Sonoma State University

Division of Natural Sciences
707 664-2171

Department of Biology
June 27, 1979

Dear Don:

It was very nice to talk to you on the phone the other day. I haven't forgotten that I said I would send you information about glass knives used to cut sections for electron microscopy. This article by Lindner and Richards seems to give a nice review of the whole business and this add by LKB will show you how someone is capitalizing on it. This stuff along with a price list arrived today. We have an LKB KnifeMaker 7800 and thus we are on the LKB mailing list.

I will send you additional stuff on glass knives as I come across it.

Small world: The Bennett mentioned here had a daughter who went to high school with me. She was choice. Bennett was the founding chairman of the Anatomy Department of the Medical School in the University of Washington way back in the Dark Ages when I was growing up in Seattle.

Julian is recently off to Texas again. We haven't heard from him since he left. Perhaps he is back by now. He has really been covering a lot of territory recently.

Best regards,

Colin

Colin O. Hermans

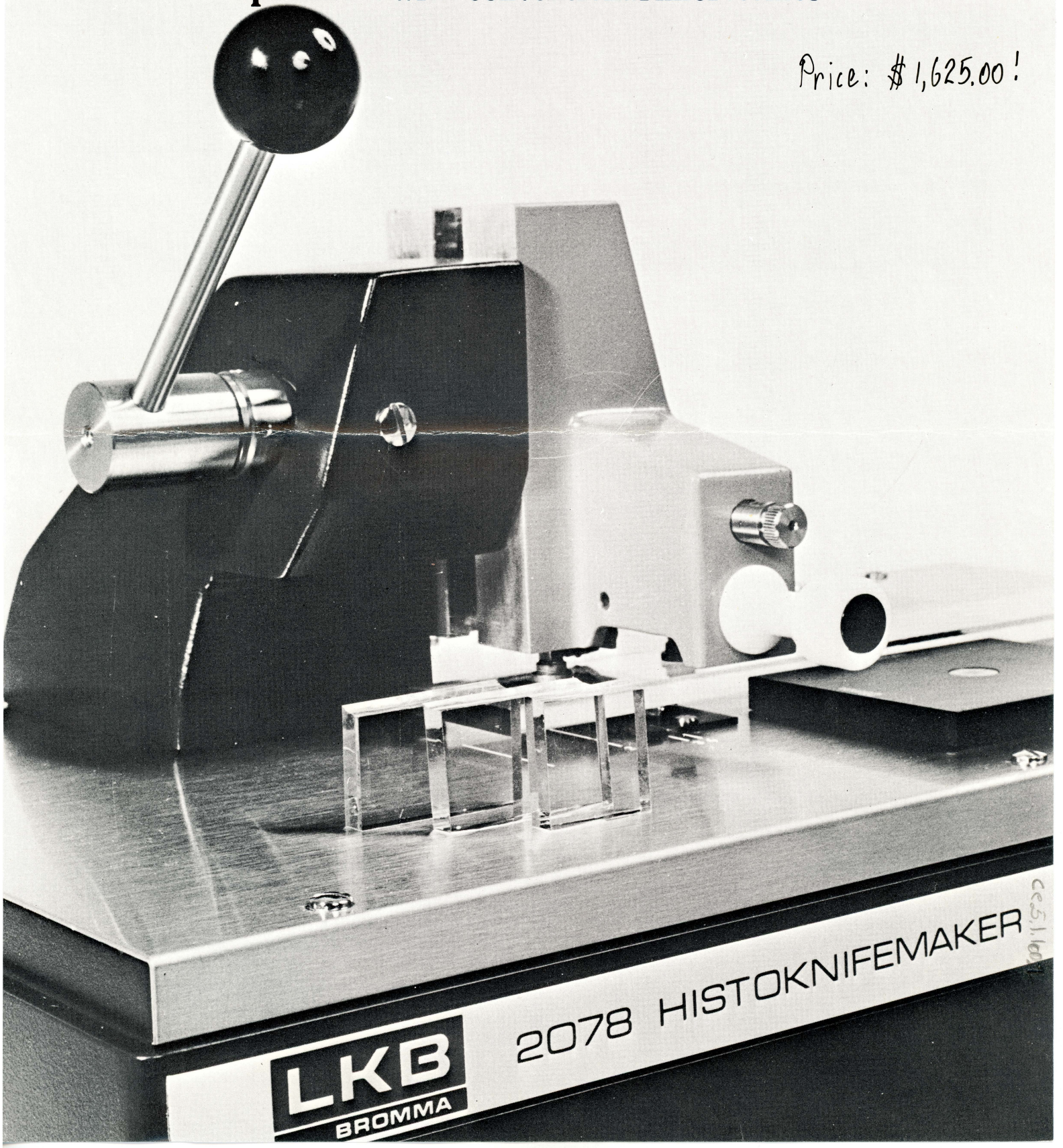
2078

LKB

LKB Histo KnifeMaker

Gives you a new break for the sectioning of plastic-embedded specimens with conventional microtomes

Price: $1,625.00!



The new LKB 2078 Histo KnifeMaker enables you to produce super-long (25 or 38 mm), extremely-sharp, disposable glass knives at a very low cost. These so-called 'Ralph' knifes* are fitted to the most-commonly used microtomes by means of a simple holder. The combination of super-long glass knives with conventional microtomes means that you can now cut large areas of tissues embedded in resins such as methacrylate, Epon or Paraplast. In this way, you obtain high-quality semi-thin sections for light microscopy, with improved resolution far beyond that which is possible with steel knives and paraffin embeddings.

