the motor contains a gimbal joint in which the ring is of neoprene to prevent vibrations passing via this avenue.

In an ultramicrotome where the tissue block is mounted on a rotating member, the bearing clearance, which is necessary for its proper functioning, introduces a small (0.0005 inch) but submicroscopically large (125,000 Å) and troublesome sideward play. In all instruments which have a "reproducibility" of better than 200 Å, the rotating members have been spring loaded to bear always in one direction against the bearing surface (for example, Gettner and Ornstein, 1951, 1953; Hillier and Chapman, 1953. See Figs. 5 and 11).

The total error is the effect on the product of the sum of all these random changes taking place in the various parts of an instrument. Since random change follows statistical law, one change may cancel another change at one time but complement it at another time. Reproducibility of an ultramicrotome is the magnitude of section thickness desired plus or minus the root mean square of all errors which may be expected to accrue in an aver-

age section cut during an average sectioning period. The ultramicrotome should have a knowledge of the mean total error of his instrument so that he may know with what probability his instrument can cut sections of a certain thickness. It probably would be desirable if all ultramicrotome manufacturers would state the mechanical limits of their instruments. So far as is known, only two manufacturers make available any such data.

IV. Knives and Knife Sharpening

Knife sharpening has always been and is yet today a problem with microtomists. In common practice today is the basic technique of Hugo von Mohl (1857) of moving the knife over a roughened glass surface upon which is placed an abrasive compound suspended in some liquid. A second in common use is that of sharpening on a stone and then polishing by stropping. Still a third method is that of grinding the knife against the edge of a glass wheel. For a history of these methods and their variations, the reader is referred to a discussion by Uber (1936). (See also Richards, 1949).

As the requirements on the microtome became at once more refined and critical with the advent of ultramicrotomy of tissues for electron microscopy, the problem of producing an adequately sharp knife became acute. Fullam and Gessler (1946) used a part of an ordinary razor blade mounted in the rotor of the high-speed microtome. Claude (1948) used an ordinary razor blade in his double screw microtome. Pease and Baker (1948) used a standard histology knife in their modified Spencer microtome. Hillier (1951a) has refined the old technique of von Mohl to produce a knife sharp enough to cut $0.1\text{-}\mu$ sections of a paraffin-collodion embedded tissue, and Gettner has made refinements on Hillier's technique to cut $0.02\ \mu$ sections of polymethacrylate with a standard histology knife (see below). Razor blades (Sjöstrand, 1953a,b), stainless steel knives (Ekholm *et al.*, 1955), and a beryllium-bronze knife (Fabergé, 1949) have been sharpened for ultramicrotomy, and glass can be broken to produce edges sharp enough to cut $0.02\text{-}\mu$ sections and sometimes less (Latta and Hartmann, 1950). Several ultramicrotomists have envisioned the theoretically ideal edge as being a polished homogeneous single crystal. Gettner and Beyer (1950) have made a knife of fused quartz, and Fernández-Moran (1953, 1956) and Haanstra (1955b) have produced knives of diamond. Some of the above knives and sharpening procedures will be discussed because they are attempts to push sharpness to the theoretical limit.

1. STEEL KNIFE SHARPENING

a. Method

In Hillier's method for sharpening a standard histology knife (Hillier, 1951a), a small hardened steel back $\frac{1}{2}$ inch long is firmly and permanently

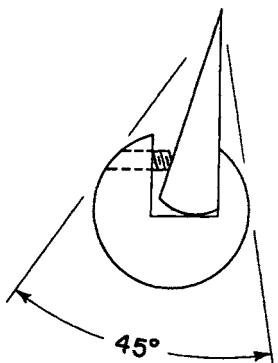


FIG. 15. Steel knife with sharpening back attached

fastened to the base of the knife, and this assembly worked over the surface of a slightly roughened thick glass plate. An abrasive, Linde A powder (synthetic sapphire hexagonal crystals approximately 0.3μ in diameter; Linde Air Products Co., New York) is suspended in water and the mixture used as a lapping compound. The knife is moved over the glass surface in a zigzag sideward manner with a concurrent gradual excursion down the length of the glass plate, the edge leading. If the edge is allowed to follow (edge trailing), it is immediately roughened. Although the facet angle was kept at 30° , sections no thinner than 0.1μ were obtained with collodion-paraffin embedded tissues (the paraffin compressed more than 50% with thinner sections).

Some refinements of the above method, embodied in the following three step procedure, are in current use in our laboratories and yield edges that will cut sections of polymethacrylate 0.02μ and less. Two removable knife backs are used, one giving a wedge or total facet angle of 42° and another giving a total facet angle of 45° as shown in Fig. 15. A glass plate 1 inch by 11 inches by 17 inches is initially prepared by scratching the surface lengthwise with Linde B powder (synthetic sapphire cubic crystal approximately 0.1μ on the side; Linde Air Products Co.) using the round surface of one of the knife backs. Any metal chips that may become embedded in the glass are removed with a 50% aqueous nitric acid solution. The sharpening procedure is begun with a thorough cleaning of the glass surface and a rinsing with distilled water. A lapping compound, consisting of $\frac{1}{3}$ Linde B powder to $\frac{2}{3}$ by volume of a solution of 10 ml of a filtered 0.1% solution of sodium chrome glucasate (Haering Co.) in distilled water, 8 ml of glycerine, and 2 ml of acetone, is mixed and poured on the glass surface. The 42° knife back is rigidly clamped to the center of the knife. The knife is placed in the lapping mixture and worked over the glass surface with a zig-zag and gradually advancing (edge first) motion as shown in Fig. 16. After about 5 min of working each facet alternately, the knife and glass plate are rinsed in distilled water, but not washed.

The second step of this procedure consists of carrying out the identical motions for the same length of time but with a lapping mixture consisting of 8 ml of the filtered corrosion inhibited distilled water, 10 ml of glycerine, and 2 ml acetone. In this step the 42° knife back is kept on the knife but no Linde B powder is used. The glass plate may be acid-rinsed to remove any metal that has been left on the surface, giving a final rinse to both glass and knife with distilled water only.

