Common Problems and Mistakes

by H. Sitte

MICROSYSTEMS

ULTRAMICROTOMY - COMMON PROBLEMS AND MISTAKES[+)]

by H. SITTE [x)]

Summary

Unfortunately, nowadays it seems to be common usage to publish good results exclusively and to be bashfully quiet about all problems and mistakes, which are often connected with new methods and instrumentation. Therefore, many problems usually arise for the beginner or the less experienced scientist, since expectations do not agree with reality. Many laboratory beliefs are based on this uncertainty. In many cases these beliefs are wrong, but cannot be refuted, since a lot of experience and instrumentation is needed to do this. With the presented paper, an attempt is made to discuss the main problems and failures within ultramicrotomy on a simple level especially directed to routine work. The paper, therefore, includes mainly the common sectioning artifacts (uneven section thicknesses, as well as vibrations, knife marks and wrinkles in the sections) and the general problem of section contamination, which deserves much more attention in electron microscopy than in light microscopy. The cited literature may serve as background information. The excellent handbook of FAWCETT is still recommended today as a standard for perfect routine ultramicrotomy.

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1. Preliminary Remarks

With suitable operation, modern ultramicrotomes permit the preparation of regular, satisfactory sections in the thickness region around 70 nm (= 700 Å, corresponding to "silver" interference in the reflection of the sections floating on a liquid) without using any special expedients. The prerequisites for this are:

(1) satisfactorily embedded, easily cuttable specimen
(2) correct shape ("trimming")
(3) satisfactory knife-edge
(4) appropriate cutting method

Since good sections in the collecting boat do not necessarily assure good images in the electron microscope, the following additional prerequisites should be fulfilled:

(5) elimination of the section compression through spreading out
(6) appropriate collection of the sections on the grids
(7) careful staining of the sections
(8) absolute cleanliness of all instruments and solutions used (contamination)

Accordingly, most of the possible faults and errors frequently observed in practice in the preparation of ultrathin sections are attributable to an erroneous method in one of the regions listed above. If the method corresponds to all of the criteria mentioned above, failures could be due to two additional circumstances:

(9) specimens difficult to cut (for example, inhomogeneous specimens with hard inclusions)
(10) faulty functioning or erroneously set up ultramicrotome

Difficultly cuttable specimens require special expedients - faulty functioning of the instrument, as a rule, calls for the participation of the authorized service organization. Within the scope of this lecture, it is hardly possible to cover completely, even approximately, all of the questions arising in the regions (9)

and (10). However, experience has taught us that extremely difficult to cut specimens are as rare in the biological-medical field as faulty functioning of ultramicrotomes, which, on the whole, have turned out to be much more robust in routine practice than was at first expected. Accordingly, the subject of this lecture is predominantly the standard ultramicrotomy of biological-medical specimens, in which the material is dehydrated after the customary glutaraldehyde-OsO_4 fixation and embedded in epoxide, cut about 70 nm thick in areas between 0.5 and 1 mm^2 and, after the cutting, stained on the grid with uranyl acetate and lead citrate according to REYNOLDS. These preparations and requirements cover the predominant number of studies that are carried out today in the field of ultramicrotomy.

2. Goals and Problems of Routine Ultramicrotomy

Viewed as the goal of this standard section preparation outlined above is that one can introduce regular sections in the electron microscope, which, aside from a uniform thickness, are free of vibrations, knife marks and other cutting artifacts, as well as free of impurities, so that every place of every section can be photographed and, therefore, every morphologically interesting part of the section can also be published and, if necessary, evaluated morphometrically. With the use of a suitable method and with thoroughly clean work the practitioner can meet these requirements without great effort. On the other hand, beginners or insufficiently instructed employees are frequently confronted with problems which result not only from their inadequate knowledge of the situation, but frequently also from laboratory traditions and incorrect advice. Since many steps in the course of preparation are not capable of a satisfactory scientific explanation or checking, such house recipies and advice often lie more in the region of faith than of scientifically and empirically based facts.

The situation is complicated by the fact that many phenomena with identical manifestations can have entirely different causes. Vibrations (waves in the section), for example, can be produced by an incorrect embedding, or by a faulty trimming of the specimen, as through a worn or too pointed or an erroneously

adjusted knife. However, they can also be caused by an insufficient clamping of the knife or the specimen or by external influences (building vibrations). Finally, a defective or erroneously set up ultramicrotome can represent the source of the error. Without an adequate knowledge of the situation and years of practice, it is practically impossible to differentiate clearly between the different causes.

An additional problem results from the partially Babylonian language confusion, which often makes it difficult for the expert to present a clear diagnosis. The vibrations (synonym: waves, undulations, chatters, chatter marks) give a good example of this. The terms mentioned - mostly without an accurate definition of terms - cover a multitude of different phenomena, which are produced by oscillations in the frequency range between about 10 and 10,000 Hz (1 Hz = 1 Hertz = 1 cycle per second). Without a determination and statement of this frequency, a diagnosis is impossible. On the contrary, if the frequency range is known, in most cases a clear differentiation between building vibrations, instrument errors or methodological causes can be made and the fault can often be eliminated rapidly. The same applies to irregular section thicknesses. In the following (Sections 3 and 4), therefore, an attempt will be made to define the terms "vibrations" and "nonuniform section thicknesses" more clearly and differentiate them.

Finally, the individual, typical disturbances in the course of making the preparation will be discussed, whereby the numbers in the context of the first section on page 2 will be used (Section 5 with items 5.1 to 5.10). Problems which result in the regions of ultramicrotomy outside the normal routine, will be outlined in a separate section (Section 6). Finally, a check list should facilitate the finding of erros (Section 7).

3. Vibrations

One speaks of vibrations (waves, undulations, chatters, chatter marks) in all cases in which the section displays periodic wavy changes in thickness, whereby the alternating thicker and thinner zones run parallel to the knife edge and perpendicular to the knife marks (compare Section 1). Vibrations are produced

by periodic oscillations of the building or the entire ultramicrotome, or components of the ultramicrotome, the knife and the specimen or in the microarea of the knife edge or the surface of the specimen. Thereby the frequencies increase with decreasing vibrating masses. Whereas the inherent frequency of buildings as well as ultramicrotomes, with appropriately damped set up, lie in the region between 5 and 20 Hz, parts of the ultramicrotome can reach frequencies of up to about 500 Hz, knife or specimen frequencies can reach up to about 1,000 Hz and vibrations in microareas in extreme cases can reach frequencies above 10,000 Hz, since the vibrating masses decrease in the sequence "total apparatus (50 to 100 kg) → instrument parts (0.1 to 10 kg) → knife/specimen (1 to 20 g) → microareas (order of magnitude in the mg region)". Therefore, on the sections floating on the liquid surface of the collecting boat only waves can be perceived in the stereomicroscope, which are caused by vibrations of the building, the microtome, or certain ultramicrotome parts (entire knife holder or entire specimen holder). In contrast to the stereomicroscope, which operates in the weak magnification range up to about 50 : 1, in the light microscope vibrations of higher frequencies up to about 1,000 Hz can be identified. High-frequency vibrations in the region above 1,000 Hz can first be made visible in the electron microscope. In contrast to the vibrations in the frequency region below 1,000 Hz ("low-frequency chatter"), which are due to the apparatus, in the case of frequencies above 1,000 Hz ("high-frequency chatter") faulty functioning of the apparatus can be completely excluded: They are always attributable to methodological errors (poor embeddings, erroneously selected cutting parameters; compare Sections 5.1 and 5.4). Frequently, different vibrations are observed alongside one another - for example, high-frequency chatter in the electron microscope alongside lower-frequency chatters, which were already visible in the stereomicroscope or the light microscope. Likewise, low-frequency chatters in the frequency region around 100 Hz, under stronger magnifications, often display a superimposition by higher frequency beats. In general, it may be stated that low-frequency chatter, due to the large distances between the individual wave peaks, in comparison with the high-frequency chatters, under strong electron-optical magnifications, display no noteworthy disturbances, since, under magnifications greater than 5,000 : 1, an impression of a completely uniform section thickness is already obtained.

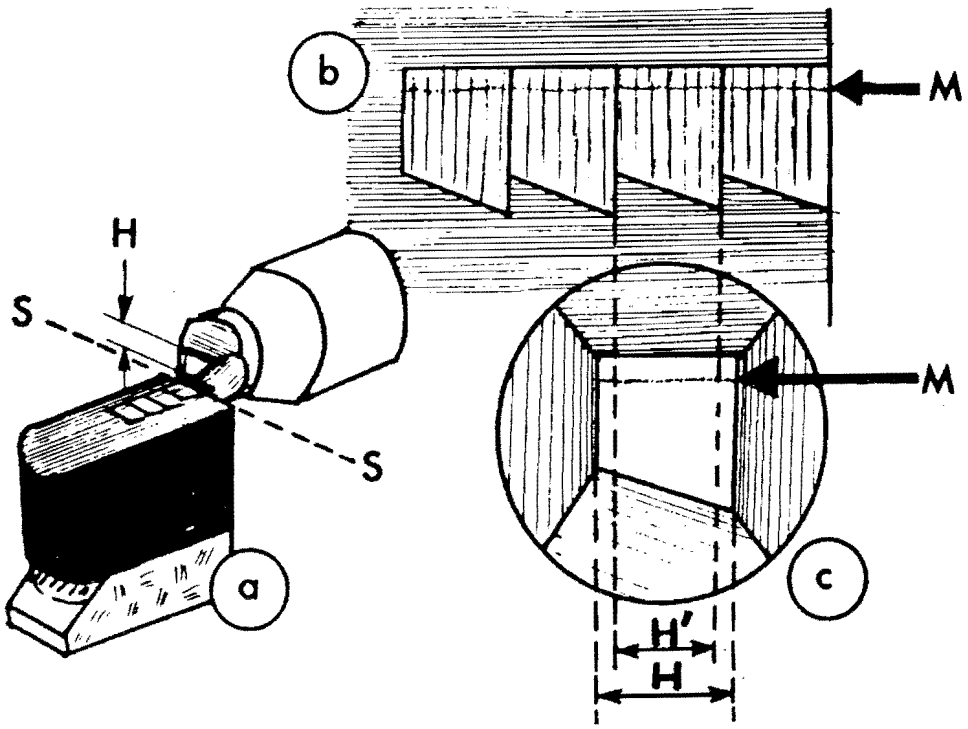

Figure 1 Vibrations and compression in the section: (a) Ribbon of sections on the water surface in the collecting boat, which is fastened at the glass knife. - (b) Ribbon of sections in top view. The waves run parallel to the edge SS of the knife and perpendicular to the knife marks M. - (c) As a consequence of the compression, the height H' of the sections does not correspond to the height H of the surface being cut on the block (H>H'). In this connection see also Fig. 8.