Histo KnifeMaker is the second new and exciting instrument which LKB has introduced for convenient and reproducible knife making. Its design is based upon experience obtained with the successful LKB 7800 KnifeMaker, which was introduced in 1962. LKB KnifeMaker is regarded as essential to every electron microscopy laboratory, and we believe that LKB Histo KnifeMaker will find a corresponding place in every optical microscopy laboratory. Here are some of the reasons why:

- Histo KnifeMaker produces 25 or 38 mm-long knives: this means that you can cut large-area sections up to about 30 mm.
- Histo KnifeMaker is inexpensive, and so is within the reach of every laboratory.
- Histo KnifeMaker is easy to operate: no previous skill or experience is needed to produce perfect knives every time.
- You can obtain hundreds of sections from the same Ralph knife, because of the very durable cutting edges.
- The knife edges are very sharp, and so the quality of sections even as thin as 0.2 μm is very high.
- Low cost per knife, because of low wastage of glass.
- Ralph knives can be used with currently-available microtomes, sledge or rotary.
- Disposable glass knives means no tedious re-sharpening of steel ones.
- Ralph knives are also suitable for trimming electron microscopy specimens, for example, in your LKB 2128 UM IV Ultrotome.

Technical specification

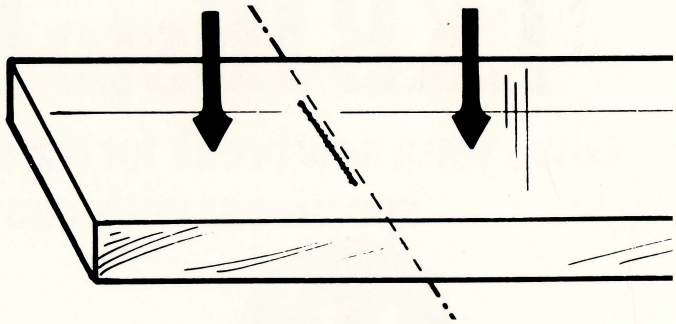
Accepts glass strips with dimensions	width 25mm or 38 mm thickness 6 mm
Cutter wheel	sintered tungsten carbide
Dimensions (LxWxH)	480x300x340 mm
Net weight	Histo KnifeMaker alone: 15 kg Histo KnifeMaker plus standard accessories: 21 kg
Gross weight (incl. packaging)	22.5 kg

Ordering information

LKB 2078 **Histo KnifeMaker,** complete with glass cutter, clamping device and breaking tool combined in one unit. Includes one packet of LKB 2078-038 38 mm glass strips for Ralph knives, three extra cutting wheels and ten breaking plates, and adjusting tools.

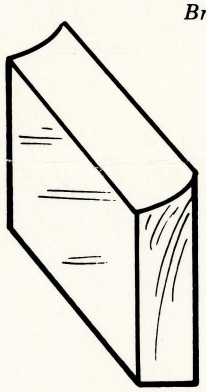
*Bennett et al., Stain Technol. 51(1976)71.

See how easy it is to make a sharp new knife!



Place the glass strip in the Histo KnifeMaker. Clamp the strip. Score.

Break.



A characteristic Ralph knife ready for sectioning.

Optional accessories

LKB 2078-025	Glass strips for Ralph knives: 400x25x6 mm, pkt. of 30.
LKB 2078-038	Glass strips for Ralph knives: 400x38x6 mm, pkt. of 22.
LKB 8890-20	Wax for mounting Ralph knives, pkt. of 3.
LKB 2208-010	**MultiPlate,** for mounting knives on holders and for melting wax. For 110 V, 50–60 Hz. 25 W.
LKB 2208-020	**MultiPlate.** For 220 V, 50–60 Hz. 25 W.
LKB 7894–01	Cutter wheels, pkt. of 3.
LKB 94 91 0883	Adjusting tools kit.
LKB 90 01 0622	Breaking plates, pkt. of 10.

For details of Ralph knife holders for LKB 2128 UM IV Ultrotome and most commercial microtomes, see separate price list.

Patents applied for.

For details of LKB KnifeMaker, please ask for Data Sheet 7800.

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LONG-EDGED GLASS KNIVES ('RALPH KNIVES')–THEIR USE AND THE PROSPECTS FOR HISTOLOGY

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(Received September 11, 1978)

ABSTRACT

The general use of plastic embedding media in histology may be inhibited by the frequent steel-knife sharpening that it necessitates. This article considers some of the problems of section cutting and knives, and describes experiments using machine-broken long-edged glass knives ('Ralph knives'). The pros and cons of plastic and paraffin embedding are discussed, and a role for Ralph knives–which can cut both media–is suggested.

In 1976, Bennett et al. published an article which makes a considerable contribution to the technology of section cutting. They described a method for making long-edged glass knives, theoretically of unlimited edge length, which they called 'Ralph knives' in honor of the late Dr. Paul Ralph, who invented the technique. Fig. 1 compares a triangular glass knife of the type introduced into electron microscopy by Latta & Hartmann (1950) with a Ralph knife. Bennett et al. (op. cit.) used their knives for histology rather than ultramicrotomy, and this is also our application.

In late 1977, one of us (M.L.) commenced an engineering M.Sc. project to produce a device for the machine-breaking of Ralph knives (the previous method was by hand). A working machine was produced in early 1978, but this did not possess a glass-scoring mechanism. At this stage we contacted LKB-Produkter AB in the hope of obtaining some of the scoring wheels used in their triangular glass knifemaker (*KnifeMaker* 7800). We discovered that a prototype LKB Ralph-knifemaker (now the HistoKnifeMaker 2078) using the same principles as our own machine had been built by Mr. Lennart Kindel at Bromma. The parallel development of similar machines thus became united in its later stages.

This article attempts briefly to describe some of the properties of knives in general, the problems of the cutting process, to present some of our preliminary findings on the use of Ralph knives in ordinary microtomes, and to indicate how we think these knives might contribute to our view of trends in histological technique. We apologize in advance to histologists in the hope that they will not feel insulted by the naive histological thoughts of a mechanical engineer and an erstwhile zoologist!