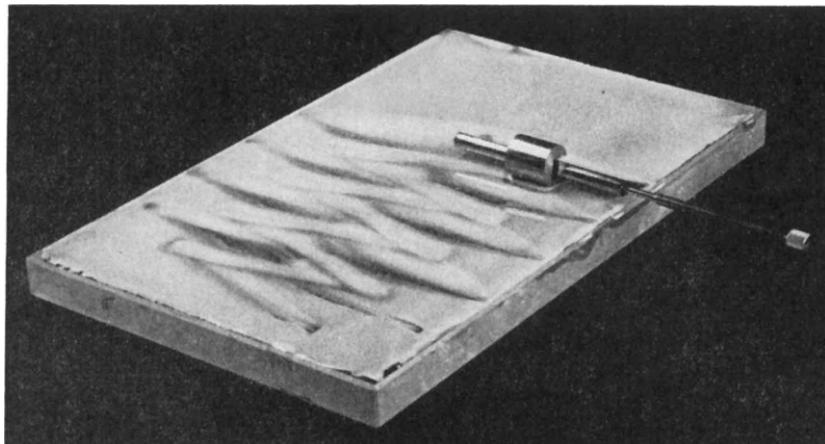


FIG. 16. Motion used for sharpening a standard steel histology knife on a plate glass lapping surface.

The third step is the edge-producing step and should be carried out with extreme care. A lapping solution of 6 ml of the filtered corrosion-inhibiting distilled water, 12 ml of glycerine, and 2 ml acetone, is placed on the central area of the glass plate. The knife, now with the 45° knife back solidly and centrally clamped to it, is worked over the glass surface for only a very short time. A total of not more than 8 excursions of 4 sideward strokes on each side is performed applying a force to the knife that is only sufficient to move it across the glass surface in a *smooth, controlled motion* as indicated in Fig. 16. This *final motion should be slow*, taking about 2 sec for each sideward stroke. After 8 excursions in 2 min of lapping time, a facet 2 μ wide is produced. The knife is rinsed with the corrosion-inhibiting distilled water and dried with an absorbent paper *without touching the edge*. The glass plate is prepared for storage by spreading the last lapping mixture over its entire surface and covering it to prevent evaporation and dust adhesion, e.g., with a paper towel.

The method of Sjöstrand (see Chap. 6 of this book) is that of clamping a razor blade in a rigid holder and working this assembly, edge first, over a glass upon which there has been placed a suspension of Linde B powder and soap solution. The assembly of razor and knife back forms a line and point contact when placed on the glass plate, the angle between the facets being approximately 53°. The motion adopted by Sjöstrand is a forward motion, edge first, of 12 to 20 excursions down the length of a new or optically flat glass plate, varying the velocity from beginning to end from 2 cm/sec to 0.5 cm/sec with a concurrent reduction in pressure from 150 g to 50 g. With this technique Sjöstrand has obtained some of the thinnest

sections yet cut of polymethacrylate, being reported as thin as 0.007μ (70 \AA).

Ekholt *et al.* (1955) using a carbon-chromium-steel knife in a two-step procedure lap a 30° wedge angle against a revolving iron lap, and then a 50° angle on a revolving glass plate. A lapping paste consisting of chromium oxide in oil is applied to the cast iron lap, but an abrasiveless lapping fluid of 3% sodium bicarbonate and detergent is used on the glass plate. After 2 to 3 min of final lapping, a 10 to $15\text{-}\mu$ facet width is obtained with the desired sharpness.

b. Theory

The procedure of sharpening the very keen edges used in ultramicrotomy is essentially that of lapping one material on another, with the special condition that attention is focused on the edge in the hope that it will be the ideal locus of two plane intersecting surfaces, the facets. Though the actual mechanism is not fully understood, some evidence does exist that allows us to assume that lapping and polishing are achieved when material from high areas of the surface are heated by friction and flow or spread out under the force of friction to the adjacent low areas (Bowden, 1945). If an abrasive is used (a powder with a higher melting point but not necessarily harder than the material to be lapped), local "hot spots" will develop where a high point of the lapped surface contacts an abrasive particle. If an abrasive is not used, the high microscopic areas of the lapped surface which intimately contact those of the lap will be heated by sliding friction. Very high temperatures lasting for only a few tenths of a millisecond have been recorded, as high as 1000°C for sliding speeds of 300 cm/sec and 500°C for speeds of 30 cm/sec . It is well known that mechanical properties such as the shear strength and yield point of a metal will greatly weaken at relatively low temperatures; only a few hundred degrees is needed for plastic deformation to occur under reduced stresses for most steels (see Fig. 17). Furthermore, higher temperatures are more readily reached if the material of the lap has a low thermal conductivity, and are less frequent if lubricants, especially of high molecular weight and long chain length, are added. Although these lapping phenomena must be considered in attempting to define the conditions for knife sharpening, a *highly polished facet* under any light optical magnification is not, by itself, an indication of the quality of the edge. A facet may be given an exceptionally high polish by lapping with great force and high velocities, and yet it will not be sharp enough to cut $1.0\text{-}\mu$ sections.

The force which is applied to the knife and the pressure with which the knife facet bears against the glass plate may not be as important as has been thought. The intimate contact pressure with which one surface is pressed against another rapidly approaches a constant value; for one type

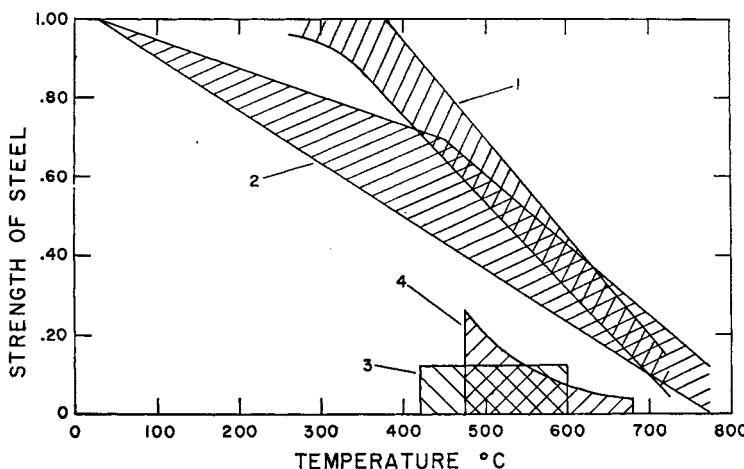


FIG. 17. Decreasing strength of carbon steels with increasing temperature. 1, Decrease of tensile strength from a maximum at room temperature. 2, Decrease of yield strength. Graphs 1 and 2 are drawn from and include data of all carbon steels given in Everhart *et al.* (1943). 3, Temperature range in which most glasses become ductile (Preston, 1935). 4, Temperature range in which the viscosity of the soda-lime-silica glasses decreases from 10^{15} poises to 10^7 poises (Sharp and Badger, 1953).

of soft steel this has been reported to be 8000 to 10,000 kg/cm² (Bowden, 1945), which may have been the upper yield point of the particular metal. As greater loads are applied to the objects in contact, the number of intimate contacting areas increase in such a way that the ratio of the load to the sum of intimate contacting areas remain constant. Applied to knife sharpening, this at first suggests that while the over-all speed of metal removal may increase with pressure, the *rate of metal redeposition* from each contacting area and, therefore, the quality of the facet, and of the edge, are not affected. The authors have tested this, applying a force to the knife approximately 100 times greater than the weight of the knife but sliding the knife over the glass lap with a velocity no greater than usual; this results in an edge essentially *no different* from that produced by the procedure given above.