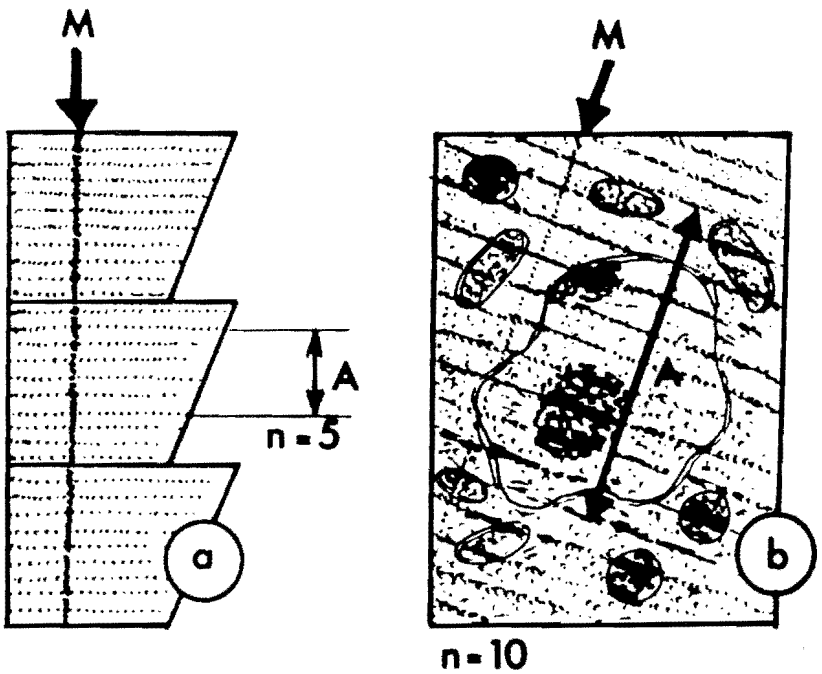

Figure 2 Determination of the frequency ν of vibrations: Sections are prepared with accurately known cutting speed v_S (for example, 2 mm/sec). In the picture, one determines the distance A between n waves (for example n = 5 or n = 10) in spread out sections. The formula for calculating reads: $\nu = n : v_S / A$. Hz(= vibrations per second). A poor spreading makes the measurement erroneous and yields too high values. It can be corrected according to Fig. 1 by the factor H'/H, which is determined by comparing the height dimension H on the block and H' on the section. The corrected value results according to the formula: $\nu = n \cdot v_S \cdot H' / A \cdot H$ Hz. The schematic figures show low-frequency vibrations (a), which can be measured with the light microscope, as well as high frequency vibrations (b), which can be measured from the electron micrograph.

For the reasons mentioned, a calculation of the frequency ν in the sections is of decisive importance for differentiating the different phenomena. It is carried out, according to Fig. 2, on sections, which where produced with an accurately known cutting speed v_S and were satisfactorily spread out according to the given formula (Fig.2). The irreversible compression remaining after the spreading out, falsifies these ν-values but slighly: As a rule, they are only about 5 to a maximum of 20 % too high and, therewith, give the order of magnitude of the frequency with sufficient accuracy.

4. Irregular Section Thicknesses

Just as in the case of vibrations, irregular section thicknesses can have very different causes and can appear in very different manifestations. If the periodic change in thickness in vibrated sections is disregarded, according to Fig. 3, essentially three phenomena can clearly be separated from one another:

(a) Different thicknesses from section to section with uniform thicknesses within the individual sections,

(b) zones of deviating section thickness parallel to the knife edge SS, as well as

(c) zones of deviating section thickness, which do not run parallel to the knife edge, but follow a specimen structure.

Naturally, the phenomena according to (a), (b) and (c) can overlap, like the different types of vibration.

As a rule, an irregular section sequence with a uniform section thickness within the individual sections (Fig. 3 a) is attributable to manipulations and external influences on the ultramicrotome (compare Section 5.4), whereas zones of different thickness parallel to the knife edge (Fig. 3 b) are attributable to individual impulses during the taking of the section concerned. Here, the same phenomenon takes place in the subsequent section in the "negative" - a zone of increased thickness is followed in the subsequent section in a zone of reduced thickness. Zones of variable thickness running irregularly within a section (Fig. 3 c) are

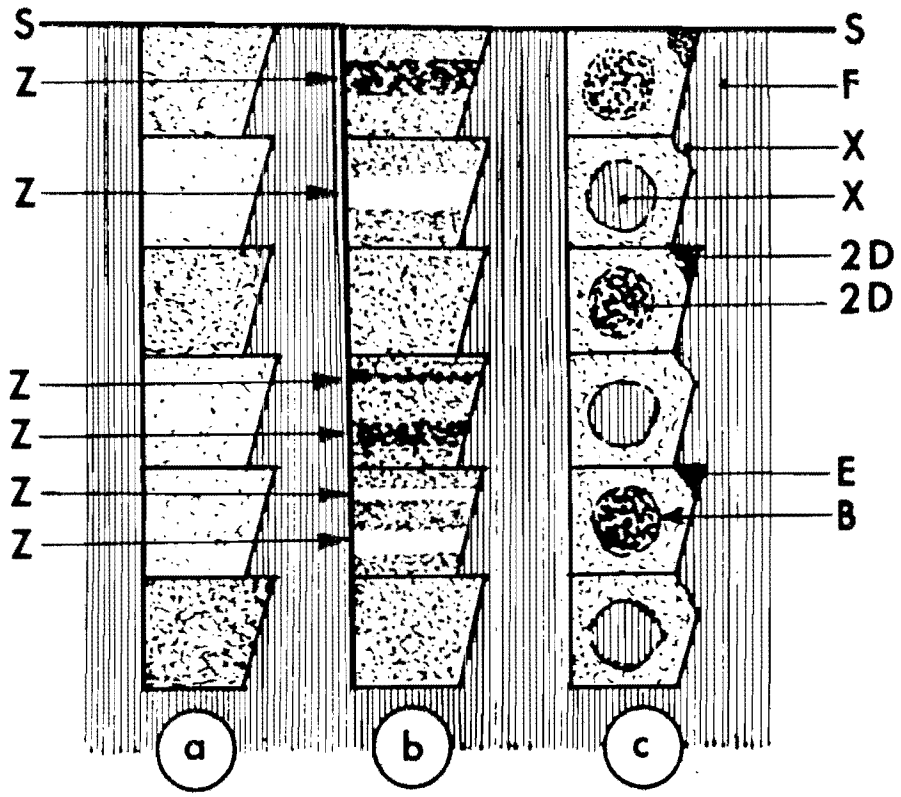

Figure 3 Irregularities of the section thicknesses. The ribbons of sections on the liquid surface F of the collecting boat are illustrated analogous to Fig. 1 b: (a) Variable section sequence with uniform thickness within the individual sections. - (b) Zones Z of variable thickness in the individual sections, which run parallel to the knife edge SS. - (c) Regions of different thickness corresponding to structures with different cutting consistency, for example, blood vessel B or free embedding medium E. Here, the corresponding parts often are missing (X) and then appear with double thickness (2 D) in the subsequent section. In this connection compare Figs. 4 a and b.

caused by zones of variable cutting consistency in the specimen. Thus, free embedding medium mostly displays a completely different cutting consistency than an enclosed object. For example, if a larger blood vessel is located in the cutting area or at the object edge there is a zone with free embedding medium, such irregularities can hardly be avoided with the use of glass knives. Here, only the diamond knife provides a remedy, which, as a rule, cuts the inhomogeneous preparation smoothly and without problems. In contrast to the types (a) and (b) according to Fig. 3, the type (c) is never due to faults of the ultramicrotome, since an ultramicrotome mechanics can never cut clearly delineated thicker regions directly adjacent to thinner ones. Also in the types (a) and (b), faulty functioning of the ultramicrotome mechanics, is far rarer than operating errors and external influences.

5. Frequent Problems and Errors in the Section Preparation

5.0 Arrangement: As already established above, it is difficult to refer the individual phenomena to the various possible causes. Therefore, based on the list on page 2, the problems appearing frequently in the individual steps of the course of preparation (5.1 to 5.8) and those occurring in the individual steps, will be discussed in the following. In two subsequent sections (5.9 and 5.10) instructions will be given for objects difficult to cut and faulty functioning or faulty set up of ultramicrotomes. The stepwise treatment of possible disturbing phenomena gives an approximately complete survey of the problems involved in the standard preparation. This survey is supplemented for the individual cases of disturbance by a check list, which refers to the most essential causes (Checklist is at end of paper).

5.1 Embedding and Specimen: Soft animal or embryonic plant tissues without dense cell-wall structures based on chitin or cellulose, as well as without hard enclosures or a high content of lipids and fats can be fixed, dehydrated and embedded in block dimensions up to 1 x 1 x 1 mm^3 (however, compare Section 5.9). After satisfactory fixation, dehydration, monomer impregnation and polymerization, the consistency of the blocks should be hard and not soft and elastic, the free plastic surface should not be tacky and dull, but smooth and shiny. The specimen should be fixed thoroughly in its interior and blackened by osmium. If this is not the case in larger specimen blocks, a satisfactory through hardening of the embedding medium in the unblackened central zones of the blocks should be checked. If this center is soft and tacky or not homogeneously impregnated (crumbling out of granular material), the block should be discarded, since such blocks cannot be cut satisfactorily even in the outer zones.

Problems result primarily through insufficient fixation, when too little fixing agent is used (rule: 1 ml of fixing agent per block) and the fixing agent is not moved continuously during the fixation (build up of gradients... insufficient exchange through the surfaces). The same is true for the dehydration, especially in the last stage. For this, it is recommended to open really waterfree analytical grade reagents in original bottles (MERCK or

similar products) first for the final state and using them freshly (using up for initial stages - no longer suitable for the final stage after opening and longer standing). A direct transfer of absolute alcohol in the epoxide monomer mixture, as a result of the slow exchange, frequently leads to poor blocks (Remedy: Propylene oxide intermediate stages). On the other hand, one can go from absolute acetone directly to the epoxide monomer. The epoxide charge must contain all components from the start, including the accelerators (polymerization catalysts, activators, etc.), since these components diffuse in only inadequately later. Great importance is given to the correct composition and homogeneous mixing of the monomer charge (weighing out small amounts, mixing with mixer, agitator or screw), since deviations of the standard mixture sometimes produce marked shiftings of the section consistency. Here, too, a correct impregnation can be achieved only through a continuous convection (Optimum: Turntable or automatic embedding - as a replacement: Frequent shaking). Of decisive importance is the exclusion of moisture in the final stages (Transferring of the specimens in fresh monomer in well predried containers or capsules). Frequently, the cutting consistency can be improved by dissolving of the capsules or molds and continued thermal treatment in the incubator.