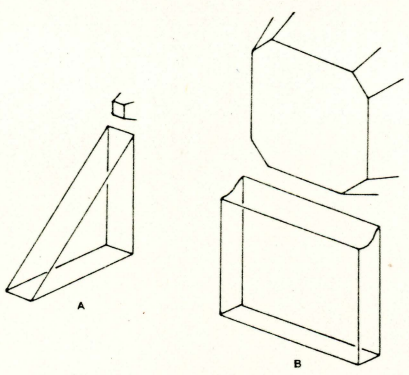


Fig. 1. A–Latta & Hartmann knife. B–Ralph knife.

KNIVES AND CUTTING

General

Little is known of the cutting process. Wachtel et al. (1966) propose a scheme of events in the specimen block at the molecular level. They claim that cutting is the rupturing of molecular bonds which lie in the path of the advancing knife edge. A wave of compression precedes the edge and exerts transverse tensile stresses parallel to it. As the knife advances, the tensile stresses increase, and the molecular chains first undergo an elongation along the lines of tension. They then rupture and slip over each other (plastic flow) tearing out from the body of the material as the yield point is exceeded.

The above scheme does not help, however, in defining the best edge to rupture molecular bonds, nor in explaining empirical findings concerning knife effectiveness. Thus, Collins (1969) found that etching carbon-steel microtome knives for two minutes in 0.1N nitric acid increased their paraffin sectioning abilities. Roberts (1974) found that glass knives for ultramicrotomy were improved considerably if tungsten was evaporated onto their cutting edges. Collins (op.cit.) suggested that her improvement was due either to the shape of the serrations on the etched edge, or that the carbide particles exposed on the bevel facet (see Fig. 2) produced less friction than the microscopic grooves on the facet of an untreated knife. Roberts (op.cit.) suggested that improvements in cutting with his tungsten-

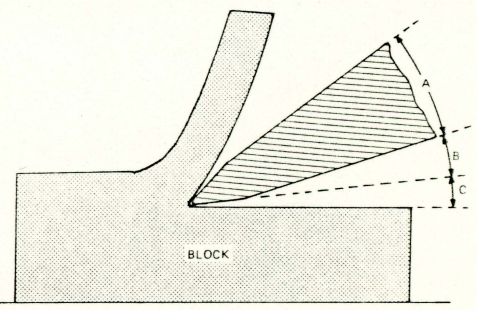


Fig. 2. The knife edge. (A) Bevel angle. (B) Facet angle. (C) Clearance angle.

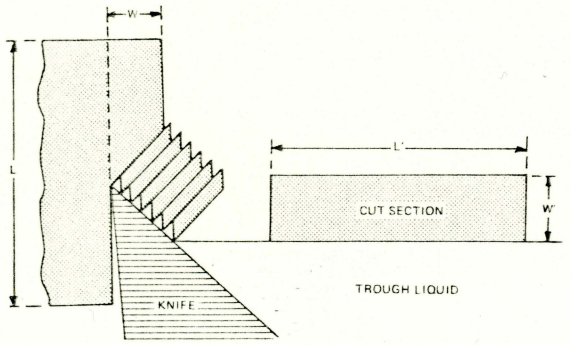


Fig. 3. Nonrecoverable compression can be expressed in terms of L–L' and W–W'.

coated glass knives could be due to either a lowered coefficient of friction between knife and specimen, or the tungsten particles along the knife edge forming a microscopic saw, or the possibility that by now having a pseudometal edge any heat produced by the shearing effect of the knife through the specimen was transferred away from the edge by the conducting surface (however he found no improvement to glass knives which he coated with gold or aluminium).

Marengo (1967) found that steel knives with a facet angle of 21° caused less compression in cutting 4 μm paraffin sections than did those with a facet angle of 30°. With triangular glass knives, Ward (1977) found that knives made with an angle of 55° rather than the customary 45° had more usuable edge and at the same time cut epon sections as satisfactory as those obtained with 45° knives. Helander (1969, 1973 *a*, *b*, 1974) has done the most sophisticated recent studies on glass-knife edges and the topography of ultrathin sections and their parent blocks.

Assessment of knives

Knives can be assessed in two main ways. One way is to examine them visually, the other is to use them.

(a) Visual examination

Visual examination is used to see if the edge has any flaws or 'nicks'. With steel knives, quite high magnification is recommended for this examination; ×200 or ×400 and "the use of a better instrument than, for example, a stereomicroscope!" (Haselmann, 1971). Hallen (1954, 1961) used an interferometric technique to view steel knives. Methods for examining glass knives are given in Reid (1975, pp. 259–261).

(b) Section cutting.

Most authors agree that the surest test of a knife edge is to use it to cut sections. This is to find: (1) If the knife will cut sections of the required thickness. (2) If the sections produced have scratch marks. (3) If serial sections are of uniform thickness, i.e., alternate thick and thin sections are not cut. (4) If there is much section deformation ('compression').

The first two questions require simple yes–no answers, and there can be little argument about them. Questions (3) and (4) are more complicated. Thus, Steedman (1960) states that alternate thick and thin sections can be caused by a blunt blade. Wachtel et al. (1966) state that such a phenomenon can be caused by the elasticity of the block. Yet others have found that the speed of the cut can alter the section thickness,

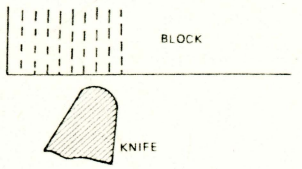


Fig. 4. Close-up of knife edge and block. (After Steedman, 1960.)

whilst Hallen (1961) considers cutting speed has no effect. Compression was first studied quantitatively by Dempster (1942, 1943). It seems to consist of two types; recoverable compression which can be corrected in the process of flattening the section on the slide or in the trough, and nonrecoverable compression. The latter compression is described well by Reid (1975) (see also Fig. 3), and both she and Helander (1974) relate microtome section cutting to metal cutting. They describe nonrecoverable compression in terms of 'slip steps'.