On the other hand, the velocity with which one surface slides over another largely determines the surface layer temperature of the intimate contact areas. Glow temperatures have been observed occurring at the surface of a metal sliding on glass with a velocity of only 30 cm/sec (Bowden, 1945). As surface temperatures increase, the thickness of the layer which exceeds the yield point and spreads out to adjacent areas must likewise increase. Calculations show that as much as 5 w/cm² of heat may be generated by a 2 μ facet moving over a glass surface at 4 cm/sec with no externally ap-

TABLE III
Facet and Lapping Characteristics of Some Knives

Knife	Facet width in microns	Facet angle	Lapping velocity ^a in cm/sec	Apparent lapping pressure ^b in kg/cm ²
Sjöstrand ^c	6 ^d	51°	0.5	43 ^e
Gettner	2	45°	4	28 ^f
Ekhholm <i>et al.</i> ^g	10-15	50°	4-10	
Hillier ^h	large	30°		
Richards ⁱ	large	27°-32°		
Commercial razor blade	100-300	13°-20°		
Glass knife ^j		70° and up		
Diamond knife		60° ^k		
Diamond knife		54° ^l		

^a That lapping velocity which produces the final knife edge.

^b Apparent pressure between the final knife facet and lapping surface.

^c From Sjöstrand (1953a,b).

^d Schick razor sharpened by La Fontaine (1955).

^e With the recommended externally applied load of 50 g.

^f No externally applied load.

^g From Ekhholm *et al.* (1955).

^h From Hillier (1951a).

ⁱ From Richards (1949).

^j From Latta and Hartmann (1950).

^k From Haanstra (1955b).

^l From Fernández-Moran (1958).

plied downward force. If it is considered that the area involved in this calculation is only the apparent area and not the real intimate contact area, much greater local heat would be expected to develop. The fact that the sharpest edges have been made by lapping at 4 cm/sec, or considerably less (see Table III), suggests that velocity is a critically important consideration in knife sharpening. Presumably the radius (sharpness) of the final edge will be dependent upon the thickness of the layer of softened metal.

That the knife must be moved edge forward over the glass lap has been stated from empirical experience many times (Uber, 1936; Richards, 1949; Hillier, 1951a). It is conceivable that the adjacent area over which the heated layer of metal will be spread is largely determined by the relative direction of motion of the two surfaces. If the temperature rise is not very great, the metal film will be pushed in the opposite direction from the motion and therefore away from the edge in an edge-forward motion. If the edge trails, the softened metal will build up on the edge and produce a burr effect.

Not much is known about the contribution of the texture of the lap surface to knife sharpening. A keener edge is obtained if a lubricant is used

(Hillier, 1951a; Sjöstrand, 1953b; Ekholm *et al.*, 1955). It is entirely possible that the lubricant supports a considerable portion of the pressure from the knife facet and thus reduces the number of points of intimate contact. When such contact does occur, however, the lubricant may be decomposed by the heat generated in friction. The most efficient lubricant is one which will form chemical bonding with the lap and offer a low shearing substance to the material sliding over it (Landau, 1945). To obtain this type of lubricated lapping surface, the present authors applied a silicone fluid with a viscosity of 50 centistokes (Dow Corning 200 fluid) and obtained excellent sharpening characteristics and very keen edges. The silicone fluid was, however, very difficult to remove from the knife facet and a glycerine solution of equal viscosity has given comparable results.

As the knife is lapped on the glass plate, it is highly probable that the glass is also lapped by the metal; i.e., both glass and steel soften at comparable low temperatures (Preston, 1935; see also Fig. 17).

The knife edge becomes stronger and can be lapped to a sharper apex as the facet angle is increased. The factors limiting the upper size of this angle are the compression of the embedding material, if paraffin (Dempster, 1943) or paraffin-collodion is used, and the ability of the liquid to wet the facet, if a trough is used to float the sections. Table III lists the facet angles for various knives used in microtomy.

The more narrow the facet, the greater will be the chance for successful sharpening. This has been a disadvantage to steel knife sharpening because the facet becomes broader as sharpening continues and succeeding attempts will take longer (more lapping strokes) to achieve the same end results. This lengthening of the time of sharpening increases the chance for chipping and other accidents to occur. The use of a 42° knife back in the first two steps of the authors' procedure, and the 30° initial lap of Ekholm *et al.* achieve the result of reducing the breadth of the final facet. Sjöstrand maintains a minimum facet width by choosing a new razor blade each time sharpening is required. In the final analysis it may be true that to achieve the sharpest steel edge, a certain facet width must be obtained such that, with the force applied to the edge and the given velocity of movement, a speed of sharpening ensues to enable the production of the optimum edge in a minimum of lapping strokes.

The particle size of the abrasive compound is, of course, important when an abrasive is used in lapping; the smaller the particle size, the smoother the lapped surface. In the rinsing of the glass plate in the authors' method, only the abrasive particles that are pressed and embedded in the glass plate remain. These are submerged partially below the surface and give the effect of a much reduced particle size during the second and third steps of the procedure. There is yet another abrasive untried at present (Linde Boule

Powder, a synthetic sapphire by Linde Air Prods. Co.) which, if levigated, promises to yield a particle much smaller than even Linde B, (0.01 to 0.02 μ estimated particle size). The fact that Ekholm *et al.* (1955) achieve their final facets on a glass surface entirely free of abrasive particles suggests that the abrasive particles of the other procedures have little to do with the attainment of edges with less than 100 Å radius of curvature especially if those particles are a minimum of 1000 Å across.

Cleanliness is an important aspect of successful knife sharpening. A particle of dust may descend upon the lapping surface from the room air, or a cinder particle may fall out of the microtomist's hair (having been collected during a previous exposure to the city's street air). These particles will be much larger on the average than the particles of abrasive and will produce huge gouges in the knife edge. When the glass plate is cleaned, it should be rinsed with distilled water to insure against the retention of various impurities of tap water. After a period of sharpening, the glass lap should be cleaned with an acid rinse to take away the metal that may be embedded in or deposited on the surface. Such metal deposits will increase the friction between knife and lap and may be responsible for some gouging and chipping of the edge.