5.2 Initial Cutting (Trimming): Many problems in cutting can be eliminated by a correct intitial trimming. In all nonhomogeneous tissues, which contain cavaties (blood vessels, alveolas, intercellular spaces) firstly the production and inspection of a well coloured semithin section is recommended. Based on this inspection in the light microscope, if possible, all cavaties and edge portions (see Fig. 4 a), which contain predominantly or exclusively free embedding medium, should be completely removed, as well as hard or difficulty cuttable inclusions (fat cells, collagen). If interfaces are of interest, the extent of the free embedding medium should be kept as small as possible (compare Fig. 4 b). In this manner irregularities according to Fig. 3 c are avoided or prevented. Normally, the section area should not substantially exceed 1 x 1 mm^2 and trapezoidal shapes (comp. Fig. 4 c), contrary to a frequently presented opinion, should first strike the knife blade with the narrow edge K - not with the long edge L - since the shock on striking the blade is propertional to the width of the edge.

Continuous ribbons of sections are obtained with sufficient contact between the successive sections. Therefore, it is recommended to align the upper and lower block edges exactly parallel to one another and both edges exactly parallel to the knife edge. With respect to the cohesion of the sections in the ribbon, rectangular initial sections are arranged according to Fig. 4 d. An assignment of the structures in the electron microscope is facilitated by a cut of corner (likewise, comp. Fig. 4 d, arrow).

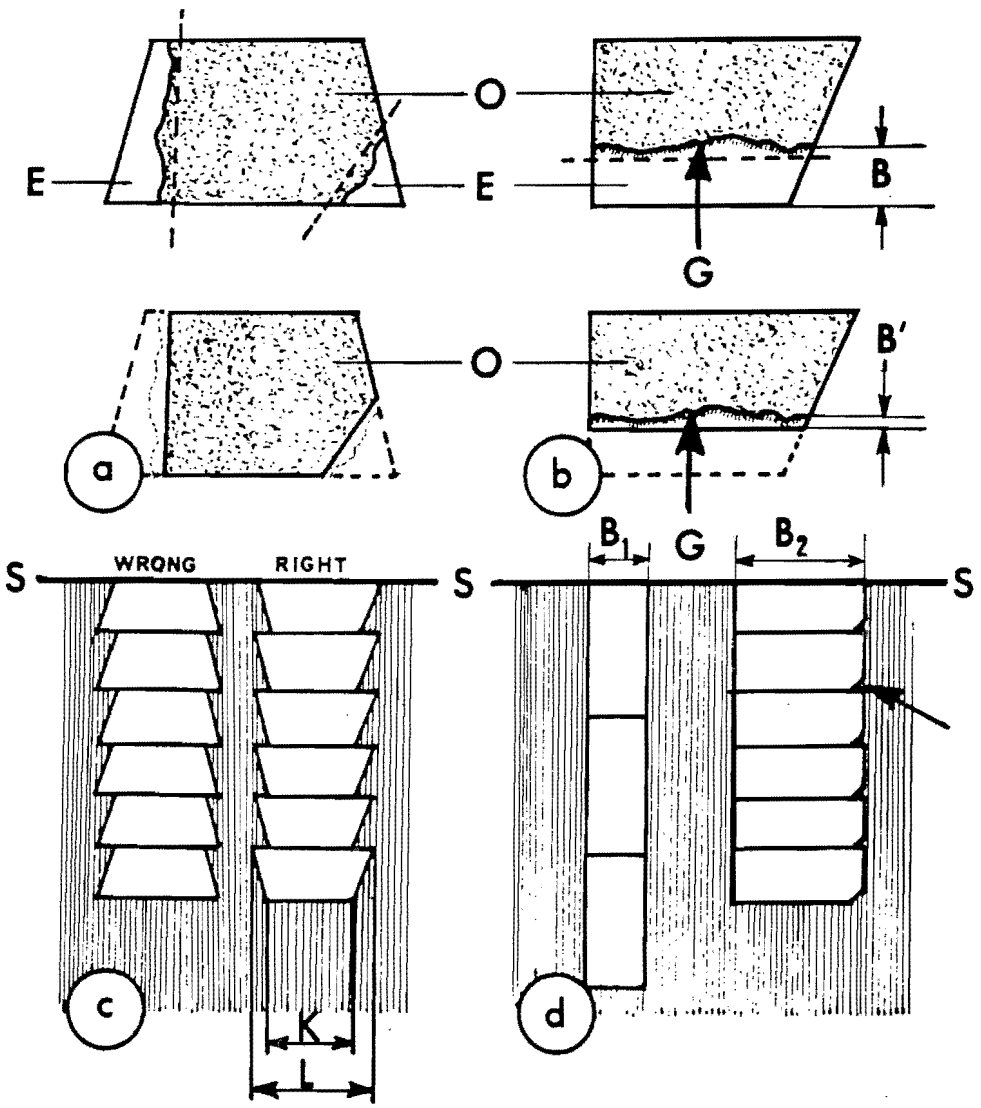

Figure 4 Trimming and orientation of section surfaces: (a) Free embedding medium E without specimen O is generally to be removed. - (b) If the free interface G is to be examined, the width B of the adjacent specimen-free zone E should be shortened to the minimum B'. - (c) Trapezoidal shapes or irregular rectangles should always first contact the blade SS with the shorter edge K, not with the longer edge L; compare Fig. 11 b. - (d) The cohesion of the individual sections in the ribbon of sections is determined by the width B and the linearity of the contact zone. Therefore, in the normal case, the setting on the left is erroneous (B_1), the setting on the right correct (B_2), however, compare Fig. 11 a. The removal of a corner (arrow) facilitates determination of the position in the electron microscope.

5.3 Knife-edge: Very controversial views exist regarding the glass knife-edges. This is true both with respect to the usuable region of the edge and with respect to stability on storage and resistance of glass edges after the break. Even if the glass knives are produced by the generally customary manner today with a knife maker, from the glass (strips or plates) specially selected for this and supplied by the manufacturer of the instrument concerned, there still exist numerous possibilities for faults. Such faults act catastrophically because satisfactory sections can be produced only with absolutely satisfactory knife edges. Decisive is the use of absolutely clean glass strips or plates free of fingerprints: In this connection the cleaning is not carried out with organic solvents, but exclusively with water and soap (any toilet and terpentine soap). It is rinsed well with water (Glass must wet satisfactorily) and dried with a clean linen cloth, household or cosmetic paper. Thereafter, the glass should only be touched with absolutedly clean, dry hands (Best with cotton gloves like those used in cathode exchange on the electron microscope) and in no case should the side edges be touched. The path of the scratch for breaking the glass square into two triangular knives (Fig. 5 a) is to be adjusted so that the edge of the knife runs linearly and the two longitudinal borders of the area F at the base of the knife (Fig. 5 b) run parallel at a minimum distance ($h < 1$ mm). If this condition is not fulfilled (Fig. 5 c), then the edge S'S', as a rule, is highly curved and, therefore, not very suitable for the preparation of sections.

In the normal case, 45° is used as the scratching angle α (Fig. 7 e). To be warned against are scratch angles of less than 40°, which can be set directly with the corresponding instruments, but which, as a rule, bring only disadvantages. In this case the effective facet angle is hardly reduced, since the break runs curved in the last section. Finally, the stability is missing that is neccessary with the customary clearance angles ε (comp. Fig. 7 e) for the taking off of section areas $> 0.5 \times 0.5$ mm^2. Therefore, with a $\alpha < 40^{\circ}$, one often obtains vibrated sections.