Glass versus steel

The most important region of all knives, the edge, is formed by the line of intersection of two surfaces. Viewed from the side, the 'apex' may be curved, and the radius of this curvature is a measure of its sharpness. Steedman (1960, p. 133) shows this feature rather well in a diagram redrawn in Fig. 4. This shows a blunt edge relative to a section of 9 μm and indicates the way in which the outer regions of the section will be damaged.

Wachtel et al. (1966) find that for steel knives the edge becomes stronger and can be more easily lapped to a sharper apex as the facet angle is increased. With glass knives, edges are obtained not by sharpening, but by fracture. The curvature of broken glass edges can be in the order of molecules. The dimensions of the edges which can be obtained with steel knives are not clearly stated in the literature. It seems likely, however, that the radius of curvature of a steel edge cannot become smaller than the granulation of the abrasive medium used in its sharpening. If an abrasive is not used, the high microscopic areas of the lapped surfaced which intimately contact those of the lap are heated by sliding friction. This can be detrimental, and the omission therefore of an abrasive is not necessarily the answer.

Glass at room temperature exhibits the typical properties of a supercooled liquid; it has no crystal structure and breaks without plastic deformation. Steel has a granular structure, and this produces varying microsharpness. Bragg (1927) discusses this in considering 'Damascus blades', which were so effective against the Crusaders. He says that it is very likely that much of the keen edge of these swords was due to the presence of very hard cementite particles embodied in the softer iron. The edge would be like a "saw with extremely fine teeth". This explanation is similar to one of Roberts's (op. cit.) suggestions for the cause of the improvement in his tungsten-coated glass knives. From these considerations it would seem that a serrated edge makes the best cutting tool. The 'granular' steel knives have an intrinsic advantage here, but empirically they are less effective for ultrathin sections, perhaps because their 'teeth' are too big. The steel teeth (ridges, crystals?) bear the same relationship to ultrathin sec-

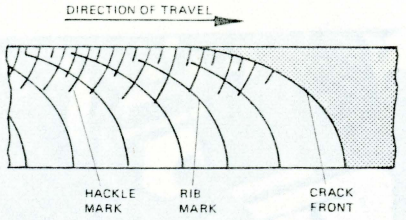


Fig. 5. Schematic drawing showing the formation of hackle marks and rib marks in glass-knife breaking.

tions that Steedman's blunt edge has for the 9-μm sections, and striations are formed. This is reinforced by the fact that it is the serrated (hackle marked) triangular glass knives which experience has taught the ultramicrotomist to avoid when making his glass-knife selection.

Perhaps 'molecular' or even 'atomic' size teeth are required, and this is what Roberts has achieved with his knives and what diamond knives achieve naturally. It has become evident to us that neither histologists, nor mechanical engineers, nor zoologists will explain the answer to 'best knife', empiricism is our wisest course; the answer to the theory presents a very complex problem for the materials scientist.

THE FRACTURE OF GLASS

In glass, fracture occurs as soon as something called the 'ideal tensile stress' is reached at the root of a sharp and narrow crack (Griffith, 1920). When a score mark is made on glass, this artificial crack is much longer than the other smaller cracks on the surface of an imperfect piece of glass (all glass apart from special laboratory preparations is imperfect–see Gordon, 1976), and fracture will always occur from this line. When tensile (pulling) forces are applied, extension of the crack beyond its original length provides an ever increasing driving force for further unstable propagation of the crack. If the forces acting on the glass remain constant during breaking, the stress in the fracture plane will increase steadily as the crack travels along, and this results in the sharp acceleration and high propagation velocity in the order of the speed of sound towards the opposite surface. The maximum velocity that a crack can reach in glass is called its Rayleigh-wave velocity, and when this speed is approached, distortions in the stress field can occur. These cause the crack front to deviate randomly above or below the extension of the crack plane. These deviations cause ridges called 'hackle marks' (McClintock & Argon, 1966). Sometimes there are also traces on the fracture surface called 'rib' marks (see Fig. 5). Both types of mark can be found in glass knives. Rib marks do not normally cause trouble as they do not reach the edge, but whenever hackle marks appear they will intersect the edge and diminish the quality of the knife. Hackle marks are in fact the 'serrations' on the part of the edge which the ultramicrotomist normally considers unsuitable for ultrathin sections. It is to avoid hackle marks that Persson (1965) and Sutton (1969) recommend slow crack propagation.

SOME PRELIMINARY EXPERIMENTS IN THE USE OF RALPH KNIVES

Experiments were carried out using Ralph knives made with a prototype of the LKB HistoKnifeMaker. All the experiments described here used knives made from 38-mm glass strip. We cut blocks made with three different types of plastic and a range of different paraffin-based waxes. Blocks were produced so that each embedding medium was represented by at least one block containing a fixed tissue and one containing no tissue. Microhardness measurements were made of each block, and as the range of hardnesses we used was found to encompass that given by Wachtel et al. (1966, p. 187) we feel that this compensates for not using a greater range of plastics (a more detailed paper is in preparation).

Knives were visually assessed in the fashion described by Reid (1975). We used virtually all the knives we broke, the philosophy being that knives with excess hackle marks could be used for trimming and for thick (3 μm or more) sections. Such knives normally could cut 1-μm sections, the degree of section scratching permitted, however, depends on the requirements of the user. When a certain amount of scratching (which does not affect the bright-field image unduly) is allowed, the entire edge is regarded as usable (i.e. 38 mm). If perfect sections are required, knife selection should be as rigorous as for electron microscopy. Although we have not experimented with this use, from visual assessment the best knives had over 50% hackle-free edge, and may have been suitable for ultramicrotomy.