The nature of the steel in the knife is an important factor in the ultimate sharpness which is possible to achieve. The hardness of the steel, the minuteness of the crystals, the cleavage strength or crystal to crystal bonding force, and the direction of grain are directly related to the ability of the steel to hold an edge, although the exact relationships are not known. The texture of the knife edge will never depart from a certain inhomogeneity, being composed of hard high-carbon steel bonded with softer low-carbon steel. All of these properties will, moreover, change as soon as sharpening begins, because the edge will be composed of a layer of newly softened metal which will have a hardness determined by the ultimate temperature reached and the rate of cooling that follows.

Tests for knife sharpness have been of two types: (1) a light microscopic observation of the edge is an essential preliminary test, and (2) the thinnest section that can be cut, which is the final test, is self explanatory.

There are several techniques for observing and judging the quality of the knife in the light microscope: (1) The edge is observed at highest dry magnification and the straightness and extent of random indentations or knicks noted. (2) The edge is observed at highest dry magnification with the light beam blocked off from the substage condenser but directed horizontally at the knife edge. The intensity of light which is scattered from the edge is noted (Richards, 1949, 1950; Hillier, 1951a). The edge is sharpest when the intensity of scattering is a minimum. Knicks or breaks are observed as either bright high scattering points, or interruptions of an otherwise evenly scat-

tering edge. (3) The facet is dark field illuminated and observed at high-dry magnification in a reflecting metallurgical microscope (Rhodin, 1954). Knicks appear as interruptions in the very thin newly produced facet of a razor blade.

There still remains doubt, however, about the true sharpness of an edge which reflects light uniformly or scatters the very minimum intensity of light, because it is a common observation that some relatively dull knives scatter practically no light. The real information gained from a light-scattering examination of the edge, therefore, is the presence or absence of gross irregularities of an otherwise straight but *not necessarily sharp edge*, sharpness being determined, in the final analysis, by *the thinnest section that can be cut with it*.

Some disadvantages of the steel knife are still present. The steel is subject to the corrosive forces of the room atmosphere and the trough liquid, although this may be partly overcome by air conditioning and a corrosion inhibitor in all liquids that contact the edge. The knife should be newly sharpened each day thin sectioning is attempted, if $0.02\text{-}\mu$ sections are desired, because a sharp edge may not remain so even until the next day. If, however, the knife is stored in a desiccator, this "shelf life" will be greatly extended. When polymethacrylate-embedded tissues are cut, a keen edge will lose its sharpness after as few as 25 sections $0.02\text{ }\mu$ thick. Once a thicker section has been cut, i.e., $1.0\text{ }\mu$ or even $0.2\text{-}\mu$ section, that area of the edge may be too dull to cut $0.02\text{-}\mu$ sections. A razor blade may be more durable in respect to the number of thin sections one can cut than the standard histology knife. The edge is grainy in texture as mentioned above, therefore, theoretically at least, the sections will never be entirely free of scratches, although refinement of the sharpening procedure can reduce this effect to a negligible level. On the other hand, once sharp, the entire length of the edge can be used, new areas being chosen as each preceding one is dulled in sectioning.

2. GLASS KNIVES

The glass knife (Latta and Hartmann, 1950) was introduced at a time when sharpened steel knives could not cut sections less than $0.1\text{ }\mu$. The glass knife is easy and inexpensive to make, and convenient to use, and has therefore come into wide favor for cutting sections between 0.02 and $0.05\text{ }\mu$. A glass knife is made by cutting a piece of $\frac{1}{4}$ or $\frac{3}{8}$ inch plate glass lengthwise into uniform strips from 1 to $1\frac{1}{2}$ inches wide. A series of straight parallel cuts at 45° to the long axis and about 1 inch apart will then yield parallelograms, one of the 45° apices being the knife edge. A striation-free edge can more often be obtained if the 45° cut is started about $\frac{1}{8}$ inch from one of the sides. When tested for sharpness by the light-scattering

method described above, a newly broken glass edge scatters approximately the same intensity of light as a comparably sharp steel edge.

The toughness or resistance to impact of the glass edge varies considerably with the type of glass. For some grades of plate glass the edge has shattered and nicked with the second or third section cut, while others remained sharp for several hundred sections.

Glass is subject to flow and creep at room temperatures because it is a supercooled liquid. It is reported that, in some instances, knives have lost their sharpness in one week after they have been broken (Hartmann, 1953) although they were not used for cutting. It might be advisable, therefore, to break glass knives just prior to sectioning. The true angle at the apex of the facets is more than 70° (the average of measurements by reflected light from a random sampling of glass knives broken from commercial grades of plate glass is approximately 75°). It is evident, therefore, why embedding materials that exhibit crystalline slippage, or other permanent deformation, such as paraffin, have not yet been successfully sectioned with a glass knife. The principle disadvantage of the glass knife is the uncertainty of the sharpness of its edge, and new knives have been tested only by sectioning. The result of this is that *for every new knife* (with a different rear facet and orientation of cutting edge due to the randomness of the break) *a considerable amount of material may have to be cut from a particular tissue block in the trimming operation* before a section as wide as the full face of the block is obtained. This may consume a considerable amount of time and specimen material, assuming the knife edge is not destroyed in this trimming operation.

3. SINGLE-CRYSTAL KNIVES

A single-crystal knife has been envisioned as being the theoretically ideal knife. Such an ideal knife would have properties somewhat as follows: It would be high on the scale of hardness, but more important it would have toughness and would not cleave or chip with moderate impacts. It would be homogeneous in both physical and chemical aspects in order that the tissue block would be cut by the same keenness and strength of edge at all points. It would not be subject to flow or molecular rearrangement with time at room temperatures. It would not be subject to chemical oxidation or decomposition. It would be rigid and incapable of flexure and large enough to cut any size section.

A single synthetic sapphire crystal is one material that has properties closely approaching the above properties, however no known attempt has been made to fabricate a knife of it. Several years ago Gettner and Beyer (1950) made a knife of fused quartz as a pilot investigation into the production of a sapphire knife. A technique of lapping a "perfect" edge was success-

fully developed (E. and S. Optical Co., Hoboken, N. J.). The lapping method used was the following: One face on each of two pieces of flawless fused quartz was ground and polished to an optical flatness better than $\frac{1}{20}$ wavelength of sodium light; the face of one of these (*K*) later became a facet of the knife (see Fig. 18). They were pressed together face to face without any cementing material, being joined by molecular bonding forces over a large portion of their surfaces. The other facet was then ground and polished to the same high optical standards; the piece (*B*) formed a support for the edge (*E*). The pieces were separated by applying heat to the back of one (*B*) whereupon they snapped apart. When clamped in a suitable holder and mounted in the ultramicrotome, this fused quartz knife was shown to have a blemish-free edge much sharper than any other knife known at that time. Its edge, however, having a facet angle of only 30° , shattered and chipped after only a few $0.06\ \mu$ sections of paraffin-collodion embedded tissue.