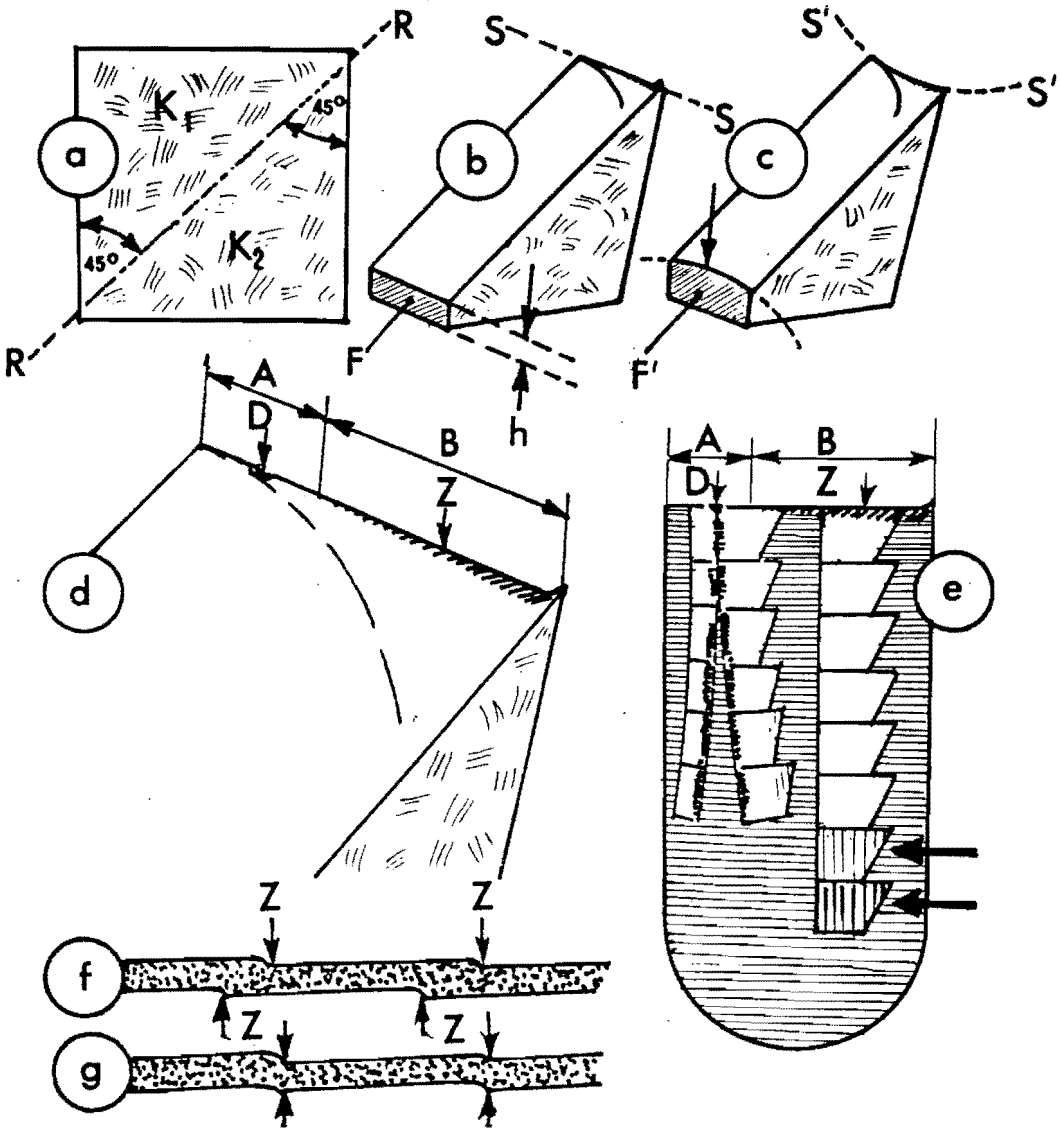

Figure 5 Preparation and handling of glass knives: (a) Standard glass knives K_1 and K_2 are prepared from glass squares, which are scratched approximately diagonally (path of scratch = RR; scratching angle α = 45°) and broken. - (b) The path of the scratch RR should be adjusted to that the area F forms a very narrow rectangle with $h <$ 1 mm. In this case the greatest portion of the knife-edge SS runs practically linearly. - (c) A poorly adjusted path of scratching RR leads to a considerably larger irregular area F′ and to highly curved cutting edges S′S′. - (d) A portion of the glass knife edge always displays saw teeth Z. The ratio A : B depends on the damping system of the knife maker. On the other hand, real knife defects D result practically exclusively through contact with hard objects. - (e) The relief of the saw teeth is seen only in the first sections of a ribbon and, with normal section thicknesses around 70 nm, usually produces no kind of disturbance (right). On the other hand, defects D (left) reduce the section quality considerably and often lead to separation of the sections. - (f) Disturbing marks through saw teeth Z rarely appear in the section image, when the relief on the top side of the section is displaced toward the relief on the bottom side (arrows!). If the two reliefs coincide (g), no disturbance of any kind appears in sections with average thickness through the saw teeth. Compare the text.

Since everyone endeavours to avoid knife marks (knife grooves) in the sections, the so-called "saw teeth" (Figs. 5 d to g) stimulate particular suspicion. They result in the breaking of the glass by a vibration, which, indeed, is damped by special elements, but cannot be suppressed completely, however. In the case of "silvery" sections, however, in the thickness region around 70 nm, the effect of these teeth is frequently overestimated. When one cuts in the saw-tooth region B (Fig.5e), the profile of these teeth is visible only in the first or second section. The following sections are entirely free of faults. The reason: The shallow relief of the individual saw teeth that is transferred from the knife edge to the section surfaces, is displaced somewhat by a resetting of the block with every interruption and resumption of the cutting on the top and bottom of the section (Fig. 5 f). After a few sections the reliefs of both surfaces are identically located again, so that the section displays the same thickness at all places (Fig. 5 g). Since the cutting edge in the saw-tooth region is not dull or defective, but sharp, no further disturbance then appears. In spite of this, one avoids the saw-tooth region on the first sections of the ribbon (Figs. 5 e and f), if one cuts only small areas.

Much more disturbing than the saw teeth are real knife defects (D; Fig. 5 d), which result through contact of the knife edge with a hard object (forceps, glass needle, fingers) or by dust grains, which are pressed by the block against the cutting edge. Similar defects result from hard inclusions of the specimen block. They produce notches which split the sections along the entire length and force termination of the cutting series. Knife marks of this kind differ substantially from the relief of the saw teeth in that they appear singly or at irregular intervals and split the section. On the other hand, saw teeth are recognized in that they produce fine streaks at approximately regular intervals, but which extend over broad regions of the section.

Finally, still two references to the durability of glass knives, which are often rated erroneously. The storability of glass knives is practically unlimited, if the knives are kept clean (protected against fats and dust). According to our own tests, under these conditions knives after storage

for several months cut as well as freshly made knives. Therefore, it is expedient to make the requirements for a month at one time. On the other hand, the "service life" of the glass knives is very small. Ordinarily, one should not produce more sections with one glass knife than corresponds to a ribbon of sections with a total length of about 15 mm. This includes the initial sections which are necessary to produce a smooth, complete section surface at the beginning of the cutting series. The number of sections that can be taken off with one glass knife edge, accordingly, depends on the height H (Fig. 1) of the initial cutting surface and the individual sections: If H = 1 mm, a total of about 15 sections can be taken off. With H = 0.2 mm there can be 100 sections, with larger surfaces (e.g. H= 4mm)only a few sections can be cut . Especially soft embeddings and tissues permit more sections, very hard blocks frequently less than given by this rule-of-thumb. However, on the whole, these limits should be observed, since the glass knife is worn very rapidly in every case and the quality of the sections descreases continuously. Thereby, it is important that the separation of the material apparently damages the knife-edge and the thickness of the section taken off in the region up to about 1 µm plays only a subordinate role. Therefore, it makes no sense to "spare" the knife-edge in the initial cutting of the block by taking off a large number of thinner sections until the complete area is reached: In this case the knife-edge is already dull before taking off the first ribbon of sections for the investigation in the electron microscope. Therefore, the knife-edge is taken care of by initially cutting a few 0.5 µm sections and then immediately taking off the ultrathin sections for the electron microscopic examination.

It should not be necessary to mention that a glass cutting edge should not be contacted in any case: Even the slightest pressure destroys it irreversibly. Since, as a rule, any contact leaves traces, every knife is checked before use with the backlighting illumination (dark field) and all knives are discarded which do not appear to be completely clean and free of defects.

The behaviour of the diamond knife-edge is entirely different. It permits not only the preparation of considerably thinner and qualitatively better sections, as well as the cutting of nonhomogeneous specimens like hard enclosures, but, in addition, has a "service life" that is several powers of ten above the service life of the glass knife-edge. In spite of this, problems frequently arise here, too. For a long time, the principal problem consisted in that the quality of diamond knives was highly variable in all manufacturers: In addition to good diamond cutting edges poor ones were often obtained. Poor diamond knives are recognized in that thicker sections (thickness $\geq$ 50 nm) display compression folds, which impart a milky and turbid appearance to the section in the image on the stereomicroscope on the water surface of the collecting boat. In the light microscope numerous folds are observed which run perpendicular to the knife marks, that is, parallel to the knife-edge. Such compression folds cannot be eliminated by spreading. As a rule, they can be avoided in such diamond knives by a drastic reduction of the initial cutting surface to less than 0.5 x 0.2 mm^2, by a reduction of the cutting speed to less than 0.5 mm/sec and, above all, by a reduction of the section thickness to less than 50 nm. Further problems frequently result in the wetting of the diamond knife-edge: Diamond surfaces are extremely water repellant (hydrophobic). Every additional impurity increases this property. Such contaminated diamond knives are best placed in water overnight and then coatings of foreign materials, including disturbing debris, removed with a small pith rod dipped in alcohol according to instructions from the manufacturer (Fig. 6). Thereby, the diamond knife-edge is not damaged if all horizontal forces perpendicular to the knife-edge are avoided: Diamonds are extremely sensitive only in this direction. After cleaning, the diamond is usually again normally wettable, whereby the collecting boat is best overfilled and then the excess of liquid is drawn off slowly with the REFLEXOMAT. Acids should be used for the cleaning only when the manufacturer expressly permits this, since most diamond holders are not resistant to acids.

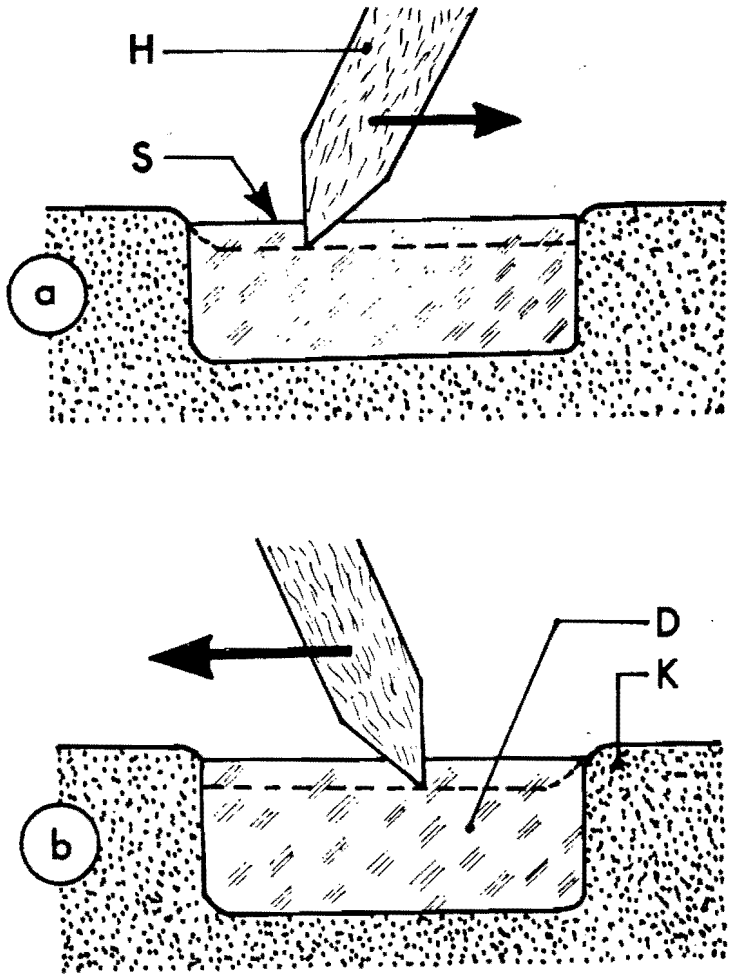

Figure 6 Cleaning the cutting edge of a diamond knife D with a small pith rod H pointed to the shape of a roof: The small rod moistened with alcohol is first placed at the left so that it is split somewhat by the cutting edge and then is drawn toward the right (a). The placing on the cutting edge must be exactly vertical, since diamond knives are very sensitive to forces acting perpendicular to the knife cutting edge S. In a second pass, the small rod H is placed in a similar manner at the right and drawn to the left (b). The cement K adjacent to the cutting edge S may not be contacted in the cleaning. With marked soiling, the entire diamond knife in its holder is placed in distilled water overnight. Operating procedure and figures slightly modified according to DIATOME, Biel, Switzerland.