A hand-cranked Jung rotary microtome with a specimen advance range of from 1 μm to 40 μm (the range from 1 μm to 10 μm being in 1-μm increments) was used for most experiments, but we have also used a Cambridge rocker microtome and a Leitz base-sledge microtome. Most of the knives were used immediately after breaking, but we found that knives which had been made up to a fortnight before use and then stored in staining jars had not deteriorated (glass as a supercooled liquid continues to flow) for section cutting in the range we were investigating. Blocks for sectioning were glued to hardwood blocks with cyanoacrylate glue ("Loctite") and the hardwood then clamped in the microtome jaws.

Attachment of knives

Bennett et al. (1976) used Ralph knives in standard microtomes by cementing them to steel strips which were of the approximate proportions of steel knives and which would fit into the knife clamps of the microtome. The LKB knifeholders have a profiled knife shape with dental wax (e.g. Econowax, Dental Manufg. Co., U.K.). We underneath. They recommend attachment of the knife to its holder with dental wax (e.g. Econowas, Dental Manufg. Co., U.K.). We also recommend this method of attachment. Szczesny (1978) uses a holder in which the knife is clamped to a steel adapter strip. We used a similar holder initially, but found that it had no great advantage except perhaps in the case of cryostat work (see later) or, as Szczesny intends, when only one holder is available (speed of knife change). We normally use two holders, so that when one knife is in use, another knife is setting in position.

Our holders consist quite simply of lengths of 1"×$\frac{1}{4}$" Duraluminium bar (this metal has favorable heat-conduction properties). The holder is placed on a hotplate at 80 to 90 °C. A piece of dental wax, about the size of a match head, is then placed on the holder in the region where knife attachment is intended. The wax melts and spreads. A Ralph knife is then placed on this region and allowed to reach the temperature of the holder. The holder with knife is then removed from the hotplate and allowed to cool (about 5 min). For the first minute or so of cooling it is possible to move the knife in relation to its holder if its position is not quite correct.

There are sound engineering reasons for using a profiled holder (i.e., knife-shaped). Our type of holder applies a force to the knife clamp screws in a direction other than that for which the clamps are designed. The screws are likely to weaken if use is prolonged. With the knife-clamp forces correctly directed (as in the LKB adapters) the attachment of the Ralph knife to the microtome is probably more rigid and therefore less likely to produce chatter when sectioning hard material.

Cutting

In our cutting tests we used a knife clearance angle of 5° and a range of knives with edge angles from 40° to 70°. We found that dry sections cut with knives whose angles were in the lower (40° to 55°) range had less recoverable compression than those cut with knives from the higher range. With the data at present available, we find no special advantage in the higher-angled knives (we first thought these might be more durable).

Knives were tested by first cutting sections with the specimen advance setting at 1-μm. A series of 500 sections was then cut with the advance set at 5 μm and then once more 1-μm sections were cut.

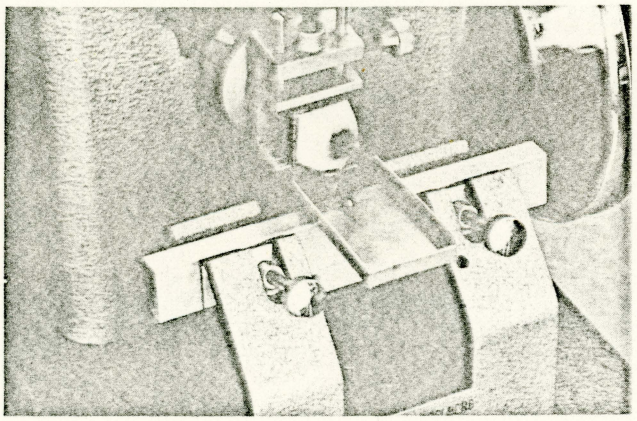


Fig. 6. The microtome used in the experiments described in the text. A trough is fitted but was not in use when the photograph was taken.

Sections from before and after the 500 series were compared. With the G.M.A. and P.M.M.A. embedded tissue (see below) we found no detectable differences as regards ease of sectioning, marks on sections, or compression. Wax similarly showed no edge deterioration. With hard Araldite blocks the edge deteriorated rapidly, and after about one hundred 5-μm sections (area = 1.5 cm × 3 cm) the number of scratches on 1-μm sections was considerable. The edge was not sufficiently strong to give acceptable 1-μm sections after a 500 series.

Araldite

The Araldite blocks used in these experiments were prepared according to the 1974 standard mixture given in Glauert (1975, p. 132 = Araldite CY212 10 ml, D.D.S.A. 10 ml, B.D.M.A. 0.4 ml). The largest area of section which we have cut with this medium to date is 1.5 cm×3 cm at 1 μm.

We cut sections either dry or onto water in a trough (see Fig. 6). Trough-cut sections were transferred to a microscope slide by bringing the slide up from underneath the section in a technique similar to that used by histologists to pick up wax sections from a waterbath. The slides were then flooded with distilled water and treated as are the dry-cut sections (see below). Dry sections tended to show a considerable amount of recoverable compression. They were transferred with watchmaker's forceps to clean slides which had been flooded with distilled water. The flooded slides (with trough-cut or dry-cut sections) were placed on a 80 °C hotplate and the sections watched until they were expanded fully. The slides were then removed to a second hotplate at 40–50 °C, where final drying occurred. We found that if slides were allowed to dry on the high temperature hotplate, bubbles of steam became trapped between section and slide causing incomplete attachment and unsightly wrinkling.