Fernández-Moran (1953, 1956) and Haanstra (1955b) have made diamond knives with facet angles of 54 to 60° and have reported encouraging results. Haanstra has cut metals as hard as aluminum (see Table II) as well as tissues at section thicknesses of $0.01\ \mu$ without noticeable change in the cutting edge. Fernández-Moran has reported 6 months of tissue sectioning using the same knife without any substantial dulling of the diamond edge. Diamond is, perhaps, not as well suited to this purpose as synthetic sapphire because cleavage resistance (chipping resistance) is lower for diamond than for sapphire (Pough, 1946). If the experiences of Fernández-Moran and Haanstra with diamond knives are indicative of extremely hard single-crystal materials, synthetic sapphire may prove to be the ideal knife material.

V. Measurement of Section Thickness as a Criterion of Over-all Performance

The error in an instrument, such as an ultramicrotome, cannot be measured while the instrument is at rest. For example, in a thermally advanced microtome it is not sufficient to know only the rate of thermal expansion of the specimen member; in a system where the flexure of all the parts due to motion under force and thermal currents may easily equal or surpass the desired incremental movements, only a measurement of the total product, the thicknesses of consecutive sections, will give a true measurement of error.

Hillier and Gettner (1950c) have proposed a criterion of reliability of an

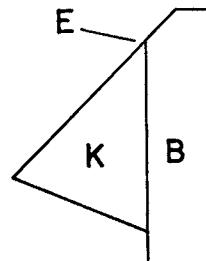


FIG. 18. Single crystal *K* backed by crystal *B* which gives mechanical support to the knife edge *E* during the lapping procedure.

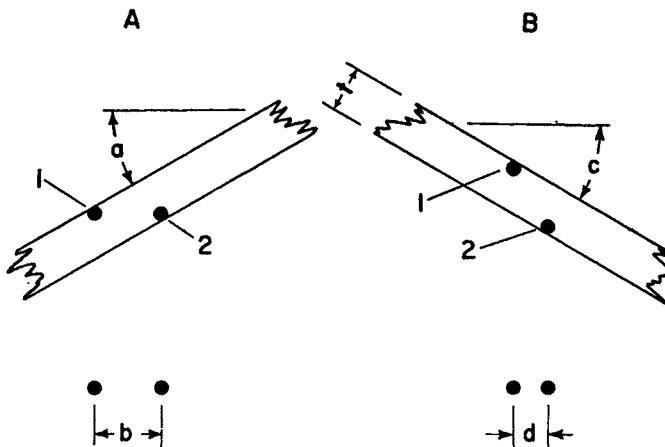
ultramicrotome based on the zero advance setting of a mechanical advance system. The microtome is first set at the smallest incremental advance movement, i.e., one ratchet tooth movement, and sections are cut. Without stopping the microtome it is then set at zero advance (no ratchet tooth movement) during which no sections or scrapings of material should be produced. After a certain time interval the microtome is again set at the previous positive incremental advance and the number of strokes of the specimen block in which no sections are cut are counted. If sections are cut or material scraped while the instrument is set at zero advance, some structural members are expanding or contracting and the true incremental advance is greater than desired. If, on the other hand, no sections are cut for a few strokes of the specimen block after it has been set for a positive incremental advance, expansions or contractions in the opposite sense are in progress and the true incremental advance is less than desired. Unfortunately this criterion is good only for instruments having discontinuous ratchet movements of a lead screw, such as the cantilever microtome (Bretschneider, 1949; Porter and Blum, 1953) and modified Spencer microtomes (Grey and Bieseley, 1953; Weinreb and Harman, 1954; Stryker, 1955) and for mechanical systems with clutch-connected continuously rotating lead screws such as that of Gettner and Ornstein (1953), and is not applicable to thermally advanced instruments. This criterion also suffers because knowledge of the total product is not positive but only inferred from the zero advance condition; it is possible that additional error is introduced with positive advance but not occurring at zero advance.

Fabergé (1949) has measured section thickness of a Carnauba wax section by a melting procedure. The section is placed on a glass slide and its area is measured in a light microscope. It is then placed on a microscopic glass fiber and slowly heated until it melts and draws into a sphere by surface tension. From a measurement of the diameter of this sphere or ellipsoid the volume and thence the section thickness can be calculated. While mathematically this method of measurement is acceptable, it is subject to grave doubts when it is considered that the area measurement may not be true due to wrinkles in the section or compression and crystal slippage. While the result would therefore give some indication of the section thickness, it would not give sufficient information of the true incremental advance "of the microtome" or its error.

Another method for thickness measurement is that of observing the length of shadow which the section makes on the supporting film (Sjöstrand, 1953a; Porter and Blum, 1953). A section is picked up on a collodion or Formvar supporting film, shadowed with a heavy metal, and placed in the electron microscope. Knowing the angle of metal evaporation and the length of shadow which the edge of the section makes on the supporting film, the

section thickness can easily be calculated. This criterion of section thickness is also open to doubt because while the method gives a true measurement of the thickness at the edge, it tells nothing of the thickness of the main body of the section. The section may easily have a wedge shape at the edges giving lower or higher values of thickness than the actual interior of the section.

A stereoscopic method (Williams and Kallman, 1955) measures section thickness at a particular point in the section. Some sharply delineated objects such as two small particles lying on opposite section surfaces are observed and their separation measured (see Fig. 19). The specimen holder is then rotated through a known stereo angle and their separation again measured. With a 60° stereo angle and with a measurement error of separation of not more than 50 \AA , the section thickness value will also contain no greater error than 50 \AA . Williams and Kallman find that thickness measurements by stereoscopy are about $\frac{1}{2}$ the value of measurements made by shadow length at the edge of the section, and suggest that embedding sublimation during exposure to the intense electron beam may account for the discrepancy. Error is introduced if the centers of the particles do not coincide with the surfaces of the section, especially if the particle size approaches the magnitude of the section thickness.



$$t = \frac{b}{\cos a (\tan a + \tan c)} - \frac{d}{\cos c (\tan a + \tan c)}$$

FIG. 19. Specimen positions for the stereoscopic measurement of section thickness. The image separation (b) and (d) are measured between two particles (1) and (2) on opposite surfaces of the section in the two stereo-positions A and B rotated through angles (a) and (c) from the normal specimen plane.