5.4 Cutting Methodology: If a satisfactorily fixed and embedded (comp. 5.1) as well as correctly cut specimen (comp. 5.2) and a satisfactory knife (comp. 5.3) are available, all prerequisites for the preparation of good sections are indeed present, but all hurdles have by no means been gone over. Serious errors can be made already in the clamping of specimen and knife. Since the cutting forces in the separation of plastic sections are considerable, a saticfactory section sequence and section smoothness are possible only with a very stable seating of specimen and knife. The specimen should not be pointed to a needle shape (Fig. 7 a) nor protrude too far out of the

holder (Fig. 7 b). In the case of normally hardened epoxide blocks pyramids ($\measuredangle \geq 90^{\circ}$ according to Fig. 7 c) or mesa trimmings (Fig. 7 d) assure freedom from low-frequency vibrations when the blocks protrude from the holder by about the amount of their diameter. Of decisive importance is an aftertightening of the clamping before the semi-or ultrathin cutting starts, since the block is always loosened after the trimming. In the clamping of the knife care should always be taken to see that all clamping surfaces of the holder are very clean, since plastic shavings or glass splinters prevent a reliable clamping and thereby often lead to vibrations. Before the cutting, all fastenings of the knife and specimen holders should again be checked.

Frequently, there are also difficulties in the now necessary preselection of the cutting stroke. According to Fig. 7 e, today the specimen is mostly cut using an ultramicrotome, where the specimen passes the cutting edge of the knife for sectioning downward in a vertical path. Afterwards the specimen respectively the knife is pulled back. The specimen returns therefore away from the cutting edge ("single pass") again vertically to the starting position. For the cutting, the specimen is moved with a cutting speed of v_S, which normally is preset between 0.5 and 5.0 mm/sec (Diamond knife... v_S about 1 mm/sec; glass knife... v_S about 3 mm/sec). Since, as a rule, the stroke amounts to about 20 mm, a specimen passage (cycle) at v_S = 2 mm/sec would last a total of 40 sec. Therefore, all modern, automatically operated ultramicrotomes work with an alternating drive, through which the velocity at the switchover points U_1 above the knife edge and at U_2 below the knife edge is varied. Between U_1 und U_2, the specimen is moved slowly with v_S (cutting stroke), and between U_2 and U_1 in the return operation considerably faster with v_R (normally 10 to 15 mm/sec). In this way the length of the cycle is reduced to less than 10 sec. For adaptation to the geometrical circumstances (knife height, location of the specimen), U_1 and U_2 can be changed mechanically or electrically. Thereby, consideration is to be given to the fact that the abrupt reduction of the velocity at the switch over point U_1 is connected with a jolt over the knife cutting edge, which initiates vibrations in the apparatus for at least 0.5 sec. Therefore, U_1 is adjusted to that at least 1 sec elapses between the switch over and the first contact between specimen and knife. If this safety period is not maintained, the sections display low-frequency vibrations (compare

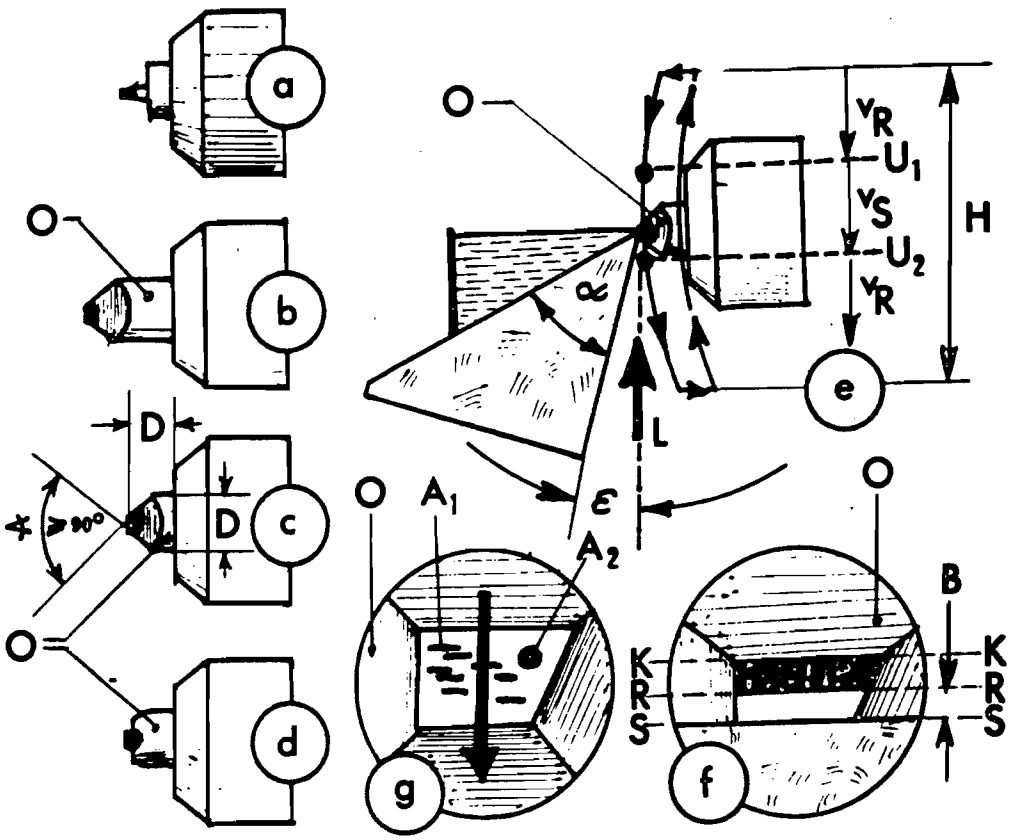

Figure 7 Clamping of the specimen block O, specimen-knife adjustment and alternating drive: Blocks that are cut too pointed (a) or protrude too far out of the holder (b) tend to vibrate. As a rule, the block should protrude from the holder by the amount of its diameter D (pyramidal trimming...c: mesa trimming...d). - In the alternating drive (e) the specimen O is moved slowly with the cutting speed v_S only in the cutting region $U_1 \rightarrow U_2$, in the remaining region of the stroke H it is moved with the return speed v_R. The switch over point U_1 must not lie too close above the knife cutting edge. The switch over point U_2 should take place immediately after passage of the knife cutting edge. - (f) Setting of the knife with the aid of the bright reflection with backlight illumination L: Cutting edge SS and reflection image of the cutting edge RR_i in the mirror-smooth precut section surface must lie exactly parallel to each other as well as parallel to the upper border KK of the block O. The width of the slit must not change in the up-and-down movement of the specimen. - (g) Cracks and fissures A_1 or A_2 are found after the taking off of thicker sections (thickness ≥ 1 µm) in the initial cutting in the form of black slits and cavities in the otherwise mirror-smooth cutting surface. They force interruption of the cutting (formation of water bridges; compare Fig. 13 a). The arrow indicates the direction of the cutting motion. The slits A_1 are perpendicular to this direction.

Section 3). Less critical is the setting of the second switch over point U_2, however, U_2 should not be located too far below the knife cutting edge, so that the duration of the cycle is not lengthened unnecessarily.

In the setting of the specimen (Figs. 7 e and f) care is to be taken to see that the specimen is located in the cutting position and not in the returning position, since in the latter case the pyramid would be destroyed already in the first cycle. The setting is accomplished best with backlight illumina-

tion L, which irradiates the specimen-knife region from below and thereby, according to Fig. 7 f, produces a bright slit between the knife cutting edge and its mirrored image in the precut section surface. The knife is adjusted so that the knife cutting edge, its reflection and the upper border of the section surface are exactly parallel. Thereafter, the specimen is adjusted in the segment arc, so that the width of the bright slit remains constant with up-and-down motion of the specimen. Only through a careful and exact specimen-knife adjustment can faults in the cutting be avoided.

In the standard ultramicrotomy discussed here the knife angle α (= scratching angle) and the clearance angle ε have only subordinate importance. One can adjust confidently using α at 45° and ε at 7°. A change by several degrees hardly changes the results. The same is true for the cutting speed, which can best be set at about 2 to 5 mm/sec for glass knives and 0.5 to 2 mm/sec for diamond knives. On the other hand, it is important that the section thickness for semi-thin sections and for the initial cutting, in the normal case, not be increased to more than 0.5 µm in the case of sensitive specimens, or above a maximum of 1.0 µm, since fissures result if the sections are too thick. Such fissures are seen on the block surface in top view or in the structure viewer as black slits or sharply delineated black areas (Fig.7g) and lead to the carrying along of water from the collecting boat and to the formation of water bridges between the cutting surface of the block and the front area of the knife. Since they usually run very deeply into the interior of the block, it is recommended to discard such specimens and to cut a new block.

After the initial cutting, the advance is set back to a value which gives silvery to pale golden sections in the reflection. Decisive in this connection is only the interference phenomenon, not the calibration, since many factors (Clamping of the block, temperature fluctuations, etc.) produce additional advances that cannot be reproduced. During the preparation of the ultrathin sections neither should the ultramicrotome be touched nore ones seating position changed. Only in this manner, in a quiet room without air draft, can a satisfactory sequence of sections be achieved.

5.5 Section Compression and Spreading: All sections display compression artifacts. According to Fig. 1, the height of the section is always smaller than the corresponding height of the cut surface. It is possible to eliminate this compression for the most part by vapors of organic solvents (xylene, chloroform, trichloroethylene, etc.). To do this, for example, a sheet of filter paper saturated with the solvent is held as close as possible above the sections on the water surface, but without touching the sections. If, among the many organic solvents available, none is found that is suitable for the plastic embedding used, one spreads with heat. Suitable hot wires are obtainable commercially ("heat pen"). They have the advantage that all plastic sections can be spread by heat. In the spreading the section extends so that its thickness decreases by about one shade of colour of the interference scale (for example: pale gold→silver). At the same time, according to Fig. 8, any deformations are eliminated which grossly falsify the section image: Ellipses again become circles, artificial preferential orientations of structures are eliminated.