We stained our sections with 1% Toluidine Blue in 1% aqueous borax solution (Pease, 1964) and store the slides with and without coverslips. We find that the mounting media D.P.X. and Euparal cause some detachment or wrinkling of coverslipped sections. Euparal also causes the toluidine stain to fade after about two days. Ameele (1976) recommends a final drying at 100 °C to prevent section detachment when using xylene-based mounting media. Roppel & Mabie (1972) developed a special drying apparatus for attaching epoxy-embedded insect tissue sections which tended to lift from the slide in hot staining solutions. Anker et al. (1974) used a mounting medium 'Malinol' to attach very-large-area, thick epoxy sections to slides. We have found that heating the slide at 80 °C for an hour or so after complete drying (at 40 to 50 °C) diminishes the amount of wrinkling and detachment caused by D.P.X. Burstones P.V.P. mounting medium does not cause any wrinkling or detachment (25 g P.V.P. in 25 ml of water overnight + 1 ml of glycerol). However it bleaches toluidine-blue stain after about four days. Humphrey & Pitman (1977) have investigated the influence of mounting media on the fading of basic aniline dyes in epoxy-embedded tissues, and reference to this paper and some experimentation to find an individual preference may be advisable.

We can summarize our cutting experience with Araldite by saying that we had no difficulty with section cutting. The edge will not cut as many sections as the other media tried without some deterioration in quality, but this is fairly usual with knife edges and Araldite. Ralph-knife sections were invariably less scored than sections we cut with freshly sharpened steel knives. (See Figs. 7–9.)

Glycol methacrylate

(also known as G.M.A. or 2-hydroxyethyl methacrylate or H.E.M.A.). Sections of a glycol methacrylate/polyethylene glycol embedding medium (Ruddell, 1967) designed for light microscopy were cut both dry and onto water in a trough. Dry sections were taken from the knife edge with watchmaker's forceps and floated out on a 60 °C waterbath. Trough sections were removed using the method described for Araldite trough sections and refloated on a 60 °C waterbath. No difficulty has been experienced with G.M.A. section flattening. Sections were removed from the waterbath by bringing a glass slide up from underneath the section as described for Araldite. Slides are dried on a hotplate at 80 °C.

The experience of one of us (P.R.) makes us feel that it is necessary to make a note of caution concerning G.M.A. media. Firstly they cannot be removed from the section, and in conjunction with this, unless the monomer is pure the medium tends to take up stains. Secondly, again if the monomer is not pure, polymerization will give blocks of unpredictable hardness. Tippett & O'Brien (1975) give a simple purification procedure for monomer batches. Cole & Sykes (1974) compared the polymerization performance of different commercial G.M.A. monomers. Our view is that a purification method like that of Tippett & O'Brien is needed for all G.M.A. monomer batches.

The extensive literature of glycol methacrylate/polyethylene glycol embedding media for light microscopy is reviewed by Cole & Sykes (1974) and Bennett et al. (1976).

Methyl methacrylate

A methyl methacrylate (P.M.M.A.)/polyethylene glycol medium modified from Hirsch & Boellaard (1958) was used. The original article on this method is in German, but the method is translated into English by Drury et al. (1967) and by Ruthmann (1970). The main modification used here is that polymerization is by exposure to ultraviolet light rather than heat. It has been found that this prevents bubble formation in and around the tissue. Bubble formation was a major problem in the original method. (A full description of this modification is in preparation.)

Generally we found that this medium sectioned very well and easily. We did have difficulties however in completely flattening large-area sections (above 1 cm square). The best method of flattening that we have found to date is to place sections in a dish in which ether (diethyl ether) has been layered upon water (in a fume cupboard, no naked lights!). The sections sink slowly through the ether layer and flatten on the ether/water interface, which has a low surface tension. The sections are then removed by bringing a microscope slide up underneath in the standard technique used for picking up wax sections. Fluid is drained from the slide, which is then reflooded with a mixture of 100 parts 95% ethanol to 20 parts of 2-butoxyethanol. The slide is then dried on a hotplate at about 50 °C. If an adhesive is required, the slide can be coated with Mayers' albumin and dried (about 20 min at 60 °C) before the section is picked up on it. (Note. No advantage is gained in using ether/water flattening when the area of the section is below 1 cm^2.)

Wax

The wax-type media used were: Paraffin wax, Paraplast, Ralwax, Paramat, Esterwax 1947, and Esterwax 1960. We dispensed with our set clearance angle of 5° and altered the angle to suite the behavior of the particular block being sectioned. When the optimum knife position was reached, the waxes behaved much as they do with steel knives and formed ribbons. There is some danger that a glass surface favors the adhesion of wax to it. It offers a larger contact area to the section than does a ground metal surface, where only roughness peaks touch the section. Our first experiments looked ominous. However, when the wax blocks were cooled beforehand (this seems to be a routine histological practice) and care was taken to make sure that both the knife and its holder had properly cooled after knife attachment, no further difficulties were experienced. It was found best to hold the ribbon away from the back of the knife edge just to make sure that sections did not stick to the glass. We also cut section onto water in a trough, but generally this seemed an unnecessary luxury.

Cryostat

Some preliminary experiments have been carried out using Ralph knives in a cryostat. The first problem we experienced was one of knife detachment; dental wax did not appear to provide effective adhesion of knife to holder at −25 °C. However, at the 'Micro 78' exhibition in London, knives in LKB holders used in a Bright cryostat were quite effectively fixed with dental wax. It would seem that

the holder's 'ledge' support to the knife helped here. The fact remains, however, that dental wax is less effective at low temperatures, and the knife may become detached. It is perhaps in this situation that a Szczesny holder (see above) could have advantages. We have overcome this attachment problem by using cyanoacrylic glue, but removal of the knife then becomes a problem!

The actual sections cut well (the material included undecalcified bone!), but further experiment is needed to find the best method for collecting them. The 'anti-roll plate' could not be used in our system, although, interestingly, the sections seemed not to roll. It is suggested that some adhesive technique (see, for example, Maxwell & Ritchie, 1971) might be required.

DISCUSSION

Paraffin wax has been the traditional embedding medium of the histologist for the last one hundred years. Most present-day histological knowledge has been obtained by the examination of material which was sectioned in wax. Paraffin wax has the great advantage that it forms ribbons for serial sectioning, but it has a number of disadvantages which become particularly evident when high-resolution light microscopy is undertaken. These disadvantages center around (1) the crystalline structure of the wax block which makes it difficult to section much below 3 μm, and (2) poor infiltration of tissues which have been fixed with noncoagulant fixatives.

