A COMPARISON BETWEEN DIFFERENT MOUNTING TECHNIQUES COMMONLY EMPLOYED IN ACAROLOGY

BY

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Acarologists are frequently distressed to find that some slide preparations of acarine material have become ruined in time, due to the aging and curing of the mounting medium. This is especially disturbing if an important type specimen is involved. Frequently one is exasperated by the fact that certain important morphological structures cannot be clearly interpreted in some slide preparations.

The recurrence of these inconveniences prompted this author to attempt to determine the reasons behind the apparent failure of many of the mounting techniques commonly employed in acarology.

NEWELL (1947) discussed Hyrax as a mounting medium for mites, stating that Berlese fluids, glycerine jelly, and balsam were unsatisfactory mounting media. NEWELL also described a way to make « permanent » preparations in glycerine. These techniques are still in use today.

CLARK and MORISHITA (1950) described the use of C-M mounting medium, which they claimed to have superior refractive qualities and permanence.

MITCHELL and COOK (1952) described the use of glycerine-jelly preparations in studying water mites. Their double-mounting method is an improvement over Newell's glycerine mounting technique.

BEER (1954) discussed the use of Hoyer's, Hyrax, balsam, and polyvinyl alcohol and lacto-phenol (PVA-LP) as mounting media. His conclusions, still adhered to today, were that PVA-LP constituted the most satisfactory mite mounting medium.

PRITCHARD and BAKER (1955) indicated a preference for Hoyer's over PVA-LP, Hyrax, balsam, and methyl-cellulose fluid (C-M mounting medium), Most European workers also use modified Berlese fluids (gum-chloral, Andre's = Hoyer's, de Faure's, etc.) (GRAY and WESS, 1950).

EVANS et al (1961), following GRANDJEAN (1949), prefer to make temporary mounts in lactic acid. Specimens are stored in vials of lactic acid or alcohol.

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A comparison of the advantages and disadvantages of the most commonly employed mounting media (Hoyer's, de Faure's media, PVA-LP, Hyrax, glycerine-jel, glycerine, balsam, and C-M medium) and techniques appears in order at this point. Comparisons will be made under four appropriate categories : 1) Clearing of specimens in preparation for study ; 2) index of refraction of the mounting medium ; 3) convenience and practicality of the techniques employed ; and 4) permanence of the final product.

1. — *Clearing of specimens.*

The specimen must be rendered transparent in order for cuticular structures to be observed through transmitted light. This is accomplished by chemically macerating and dissolving away the body tissues and products. The most generally accepted methods for accomplishing these ends involve the use of lactic acid, lacto-phenol, or various chloral hydrate solutions (Andre's, Nesbitt's, etc.). Most aqueous mounting media include clearing agents as ingredients so that delicate specimens may be mounted and cleared simultaneously.

Clearing specimens in chloral hydrate solutions includes a considerable element of risk, especially when used by inexperienced personnel. Specimens left in concentrated solutions, heated or cool, for extended periods of time will disintegrate due to an undermining of the cuticle. The advantage of these fluids (especially Nesbitt's) lies in their rapid clearing action. Specimens placed in a dish of Nesbitt's, heated to near boiling and then cooled are usually ready for mounting within a few minutes. Specimens should not be allowed to remain in Nesbitt's for more than about 1 hour, but instead, transfering them to 70 % alcohol plus 3 % glycerine in an unstoppered vial. By this means the specimens become glycerine infiltrated for storage and subsequent mounting.

Lactic acid or lacto-phenol combinations tend to act more slowly but will not damage the cuticle. Specimens may be stored in lactic acid indefinitely.

The disadvantage of the lactic acid method is that alcohol-fixed specimens tend to burst if heated in lactic acid (this tendency may be reduced by employing the clearing fluid described by Evans and Browning, 1955). Clearing requires more time, and specimens must be washed clean of the clearing solution to avoid crystallization after mounting in gumchloral media.

The use of KOH or NaOH is not recommended. These materials are dangerous to handle, take too long to clear, weaken the cuticle, and require thorough washing of the specimen before mounting.

2. — *Index of refraction.*

A critical study of the minute details of cuticular structures involves the following three considerations : 1) the refractive index of the mountant ; 2) the thickness of the preparation ; and 3) the type of microscope commonly used, either phase-contrast or transmitted light.

Index of refraction is defined as the ratio of velocity of light in a vaccum to the velocity in any given substance, determined by a measure of the sine of the angle of incidence divided by the sine of the angle of refraction. The index of refraction varies slightly with changes in temperature and wave length of light used. However, these changes are considered inconsequential, since the index of refraction of the mounting medium and that of the mounted specimen will both be altered, thereby not noticeably changing the relationship between specimen and medium.