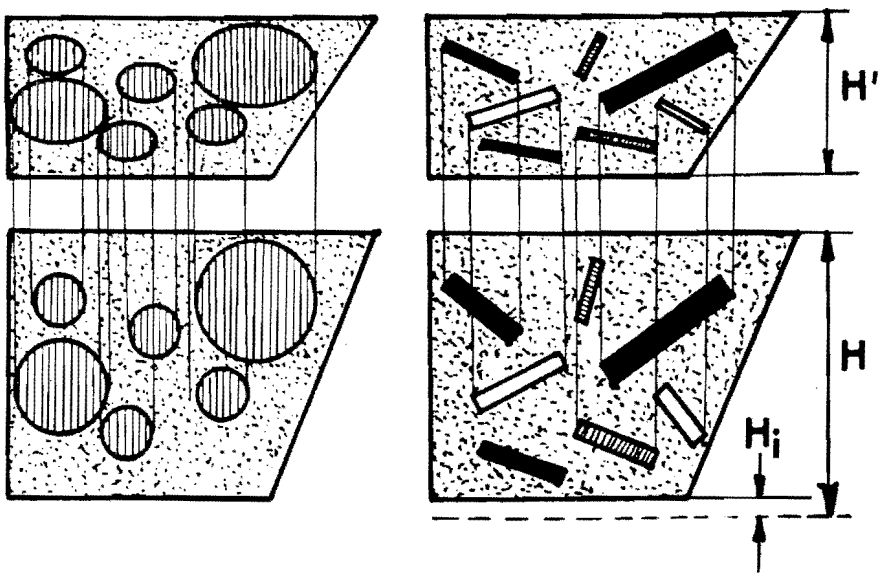

Figure 8 Section compression (above) and elimination of artificial anisotropies through spreading with vapors of organic solvents or heat (below). The compression reduces the height H of the section surface (In this connection see Figs. 1 b and c) partially to half (H' = 0.5 H). It can never be eliminated completely by spreading: There always remains an irreversible compression H_i, which, however, mostly does not interfere.

5.6 Mounting of the Sections on Grids: Frequently, the sections are transferred to the grid, according to Fig. 9 a, in that the grid is placed from above on the ribbon of sections. With normal section areas of about 1 mm^2, especially in the case of ribbons of sections, this procedure leads regularly to rejections and folds, which prevent a conclusive survey of the structures present and thwart a spacial reconstruction on a series of sections. It is recommended to avoid this fault through a "fishing from below", according to Fig. 9 b. In doing this the grid is inclined either slightly forward or backward, so that the first contact between grid and ribbon of sections takes place at either the front or rear end of the ribbon of sections and the remainder of the ribbon of sections can then be pulled up with the grid. It is important to carefully remove all adhereing water with a clean filter paper strip (Fig. 9 c) immediately after taking up the sections, since a contamination can hardly be avoided in the drying. Likewise, the cutting should be stopped immediately when the liquid surface in the collecting

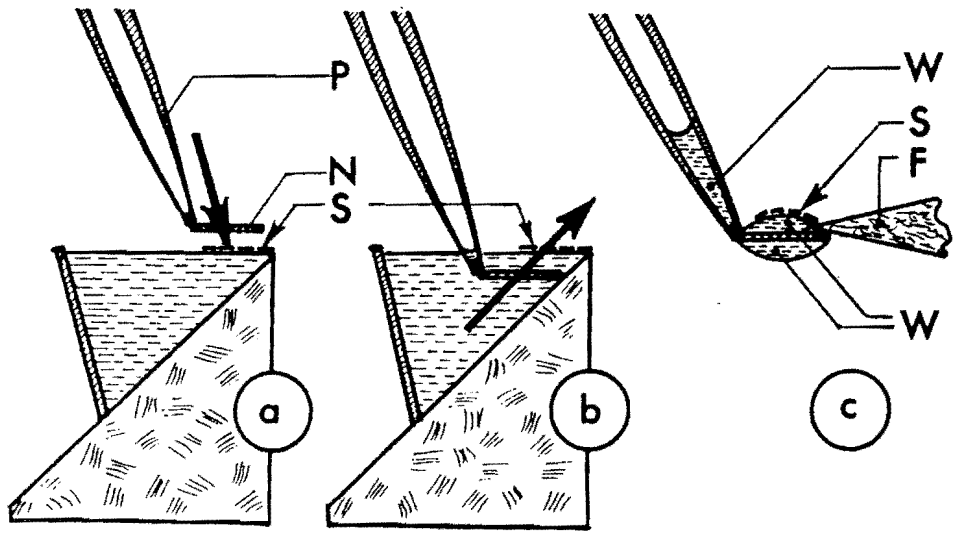

Figure 9 Mounting of sections, especially of ribbons of sections S, on grids N: The grid N, held with the forceps P, is introduced either from above (a) or from below (b) on the section ribbon. If free-floating individual sections are not being dealt with but cohesive ribbons or larger single sections, the process (b) is preferred since it assures a largely fold-free drying of the sections. If larger single sections or ribbons of sections are picked up from above according to (a), many rejections and numerous folds appear. After the mounting, the water W must immediately be sucked off with clean filter paper F from both sides of the grid, as well as between the arms of the forceps (c).

boat displays traces of a surface film. Such films frequently separate from the plastic tapes, when the boats have been filled for a longer time or spreading has been carried out repeatedly with vapors of organic solvents respectively heat.

5.7 Staining: Two problems are in the foreground on staining according to the standard procedure (uranyl acetate/lead citrate): Deficient contrasts and contaminations by uranium and lead compounds. With respect to the contrast, it is recommended not to accept the required minimum time without criticism from the literature, but determine it on your own preparation. Thereby one often makes the surprising finding that very short times (for example, for Araldit or Epon a few minutes) are sufficient to produce optimum contrasts. Hereby, according to our experience, the contrast, especially in the case of lead citrate staining, depends primarily on that the specimen be impregnated adequately by osmium (for sufficient amount of fixation solution per block, see 5.1).

Specimens fixed with higher percentage OsO_4 solutions (for example, 2% or 4% OsO_4 in the fixation solution), yield especially strong contrasts. Likewise, freshly prepared lead citrate solutions stain more intensively than ones stored for a longer time or purified by filtering several times. Finally the embedding medium plays a decisive role: As a rule, the lower viscosity media according to SPURR and to TAAB make very much longer staining times necessary than other polymers. Only with staining times longer than 20 min is it recommended to stain the sections according to Fig. 10 a in a Petri dish. Here, a wet filter paper prevents evaporation from the staining medium, sodium hydroxide beads combine with CO_2 and thereby reduce the danger of lead carbonate precipitate formation. The use of factory-fresh polyethylene films (freezing bag interiors) prevents additional contamination. After staining has been completed (required minimum time), it is washed according to Fig. 10 c only briefly in distilled H_2O, by drawing the grid once rapidly through the water and then drying immediatedely analogous to Fig. 9 c with filter paper. It is erroneous to think that impurities can be removed by a long or especially intense washing (jet stream from a plastic bottle): On doing this, one is more likely to wash

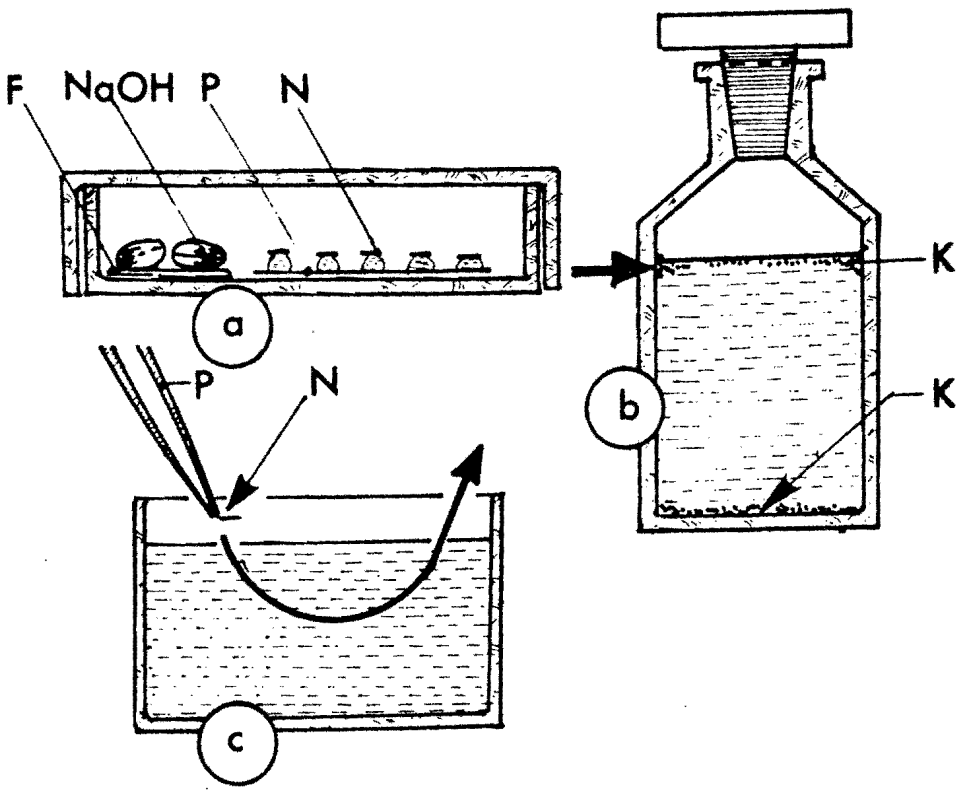

Figure 10 Staining with uranyl acetate and lead citrate (standard method according to REYNOLDS): (a) Only with longer duration $\geq$20 min, it is necessary to place the droplets for staining in a petri dish, which is made moist through a moistened filter paper F and kept free of CO_2 by beads of NaOH. The droplets with the grids N are positioned on a clean polyethylen film P. - (b) Staining solutions which display traces of crystals K at the edge of the liquid level (arrow) or a precipitate K at the bottom (arrow), should either be filtered immediately (uranyl acetate) or discarded and made up anew (lead citrate). - (c) At the end of the staining process, the grid is drawn only once rapidly through distilled H_2O and dried immediately according to Fig. 9 c.

the staining medium out of the section, since the precipitates are not very soluble. On the other hand, it is recommended to use the greatest cleanliness in making up, storing and using the solutions. If precipitates are found at the edge of the liquid level or on the bottom of the supply bottle (Fig. 10 b), a uranyl acetate solution must be filtered. If this happens in a lead citrate solution, it should be discarded and made up again. Removal of the media for preparation of the drops should be accomplished basically with Pasteur pipettes, which then may be discarded.

5.8 Contamination: Many laboratories have continuous problems with an apparently uncontrollable, scattering contamination of the section preparations. Thereby, it is frequently presumed that this is due to dust or smoke particles from the air (aerosols). However, this is only rarely the case. As a rule, these impurities stem from liquids - mostly from distilled water, which is usually highly contaminated by bacteria after standing for a week. In these cases, relief is possible only by preparing distilled water fresh continuously in your own laboratory. Recommended as best, in this case is quartz bidistillation. Ion exchange columns are completely unsuitable for this. All vessels and instruments that come in contact with this distillate must be cleaned carefully - best with chromsulfuric acid. A similar care is recommended also for forceps, pipettes and preparation needles, which are best moved about in xylene and alcohol before use and then cleaned with a clean linen cloth, household or lense paper. Finally, a similar cleaning is imperative in the case of many electrolyte grids, which are not sufficiently cleaned after manufacture. In this case, a cleaning in the ultrasonic bath is recommended. After rinsing, the grids should be placed on clean filter paper and dried in the incubator. If a REFLEXOMAT system is used to adjust the liquid level in the collecting boat, the water is exchanged every morning and the pump is refilled several times with fresh water. Thereafter, the system is used only for level adjustment. The collecting boat is always firstly filled with fresh distillate using a pipette or syringe. An autoclaving of the REFLEXOMAT in this procedure is as unneccessary as in the case of the customarily used pipettes or syringes.