Fixation

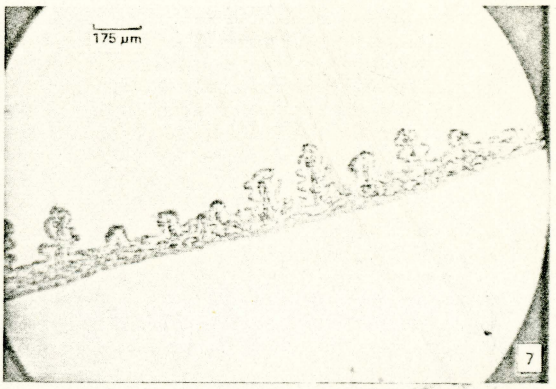
Baker (1966) expressed disappointment that as good a cytological fixative as osmium tetroxide should produce tissue blocks with such poor resistance to the distortion caused by embedding in paraffin. Paraffin penetrates tissue properly only if the proteins of the cells have been coagulated into a mesh. Baker's view is that this feature is in all probability the reason that coagulative fixatives have retained their popularity with histologists. O'Brien et al. (1973) examined living plant cells under the phase-contrast microscope. They then fixed these cells with different coagulant and noncoagulant fixatives and examined them under the electron microscope. They found coagulant fixatives generally so destructive that they have recommended that their use should be discontinued wherever possible. Buckley (e.g. Buckley, 1973) has carried out similar studies using cultured animal cells. Deutsch & Hillman (1977) have compared the shrinkage caused by different fixatives. It would appear from an analysis of their information that the noncoagulant fixatives cause less shrinkage. Pease (1964), when referring to the examination of semithin sections of material prepared for electron microscopy, states that if the conventional light microscopist appreciates that he is seeing better preserved tissue than he has ever looked at before, 'his experience will be more meaningful'.

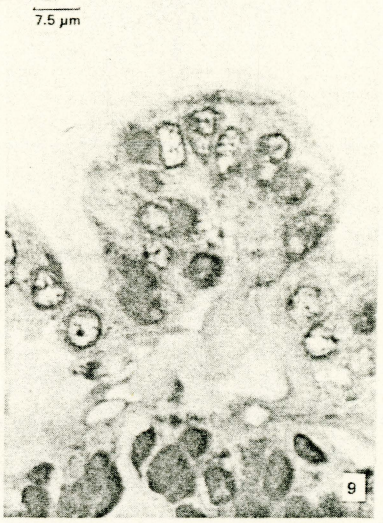
Paraffin is a poor embedding medium when used with most noncoagulant fixatives, and unfortunately these are the best fixatives.

Thin sections and 'difficult' objects

If a color transparency is held up to the light or projected, hopefully a clear image will be seen. If, however, three or four transparencies are bound together and then subjected to the same display processes, not only is the image darker (this is unimportant in this particular analogy), but if each transparency is even slightly different, a very confused image results. Wachtel et al. (1966) define a similar phenomenon for microscope images, thus: 'Specimen inhomogeneities above the object plane cause image deterioration in both systems' (condenser and objective) and 'Specimen inhomogeneities below the object plane cause the deterioration in the imaging of the condenser annulus.' In phase-contrast microscopy Ornstein & Pollister (1952) have found empirically that it is essential that sections should be no thicker than 1 μm if maximum resolution is to be achieved. With bright-field microscopy, specimen thickness is not quite so important, however, bright-field objectives have a finite depth of field (7 μm for $\times$10, 2 μm for $\times$20, 1.5 μm for $\times$40, and about 0.5 μm for $\times$100), and thin sections should provide better resolution.

Embedding media provide support for tissues during sectioning. If part of a tissue is harder than the surrounding tissues or the embedding medium, it will tend to be pushed through the rest of the section by the advancing knife edge. This causes damage. With such 'difficult' objects it is generally considered necessary for the embedding medium to be at least as hard as the hardest region of the tissue; this is not always possible with paraffin wax. Barnett (1971) recommends that the methods of electron microscopy embedding be considered when the preparation of material for light microscopy has proved difficult or impossible by other means. This is a suggestion which many workers had already followed and which they will continue to do, e.g. plant tissue (Feder & O'Brien, 1968), arthropod material (DeGuisti & Ezman, 1955; Rohde 1965), bone (Xipell et al., 1974).





Figs. 7–9. Bright-field photomicrographs of a section of guinea-pig ileum fixed in cacodylate-buffered glutaraldehyde. The section was cut at a setting of 3 μm and heavily stained with 1% Toluidine Blue to photograph clearly at the lowest magnification. Sections of the same dimensions (3 cm×1.5 cm) with the specimen advance set at 1 μm could be cut with equal facility. The embedding medium was Araldite.

Light microscopy as an adjunct to electron microscopy

It is ill advised to attempt ultrastructural work without complete familiarity with the light-microscope structure of the material. O'Brien et al. (1973), for fixation reasons already referred to, are somewhat scornful of 'parallel'-light microscope investigations with paraffin-embedded coagulant-fixed cells when the electron-microscope studies are being attempted on noncoagulant-fixed epoxy-embedded material. DeBruijn & McGee-Russell (1966) recognized a 'gap' between light and electron microscopy and pioneered techniques by which large-area tissue blocks could be prepared for electron microscopy and small regions for final ultramicrotomy selected with the light microscope. Grimley et al. (1965) prepared human and

other mammalian tissues in the form of glutaraldehyde 'immersion'-fixed tissue blocks measuring 2 cm × 2 cm × 0.4 cm, for thin light-microscope section survey before ultramicrotomy of selected areas. Boatman & Lowe (1971) used a photomicrographic technique to pinpoint areas of whole mouse lung lobes for ultramicrotomy. Jones et al. (1977) recommend perfusion fixation to obtain large tissue blocks for epoxy embedding.