Another method for measuring the section thickness at a particular point uses an obliquely cut cylindrical tissue structure whose diameter is of the same order of magnitude as the section thickness. Three measurements of the cylindrical structure are made in the image of the electron microscope or on the photographic print (see Fig. 20): the short diameter (d) of the ellipse formed by the intersection of the cylinder with one cut surface of the section, the long diameter (b) of this ellipse, and the projected or image length (a) of the top side of the cylinder. The thickness (t) of the section at this point is then

$$t = \frac{ad}{(b^2 - d^2)^{\frac{1}{2}}}$$

An example of a biological cylindrical structure which may be used is a sperm tail or cilium of which the nine circumferential fibrils form a cylinder (see Fig. 21; also Hamilton *et al.*, 1952; Fawcett and Porter, 1954). This method gives information of the thickness of the section *as it was cut* on the ultramicrotome, if the diameter of the cylinder (d) as measured on the micrograph can be assumed to be the same as the diameter of the cylinder during sectioning. [If the compression from sectioning remains unrelieved

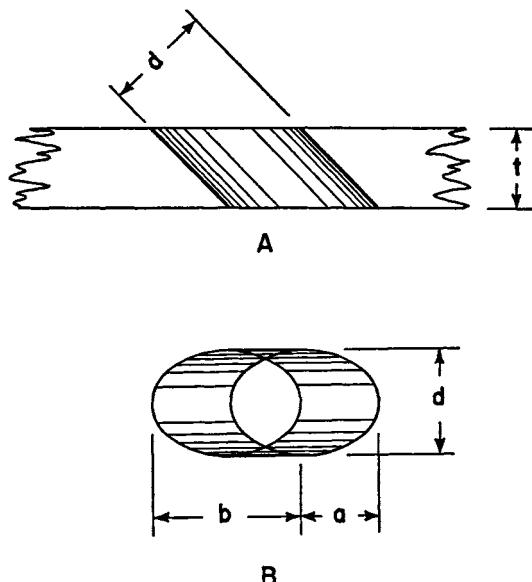


FIG. 20. Cylindrical tissue structure measurement of section thickness. A, Diagrammatic cross-section of tissue section of thickness (t) with cylindrical structure of diameter (d) passing through obliquely. B, Image or projected view of obliquely cut cylinder where (a) is the projected length of cylinder surface, (b) is the long diameter, and (d) is the short diameter of the ellipse of section.

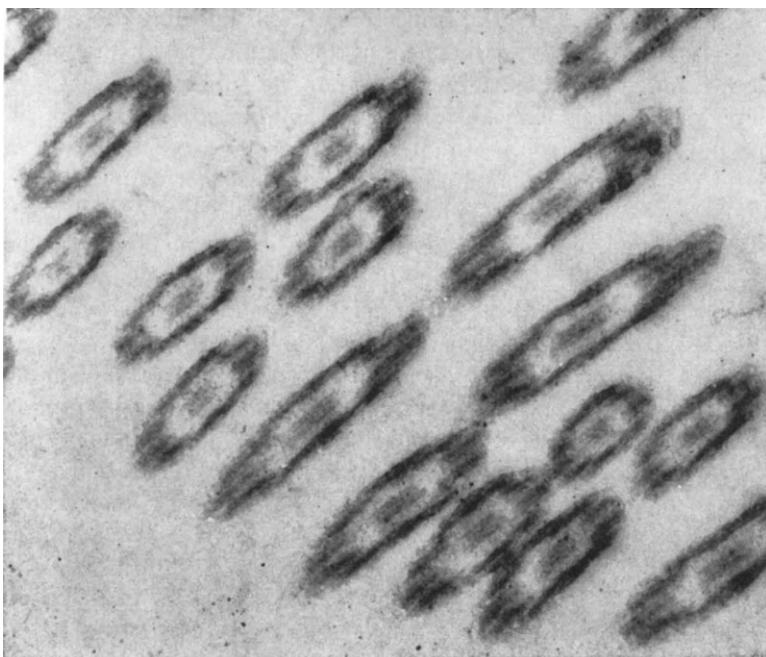


FIG. 21. Cilia from amphibian kidney (*Rana pipiens*) demonstrating thickness measurement by obliquely cut cylindrical structures, $\times 47,000$. Section thickness measures $600 \text{ \AA} \pm 10\%$. Compression, due to sectioning, was negligible; sections of spherical particles in adjacent fields (like those shown in Fig. 22A) appeared as almost perfect circles.

(see Section III,2,e), the diameter (d) would be reduced giving lower values for thickness.] The constancy of the diameter (d) would also be affected by slight lateral distortions produced by the embedding sublimation, but this error can be reduced to a negligible minimum by the use of carbon-evaporated supporting films (Watson, 1955); (see also Chap. 5 of this book.) On the other hand, if the cylinder becomes somewhat flattened as a result of sublimation, the value of the image length (a) would increase and give higher values for section thickness.

A method of thickness measurement not sensitive to slight lateral distortion is that of electron absorption measurements. Special polystyrene latex spheres of approximately 0.16μ diameter used as an internal mass reference are placed on the section and observed in a clear nontissue area of the polymethacrylate. Electron optical density measurements (Hall, 1951, 1953) are made on the polystyrene sphere, on an adjacent area of polymethacrylate, and on the supporting film without section. Assuming that the electron-scattering cross-sections of polystyrene and *n*-butyl polymethacrylate are essentially equal and vary only with their specific gravities

(approximately 1.06 and 1.05, respectively), and knowing the diameter of the polystyrene sphere, the thickness of the section can be computed. This method, however, is subject to error from the sublimation of polymethacrylate in the electron beam and from contamination buildup. On the other hand if the electron beam intensity is kept at a very low level during optical density measurements, irradiated areas will not materially increase in temperature and sublimation can be made negligible. Also if the specimen is placed in a specimen holder in which the specimen is maintained at -80°C , the net rate of contamination buildup may be reduced to zero (Leisegang, 1954). (See also Section III,3 of Chap. 5 in this volume.)