If the individual measures do not lead to success, it is recommended to carry out a consistent checking of all individual steps in the preparation with the phase-contrast microscope. This checking begins with testing the grids before mounting the sections and also after placing sections on them. Connected therewith is a checking after the uranyl acetate staining and, finally, after the lead citrate staining. In this manner it is easiest to delineate and eliminate the actual source of error.

5.9 Specimens that are Difficult to Cut: The category of specimens that are difficult to cut can be divided chiefly into two sectors: On the one hand, specimens which are difficult to cut even with optimum embedding, because they are hard, tough or contain inclusions difficult to cut for other reasons (plant or insect tissues, silicose lungs, cartilage, collagenous connecting tissues, fatty tissues, etc.) or, on the whole, are hard (bones). On the other hand, specimens which can be cut without difficulty with satisfactory fixation and embedding, but cannot be cut or can be cut only with difficulties by the normal methodology due to a poor fixation and/or embedding (In this connection see Section 5.1).

Specimens inherently difficult to cut in most cases can be cut satisfactorily with a perfect diamond knife (In this connection see 5.3). Thereby, it should be remembered that the diamond cutting edge is very rapidly and irreversibly destroyed by larger hard inclusions (for example, silicates in diameters above 0.5 µm) or by massive hard objects. Thus, the cutting of individual extremely thin asbestos fibers or hydroxyapatite crystals normally presents no difficulties. On the contrary, thicker glass fibers destroy the knife edge immediately. In contrast to Fig. 4 d, objects difficult to cut in certain area dimensions, are often arranged perpendicular to the knife cutting edge (compare Fig. 11 a), because the cutting force is substantially reduced in this manner. To exclude the shock, which results from first contact of a block edge oriented parallel to the knife cutting edge, which is then manifested as waves at the border of the section, the block can be cut to form a triangle and placed with the point against the cutting edge (Fig. 11 b). In this blank the cutting force increases from first contact of the section tip with the knife during the taking off of the section continuously up to maximum force. Therewith, the formation of vibrations is practically eliminated. If only glass knives are available, the cutting of specimens with hard inclusions should generally be avoided, after one has been convinced that the glass edge is destroyed already in the first section. If this is not the case, a precise setting often makes possible the preparation of a few usable sections. Thereafter, the position on the glass edge must be changed through lateral shifting and adjusted again. A modified blank, according to Fig. 11 b, as well as a changed position of the initial cutting

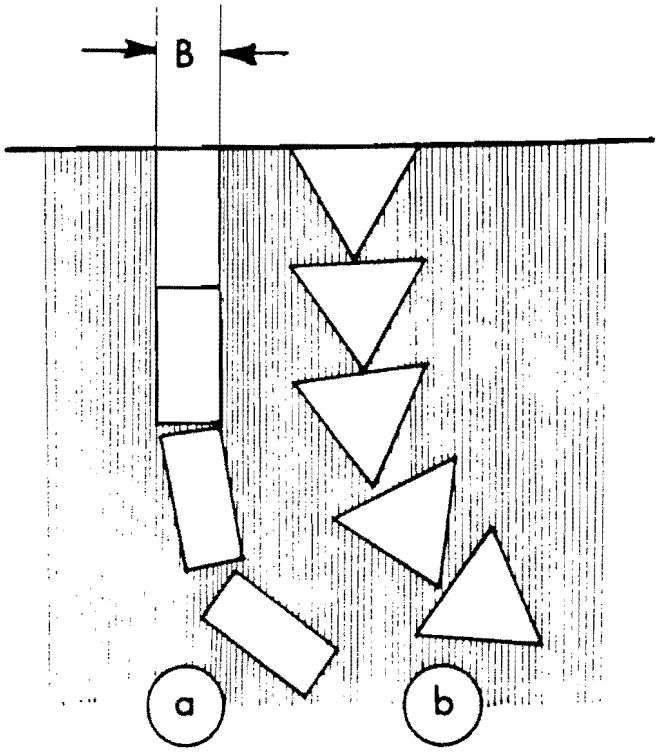

Figure 11 Position and shape of the section surface in the cutting of blocks that are difficult to cut: (a) Object placed edgewise. - (b) Triangular section (according to REID, 1974). - In both cases the preparation of usable sections has preference above the taking off of ribbons of sections (compare also Fig. 4 d) and is facilitated thru reduction of the cutting forces. The cutting forces are always proportional to the object width B. After trimming according to (b), the cutting force is increased continuously from zero to the maximum.

surface, according to Fig. 11 a, often lead to better results here. In general, in such cases, it is recommended to reduce the initial cutting area to the just acceptable minimum amount. If tough components are contained (collagen fibers, cellulose microfibrils), an increased cutting speed ($\geqslant$ 10 mm/sec) frequently leads to the goal, since the time for deformation and for pulling out is missing.

The situation in poorly fixed and/or embeddes specimens is simpler: They are discarded and new preparations are made, whereby the instructions in Section 5.1 are observed. If very rare and therefore precious specimens are being dealt with, the initial cutting area is reduced to the minimum and one cuts comparatively with glass and diamond knives at very reduced cutting speeds. Here, too, the blanks and positions according to Fig. 11 can be helpful. High cutting speeds lead to the desired success only rarely, because in these cases high-frequency chatters usually appear.

5.10 Erroneous Setting Up or Faulty Functioning of the Ultramicrotome: An erroneous setting up of an ultramicrotome frequently leads to failures - in this case to irregular section sequences, according to Fig. 3 a, to nonuniform thicknesses in the section, according to Fig. 3 b, or to low-frequency chatters, according to Fig. 1 b. A nonuniform sequence of sections is unavoidable when the instrument is stationed in a large room in which several persons work or when the instrument is subjected to irregular air movements (air drafts). The same is true for a stationing in the neighborhood of windows, doors, heaters or air-conditioning units. In these cases the ultramicrotome should be set up in a corner opposite from doors and windows and be protected from air drafts by a protective screen (paravent). Disturbing air conditioners should be shut off, at least for the duration of the section preparation, if emphasis is placed on a uniform sequence of sections. Color waves or vibrations in the section are unavoidable if the building vibrates or the instrument stands in the neighborhood of large machines (workshops, elevators, transformers, voltage stabilizers, etc.). Wooden floors in ould buildings, through transfer of step impulses, likewise lead to irregularities in the sections and are unsuitable for the installation of ultramicrotomes, even when they are covered with a modern floor covering. Whereas, in old buildings in most cases a solid wall bracket solves the problem, as a result of the solid masonry work, in new buildings one is forced to introduce heavy, air-cushioned special tables, such as those advised, for example, by EALING (EALING Corporation, South Natick, Mass. 01760, USA).

A faulty function of the ultramicrotome also occurs in quiet buildings and rooms when the instrument table is installed erroneously or the shock absorbers are not functioning or are worn. Therefore, before setting up the heavy ultramicrotome on the table, one must always be sure that all feet of the table rest uniformly on the floor. The damping of the instrument may not be short-circuited by clamped instruments, but must move resiliently in all directions. Rubber springs (resilient cushioning) must be replaced after about five years of operation.

Only when it is certain that neither methodological errors, nor a faulty installation of the instrument cause the disturbing phenomena, should one think of a faulty functioning of the apparatus and notify the authorized service organization. Entering in the accurately designed mechanical and electronic system of modern ultramicrotomes is urgently advised against: The chance of producing additional defects in such an undertaking is considerably greater than the prospect of success.

6. Problems under Special Conditions: Minimum Section Thicknesses - Large Section Surfaces - Semithin Sections

With the exception of Section 5.9, all of the foregoing sections relate to standard specimens and standard methods. New and usually more difficult problems arise when one deviates from these standard procedures, which are considered to be laboratory routine today. This is true, for example, in the region of minimum section thicknesses ("gray region"). With respect to these additional problems, before one goes over to extremely thin sections, one should convince oneself in a careful comparison whether any advantages are to be achieved through the reduction in thickness: Frequently, this is not the case at all, so that one finally obtains poorer results with an increased effort. However - for example in the preparation of crystal structures or of complex membrane structures in the macromolecular region or if better pictures are actually obtained from thinner sections, a number of requirements must be observed to avoid problems: Basically, extremely thin sections are obtained only with the diamond knife (limiting section thickness in the uniform ribbon $\geqslant 15$ nm). On the contrary, the limiting section thickness of glass knives is considerably higher ($\geqslant 30$ nm). This limiting section thickness is obtained only under optimum conditions: Best specimen composition, small, homogeneous section area, correct knife and specimen adjustment and proper trimming, optimally clean knife edge, as well as an absolute quiet workplace. To be remembered is that the advance setting may not go below a certain limit also in this case: As in the case of normal microtomy, in ultramicrotomy, on going below this limiting section thickness, every second section is skipped or an absolutely

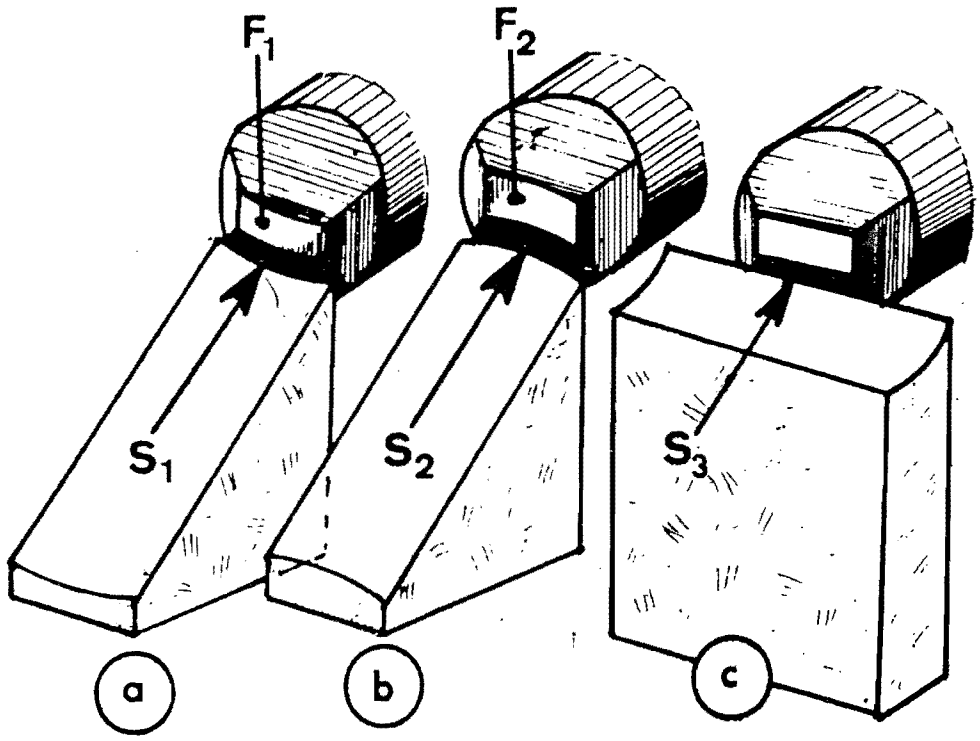

Figure 12 Problems of the cutting geometry in the taking off of large area sections: The disadvantage of curved cutting edges in the case of triangular glass knives (a and b) can be eliminated by RALPH-knives with exactly straight-line cutting edges (c). In the case of triangular knives, assistance is given in not selecting at random the knives K_1 and K_2 prepared with the knife makers according to Fig. 5 a, but places in groups exclusively knives K_1 or K_2, since the cutting geometry within the groups agrees better.

irregular sequence of sections appears. As a rule, extremely thin sections are particularly strongly compressed and must therefore be carefully spread out in every case (Section 5.6). Since the contrast of such sections is very small, it is recommended to mount these sections on unfilmed, narrow-mesh grids. If necessary, the sections can be reinforced by coating them with a thin layer of carbon.