Wax versus plastic

There is no standard plastic for electron microscope embedding. Glauert (1975) lists 38 epoxy mixtures suitable for T.E.M., nine polyester mixtures, and seven water-soluble plastic media. A 'casual' reprint collection by one of us contains 26 papers on methacrylate media (mainly 'softer' modifications for light microscopy). Comparison of the properties of some of these plastics with paraffin wax is made below:

(a) Speed of the embedding process

At present, the polymerization of plastic blocks is not as rapid as the hardening of molten wax. However, Kimmel & Jee (1975) have used a rapid setting methyl methacrylate medium whose formula bears a resemblance to the quick setting 'bone glue' used in many hip implant operations. Sims (1974) has described a G.M.A. medium which polymerizes in 30 to 45 minutes. We have little doubt that the plastics technology exists for rapid distortion-free embedding, it remains for some biologist to communicate with a materials scientist and apply it.

(b) Dehydration

Paraffin is water-insoluble, and tissue requires a dehydration process before it can be embedded. Boyde (1978) has described how dehydration affects tissue size. Several water-soluble plastic embedding media exist, and with these dehydration can be avoided.

(c) Heat labile substances

Paraffin embedding requires that the tissue be infiltrated at a temperature of (depending on the wax) between 50 and 60 °C. This temperature will, for example, denature most enzymes (Lehninger, 1975). Although most epoxy resins are infiltrated and polymerized at around 60 °C, there are several plastic media which can be infiltrated and polymerized in the cold.

(d) Ribboning

Paraffin sections form ribbons which are of particular advantage when it is necessary to make three-dimensional reconstructions of tissues. Rohde (1965) and Greany & Rubin (1971) amongst others have made modifications to plastic media to allow section ribboning. Perhaps no plastic modification is as yet as effective a ribboning substance as wax.

(e) Shrinkage

All the plastic media used in electron microscopy cause less shrinkage than does paraffin wax.

(f) Staining

Numerous authors report the superiority of epoxy embedding in electron microscopy where stability in the electron beam is important. As Bennett et al. (1976) have emphasized, however, when methacrylates polymerize they do not link with the tissue covalently. Consequently almost the full complement of dye-binding sites remain available in methacrylate-embedded tissues. This is not the case with epoxy embedding. With methacrylates, therefore, most of the classical histological stains can be used. Davie (1970) has listed the literature on histological stains applicable to resin-embedded tissue. Alsop (1974), Gori (1977), Jones et al. (1977), and Lewis & Knight (1977), give more recent light-microscope staining methods for epoxy-embedded material. With the blossoming of electron-microscope autoradiography, immunocytochemistry, and cytochemistry in recent years, we can conjecture that there are now more techniques available for identifying the chemical constituents of plastic-embedded material than there are for wax.

CONCLUSION

There can be little doubt that the techniques used to prepare tissues for electron microscopy give better preservation than those techniques standard to histology. In these good preservation techniques, plastics are essential. Although we hope it will not be taken as a controversial 'slight' on their knife-sharpening abilities, we think that many histologists believe plastics play havoc with steel-knife edges and should be avoided as much as possible. Although the triangular glass knives of Latta & Hartmann (1950) have been used in standard microtomes (e.g. Shaw 1977), 1 mm square sections collected on fluid-filled troughs seem perhaps a little esoteric in the average histological laboratory? Tungsten-carbide knives are an improvement on steel knives, but their expense (both to purchase and to sharpen) probably makes the histologist reluctant to use them for anything other than the most difficult problems (e.g. undecalcified bone). Ralph knives need bring about little change in the histologists' technique, they can be used for wax blocks as well as for plastic ones, and they do not have to be sharpened.

The traditions of one hundred years of wax embedding cannot and should not be thrown aside too hastily, but the feedback of improved preservation from electron microscopy and the need for genuine comparative light and electron microscope studies suggest that the age of plastic histology is near. Ralph knives, which can adapt the old apparatus as well as fit the new, might help introduce this new age smoothly.

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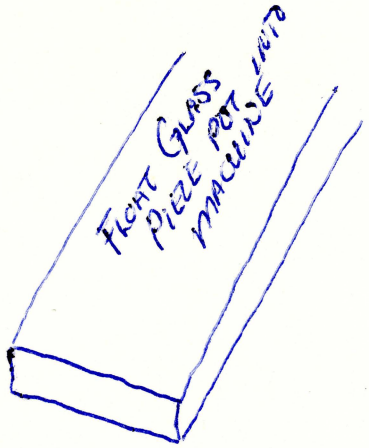

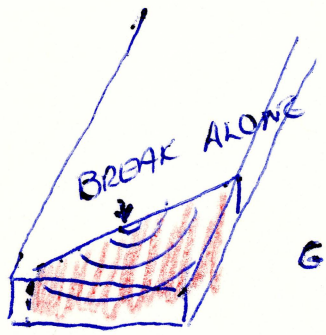

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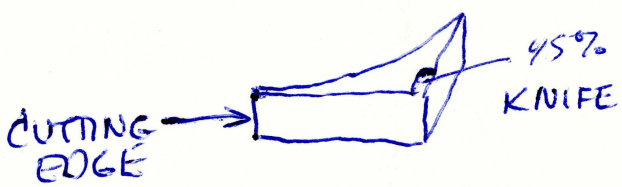

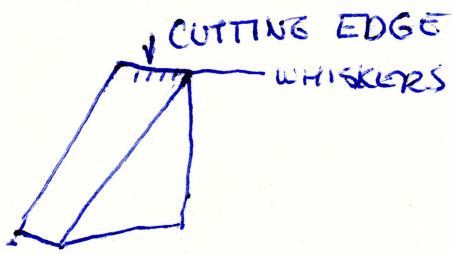

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