A quick judgment of section thickness may be made by observing the reflection of interference colors from them as they float on the trough (see Table IV). This has become popular in ultramicrotomy because it needs no additional preparation of section and a rough estimate value of thickness can be made immediately (Porter and Blum, 1953). The disadvantage of

TABLE IV
Interference Color vs. Section Thickness^a

Interference color	Section thickness in microns
Iron gray	0.013
"Lavender" gray	0.033
"Bluish" gray	0.053
Clearer gray	0.073
"Greenish" white	0.079
White	0.087
Yellowish white	0.090
Straw yellow	0.094
Light yellow	0.103
Bright yellow	0.111
Brownish yellow	0.144
Reddish orange	0.169
Red	0.180
Deep red	0.185
Purple	0.190
Indigo	0.198
Sky blue	0.223
Greenish blue	0.244
Light green	0.277
Yellow green	0.285
Yellow	0.305
Orange	0.317
Purplish red	0.370
Bluish violet	0.380

^a These values are calculated for reflected light at normal incidence for *n*-butyl polymethacrylate on water from data of Martin and Johnson (1951).

this method is that it is not accurate but highly subjective; in the range of section thickness of most interest to the ultramicrotome only the almost-imperceptible shades of gray greet the eye. Yet another doubt *may* arise from an increase in thickness of the polymethacrylate due to the imbibition of the acetone or alcohol of the trough liquid which releases the strain of compression. Preliminary measurements on this effect show that the per cent acetone absorbed decreases with decreasing section thickness and with decreasing acetone concentration.

The interference microscope may provide the most accurate means of thickness measurement at any and all points of a section (see Osterberg, Chap. 7, Vol. I; Barer, Chap. 2 in this book). Multiple-beam interferometry may also be employed, but if the technique used requires the section to be half silvered, the usefulness of the section for electron microscopy will be destroyed.

VI. Microscopic Resolution and Section Thickness

There are numerous considerations which usually come to mind when the question arises, "How thick should a section be cut for a particular purpose?" One of the more objective considerations is microscopic resolution.

Resolution in the light microscope depends not only on the numerical aperture and over-all correction of the objective lens, but on the optical properties of the path between the object plane and the objective lens and the inherent optical contrast of the specimen. Maximum resolution is achieved when the microscope is focused on the desired specimen plane without the superposition of additional specimen material above the object plane. Thus, if the detail of interest is at the top of a section rather than at its bottom, resolution will be maximum. If it is at the bottom, even though the short depth of field of high-aperture immersion lenses puts the upper planes of the specimen well out of focus, the scattering from these specimen regions (which will usually contain inhomogeneities with respect to both optical absorption and refractivity) can severely reduce contrast and resolution. For this reason, when maximum resolution is of paramount interest, specimens of the order of thickness of the depth of field of the objective are indicated. In ordinary transmitted light microscopy, this apparently severe restriction can be grossly violated with surprisingly little loss in resolution. In the phase microscope, this is not the case. Here there are two imaging systems which are critical; the object-objective-image of the object system referred to above, and the condenser annulus-condenser-objective-image of the condenser annulus in the back focal plane of the objective. Specimen inhomogeneities above the object plane cause image deterioration in both systems, and specimen inhomogeneities below the ob-

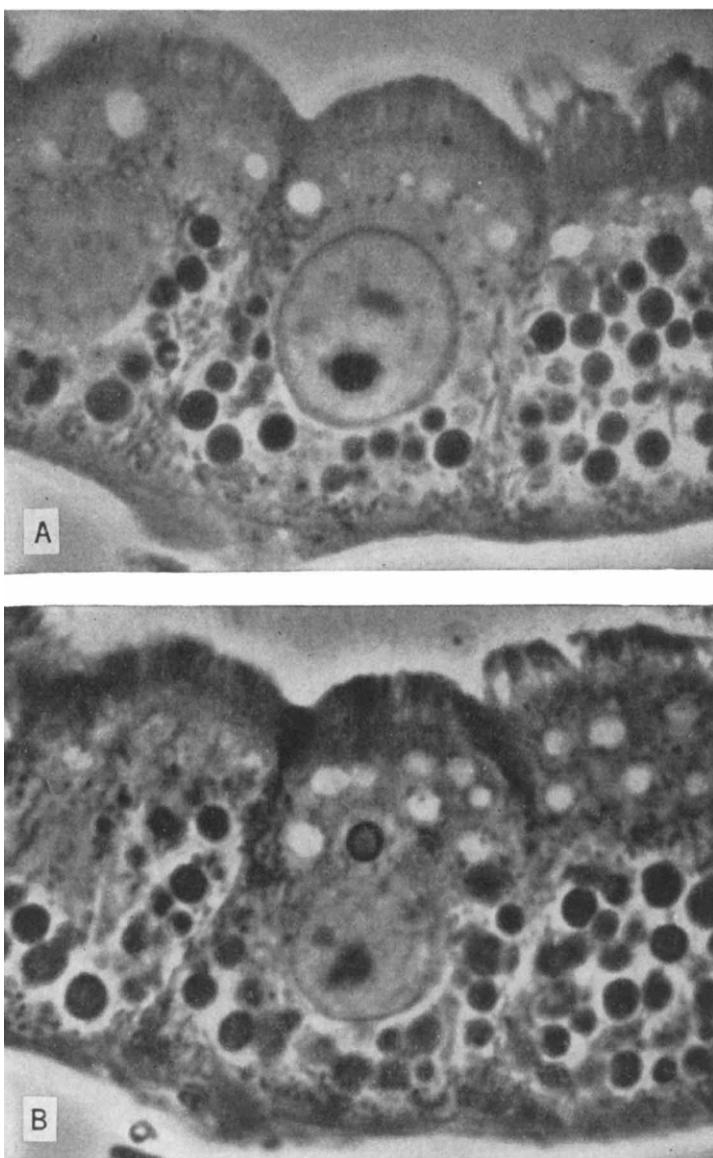


FIG. 22. Phase micrographs of serial sections of amphibian kidney cells (*Rana pipiens*). Magnification: $\times 2000$. A, Section 0.5μ in thickness; B, section 3μ in thickness. Note in the latter the partially obscured nuclear membrane and relative absence of clearly resolved mitochondria between the large dense cytoplasmic granules of the $3\text{-}\mu$ section.

ject plane cause deterioration in the imaging of the condenser annulus. The resolution and contrast in a phase microscope image depend heavily on good imaging in *both* of these systems. Section thickness in the 1 μ range is, therefore, mandatory for maximum resolution with the phase microscope (Ornstein and Pollister, 1952). Figure 22 demonstrates this dependence of resolution and contrast on thickness.

Adequate contrast is usually not limiting when the full resolving power of the best light microscope objectives is utilized. Properly selected phase objectives and proper choice of refractive index of the mounting medium for the section brings the performance of the phase objective to its limit of resolution (Ornstein and Pollister, 1952). The contrast of an object which absorbs light can be described simply as the difference between the optical densities of the object and its surroundings.

$$E_{\text{object}} - E_{\text{surround.}} = \text{Contrast}$$

Where E , the extinction (optical density) of an area,

$$E = kcd$$

is defined as

$$E = \log_{10} \frac{I_0}{I_t}$$

where I_0 is the incident intensity and I_t is the intensity transmitted by this area; k is the extinction coefficient (a constant) which depends on the particular chemical composition of the area and the wavelength of the incident light; c is the concentration of the chemical substance; and d is the thickness of the area. The presence of naturally absorbing substances or specific "stains" with extinction coefficients thousands or even hundreds of thousands of times that of their surroundings make it quite feasible to approach the theoretical limit of the light microscope in biological sections.