Entirely different types of problems arise when one wishes to prepare sections with areas greater than 2 x 2 mm^2, such as those that can be investigated today directly in the STEM system. The limits achievable with efficient modern ultramicrotomes today are located at section areas of about 5 x 5 mm^2 for ultrathin sections in the thickness region around 100 nm, and at about 10 x 10 mm^2 for semithin sections (thickness 0.2 to 1.0 µm). The use of exactly linear diamond knife cutting edges is eliminated at these dimensions. Therefore, in this region the geometric shape of the knife cutting edge plays the decisive role.

Normal standard triangular glass knives according to Fig. 5 have only limited suitability for this, since their cutting edge is always slightly curved (see Figs. 12 a and b). Since only a few sections with these large areas can be taken off before the knife edge becomes dull and therewith unusuable, the specimen must be precut with a cutting edge with the same geometry. For example, it would not be appropriate to precut the specimen with the knife edge S_1 (surface F_1, Fig. 12 a) and then prepare the ultrathin section with the cutting edge S_2 (Fig. 12 b): Before the surface F_2 would be obtained, the knife would be dull and unusable. Here, there is a solution according to Fig. 5 a in that a knife maker is adjusted very exactly and the knife is always taken only from the same side. A much better solution of the problem is offered today through the use of RALPH-knives (Fig. 12 c), which have an absolutely linear knife edge S_3 with a length of 25 or 36 mm and which can be prepared very simply with a histoknifemaker. Aside from the geometry of the cutting edges, electrostatic charges play a greater role in the cutting of large areas than in standard ultramicrotomy. They frequently lead to water bridges (see Fig. 13 a) between the block surface and the front surface of the knife, which makes the preparation of additional sections practically impossible. If no antistatic system is available (Simple antistatic pistols from the photographic and record industry are not satisfactory in all cases!), one can provide a remedy by lowering the liquid level in the collecting boat, according to Fig. 13 b, which is possible in a simple manner with the

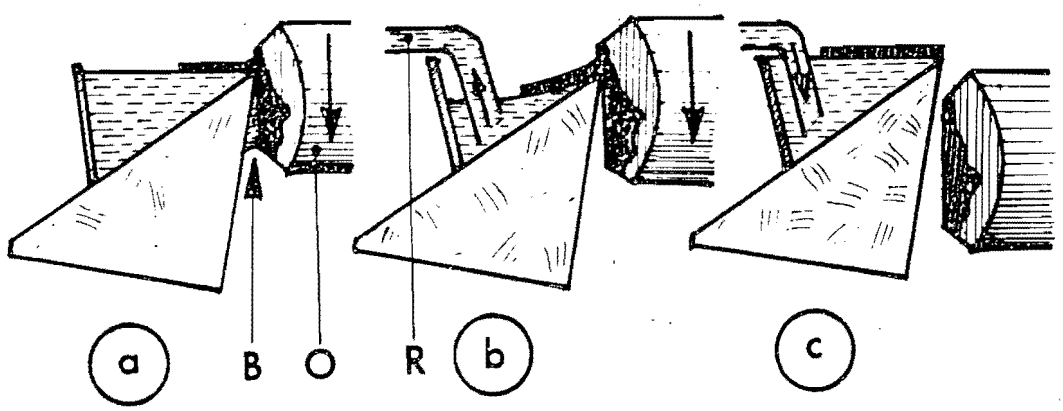

Figure 13 Avoidance of water bridges B between extremely large section surfaces and the front surface of the knife (a) through sucking water out of the collecting boat with the REFLEXOMAT-system R immediately before the cutting (b). Immediately after the sectioning process the boat is again filled to make possible a normal spreading of the section (c).

REFLEXOMAT system. The sections are transferred directly to large grids from the large collecting boats (For example: LKB-Trufs) recommended in these cases, which can then be investigated in STEM system or, with the use of side entry system, in the TEM. Semithin sections with large areas can be prepared with RALPH-knives just like large area ultrathin sections. The main problem here is not in the preparation but in the fold-free application and staining of the sections. If it is possible, sections in thicknesses greater than 0.5 µm should be used, in which these problems are easier to control than in thinner sections. A good adhesion of the plastic section to the glass slides is achieved with chromalum gelatin or through a longer heat treatment on a hot plate or in the incubator at temperatures around 100°C.

7. Checklist in the Search for Errors

The attached checklist should facilitate the search for errors in the individual case, especially make possible a differentiation of the different causes of similar or equivalent artifacts. Alongside a short description of the disturbing phenomenon, the possible causes are listed. Reference is made to the corresponding sections and figures of this paper.

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Checklist for Determining Errors in the Preparation of Sections

Disturbance	Description of the phenomenon	Possible causes	Section	Comp.
Low-frequency vibrations	Periodic waves of non-uniform thickness parallel to the knife edge in the sections, visible in the stereomicroscope or in the light microscope with dry objectives up to 40 : 1 (Fig. 1 b and 2 a). Frequency $\leq$ 1,000 Hz (measured according to Section 3, compare Fig. 2 a)	Dull or highly notched knife edge	5.3	
		Inadequately clamped knife or knife holder	5.4	
		Block cut too pointed	5.4	Fig. 7 a
		Block protrudes too far from the holder	5.4	Fig. 7 b
		Inadequately clamped specimen block (not retightened after pause or trimming)	5.4	
		Inadequately clamped specimen holder	5.4	
		Switch over point U_1 too close above knife edge (decay time = 1 sec)	5.4	Fig. 7 e
		Building vibrations in low-frequency region	3 5.10	
		Improperly set up instrument table (feet not loaded uniformly)	5.10	
		Touching the ultramicrotome during cutting	5.4	
		Faulty functioning of the ultramicrotome	5.10	
High-frequency vibrations	Periodic waves of non-uniform thickness parallel to the knife edge (perpendicular to the knife marks) visible only in the electron microscope. Frequency $>$ 1,000 Hz (measured according to Section 3, compare Fig. 2 b)	Poorly impregnated specimens or poorly polymerized blocks	5.1 5.9	
		Dull or highly notched knife edge, especially near strong knife marks	5.3	Fig. 5 e
		Too high cutting speed (>5 mm/sec) and/or too large cutting area (> 1 mm^2) and/or too large knife angle and clearance angle ($\alpha + \varepsilon$ according to Fig. 7 e greater than 70°)	5.4	
		Specimens difficult to cut, especially with a high fat content	5.9	Fig. 11

Checklist (continued)

Disturbance	Description of the phenomenon	Possible causes	Section	Comp.
Single waves or aperiodic waves in the section	Phenomenon according to Fig. 3 b	Touching ultramicrotome while taking section	5.4	
		Extreme building effects (step vibrations, strong single shocks)	5.10 5.4	
		Unstably set up ultramicrotome (faulty table adjsutment) or short circuited or defective damping element	5.10	
		Defective ultramicrotome	5.10	
Different thicknesses within different areas of a single section	Phenomenon according to Fig. 3 c (borders between areas of different thickness not always parallel to knife edge, but following specimens structures)	Nonhomogeneous section area (faulty trimming, inhomogeneous specimen) according to Fig. 4 a	5.2 5.3 5.4 5.9	Fig. 4 b Fig. 11
Nonuniform section sequence	Alternating section thicknesses with uniform thickness within each section (phenomenon according to Fig. 3 a)	Air drafts and temperature changes in room	5.4 5.10	
		Manipulations on apparatus and change of position during the cutting series	5.4	
		Advance set too low	5.4 6	
		Faulty functioning of the instrument	5.10	
Compressed sections	Section height H′ is smaller than H of cut surface (Fig. 1 b and c) and deformed structures present (Fig. 8)	Omitted or insufficient spreading out after sectioning (before mounting on the grid)	5.5	Figs. 1 and 8
Cohesion between sections in the ribbon absent	Sections float away from knife edge singly and do not adhere to one another	Upper border KK of the cut surface not parallel to the knife edge SS	5.4	Fig. 7 f
		Irregular trimming or contact zone too narrow	5.2 5.4	Fig. 4 d and 11
		Upper and lower border of the cutting surface not smooth and linear	5.4	

Checklist (continued)

Disturbance	Description of the phenomenon	Possible causes	Section	Comp.
impurities on the sections (contamination)	Sections are nonreproducibly covered with crystals or layers which disturb the investigation	Impure distilled water for preparing the films or filling the collecting boat	5.8	
		Insufficiently cleaned glass vessels	5.8	
		Insufficiently cleaned accessories (forceps, preparation needles etc.), dirty filter paper	5.8	
		Contamination by adhesive tape from the collecting boat with longer filling, frequent spreading or too high a temperature	5.8	
		Absent or too long sucking out of the water after fishing or contrasting	5.6	Fig. 9
		Contaminated staining solutions, too long a duration of the staining process	5.7 5.8	Fig. 10
Folds in the sections on the grid	Folds without preferred direction	Picking up of the sections from above according to Fig. 9 a	5.6	
Compression folds	Folds with preferred direction perpendicular to the path of the knife marks with diamond knives	Use of poor diamond knife; too high a cutting speed, too large section area or section thickness with diamond knives	5.3	