The problem of resolution in electron microscopy is considerably more involved. An electron can lose energy in passing through a specimen, and this loss results in a change in the associated de Broglie wavelength of the electron. Since electron microscopes have monochromatic lenses (without corrections for chromatic aberrations) *this change in wavelength can set the limit of the resolution obtainable for a particular thickness of section*. The chromatic limit of resolution, ℓ_c , is described by the following equation: (Zworykin *et al.*, 1945)

$$\ell_c = \frac{K \Delta v f \alpha}{v}$$

where K is a constant approximately equal to one, Δv is the change in volt-

age of an electron (in this case, on passing through a specimen), v is the accelerating voltage of the microscope gun, f is the focal length of the objective and α is the numerical aperture of the objective. If Δv is taken as the most probable loss in voltage suffered by an electron on passing through a section thickness of d Ångstroms and density, ρ , in grams per cubic centimeter, then,

$$\Delta v \simeq \frac{10^3 d \rho}{v}$$

and

$$d \simeq \frac{10^{-3} v^2 \ell_c}{K f \alpha \rho}$$

(Zworykin *et al.*, 1945) where ℓ_c and f are expressed in Ångstrom units.

This equation assumes "opaque" specimen detail on the objective side of the section. Whereas in the light microscope one relies on the enormous differences in extinction coefficient, k , to provide adequate differential contrast between structures and their surroundings, in ordinary transmission electron microscopy the microscopist must rely on differences in density.

TABLE V
Maximum Section Thickness for 20 Å Resolution for Various Electron Microscope Objective Lenses

Objective lens	K^a	f in cm	v in kv	α in radians $\times 10^{-3}$	d in Ångstroms ^b
RCA EMU-2 "Standard"	0.85	0.28	50	9 ^d	200
RCA Long focus ^c	0.9	1.2	50	2 ^e	200
RCA EMU-3	0.85	0.39	50	6.4 ^e	200
			100	6.4 ^e	800
Philips EM-100 6 mm bore	0.85	0.48	50	10 ^f	100
			100	10 ^f	400
			100	5 ^g	800
Philips EM-100A and EM-100B	0.85	0.17	50	10 ^h	300
			100	10 ^h	1200
Siemens Elmiskop 1	0.85	0.27	50	9.3 ^d	200
			100	9.3 ^d	800

^a From Liebman (1954).

^b For $\rho = 1.2$ and for $\ell_c = 20$ Å.

^c From Hillier (1952).

^d 50-μ aperture in center of lens.

^e 50-μ aperture in back focal plane.

^f 50-μ aperture

^g 25-μ aperture.

^h 20-μ aperture.

The following equation (modified from Zworykin *et al.*, 1945) describes the dependence of contrast on $\Delta\rho$, the "effective density."

$$\begin{aligned}\text{Contrast} &= E_{\text{object}} - E_{\text{surround.}} \\ &= \frac{2d\rho_{\text{object}}}{v\alpha^{\frac{1}{2}}} - \frac{2d\rho_{\text{surround.}}}{v\alpha^{\frac{1}{2}}} \\ &= \frac{2d\Delta\rho}{v\alpha^{\frac{1}{2}}}\end{aligned}$$

This equation is accurate to about $\pm 10\%$ for most organic compounds from 50 to 100 *kv* (see also Hall, 1954). $\Delta\rho$ has a maximum possible value of about 22 g/cm³. (For this reason "stoichiometric staining," as the modern histochemist knows it, is not at all promising for electron microscopy (Ornstein, 1952).

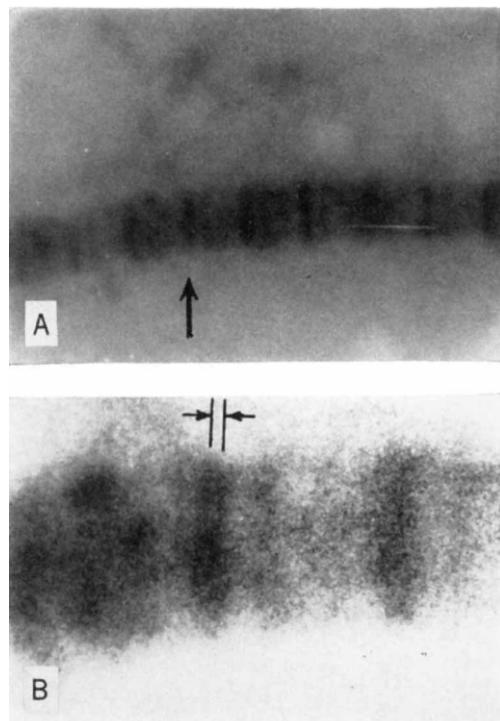


FIG. 23. Electron micrographs of an extracellular fiber found in connective tissue cells of the tadpole (*Rana clamitans*) obtained from a 0.10- μ section using a Philips EM-100A objective lens at 100 *kv* with a 20- μ aperture. A, $\times 320,000$; B, same fiber $\times 960,000$. The fine period marked by arrows equals approximately 20 Å. (See also Ornstein, 1956).

With an aberration-free objective lens of resolution considerably better than 20 Å, a particle 20 Å in diameter in the upper surface of a section with $\Delta\rho = 1 \text{ g/cm}^2$ would just be detectable (show a difference in optical density of 0.05 from the background density of the section) on a negative developed to a contrast of $\gamma = 6$, (for instance Eastman spectroscopic 548-0 emulsion developed in D 19 for 5 min at 68°C) if the accelerating voltage is 100 kv and $\alpha = 3 \times 10^{-3}$. The effective density or relative thickness of a 20-Å specimen detail must be considerably greater with real objectives since spherical aberration will degrade contrast (Picht, 1925; Grey, 1952). Differential sublimation of the embedding medium and part of the specimen as well as differential extraction of specimen substance during or after fixation aid resolution by increasing $\Delta\rho$. By these means the "opacity" required by the resolution formula may be approached.

Table V lists some properties of a few electron microscope objectives and the *maximum section thicknesses* which could yield 20 Å resolution under a few different operating conditions. Figure 23 shows an electron micrograph which demonstrates 20 Å resolution on a rather thick section.

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