The arthropod integument has been found to vary between 1.50 and 1.59. Epicuticle, the densest external portion of the integument, has an index between 1.56 and 1.59. The less dense portions, varying from 1.50 to 1.53, include the endocuticular layers, while the exocuticle varies somewhere between 1.53 and 1.56 (RICHARDS, 1951). In order for unstained exoskeleton to appear distinct from the surrounding mountant, it must have a refractive index different from that of the mountant. Cuticular structures are rendered distinct by the fact that they bend or scatter light at different angles from that of the surrounding medium. Either the membranous or the heavily sclerotized cuticular portions, or both of these cuticular components may be made optically distinct by the selection of a mounting medium with an appropriate refractive index. However, since the refractive index of the cuticle is a variable factor, varying with the specimen and the structures studied, the results obtained with different mounting media will be found to be relative.

The following is a partial listing of chemicals and mixtures which are commonly employed by acarologists, with respect to their refractive indices :

Water	1.33	(Chemical Rubber Co., 1958)
Lactic Acid	1.44	»
Glycerine	1.47	»
Gum Arabic	1.47	»
Xylene	1.50	»
Immersion Oil	1.515	»
Glass Slides	1.51	»
Gelatin	1.52–1.53	»
Balsam	1.53	»
Phenol	1.54	»
Chloral Hydrate	1.54–1.60	»
Hyrax	1.71	(NEWELL, 1947)
C-M Fluid	1.428	(CLARK and MORISHITA, 1950)
PVA-LP	± 1.44	(SALMON, 1951)

When two miscible fluids with different refractive indices are mixed, the refractive index of the resulting mixture falls between the two extremes. Addition of water (1.33) to lactic acid (1.44) will result in a mixture with an index greater than 1.33 but less than 1.43. As an aqueous mounting medium dries, its refractive index is altered by the loss of water. The refractive index for any aqueous medium is therefore relative to the composition of the mixture and to the degree to which drying has been allowed to proceed. This same principle applies to synthetic resins or balsam dissolved in xylene.

I have made temporary mounts of the larvae of a small species of *Euschongastia* (Trombidioidea) and females of *Macrocheles glaber* (Macrochelidae) in the following media with known refractive indices : water (1.33), lactic acid (1.44), glycerine (1.47), xylene (1.50), immersion oil (1.515), balsam (1.53), and molten phenol (1.54). An attempt was made to maintain a standard of uniformity in the clearing and infiltrating of specimens into the respective media. Any alteration of the refractive index of the cuticle caused by a reaction of the cuticle with the mounting medium had to be ignored, since these alterations could not be interpreted under the conditions of this crude experiment. A visual comparison was made between specimens mounted in media of known indices, with other specimens mounted in Hoyer's, deFaure's, modified Hoyer's solutions, glycerine-jelly, PVA-LP, balsam, C-M medium, and Hyrax. Observations were made with phase-contrast and transmited-light microscopy.

In this experiment, the degree of clarity of the following structures was assessed : *Euschongastia* — dorsal and ventral idiosomal striae, chelicerae and palpal claws, scutum and sensillae, solenidia and microsetae, and the appendicular scobalae. *Macrocheles* — apical setae and setal bases of tarsus I, dorsal and ventral idiosomal setae, chelicerae, deutosternal teeth, tritosternal laciniae, and unsclerotized integument of the sternum.

The results indicate that a mounting medium with an index of refraction less than 1.50 or greater than 1.55 will cause cuticular structures to stand out in sharp contrast to the background. As the index is progressively reduced from 1.50, or increased from 1.55, the degree of contrast increases. A result of this effect is that, while the viewing of some uppermost structures of the mounted specimen may be accentuated, ventral and lateral structures may be obscured by excessive light interference. This is especially pronounced in thick preparations. A heavily sclerotized structure, uncleared translucent tissue within the exoskeleton, or the presence of dust particles, will often obscure adjacent structures. Hyrax, lactic acid, glycerine-jelly, glycerine, deFaure's medium, C-M medium, and PVA-LP fit into this category. Mounting media with refractive indices approaching 1.51 to 1.54, may in some cases show a slight reduction in resolution, with respect to the viewing of membranous cuticular structures. However, dust particles, heavily sclerotized structures, and other obstructions do not tend to obscure the viewing of taxonomically important structures. Structures may be viewed with less distortion due to a lack of excessive light reflection and refraction. Hoyer's and balsam fall within this category. Gum chloral fluids have been found to vary in the refractive effects produced on arthropod cuticle, depending on the composition of the mountant. Specimens mounted in Hoyer's show a clarity in structure simulating specimens mounted in xylene for fresh slide preparations, and immersion oil for dry slide preparations. A refractive index of 1.515 (immersion oil) was found to cause about the least amount of refractive interference of delicate structures, making these structures difficult to interpret. By reducing the amount of chloral hydrate from Hoyer's formula of 200 grams (refer to recipes section below) to 100,

or 150 grams, the refractive index of the ensuing medium was lowered, producing more favorable refractive and reflective contrast in both fresh and cured mounts.

It was found that interference caused by excessive refraction and reflection induced by media with refractive indices far from that of the cuticle could be reduced by making very flat mounts. This has been found to be of value in mounting minute and thin-skinned prostigmatid mites, and dissected structures. Flattened specimens tend to show some deforming of structures, as well as causing difficulty in distinguishing upper from lower surfaces of the specimen. Thicker preparations cause less distortion and allow a clearer interpretation of structural relationships. Some of the advantages inherent in the techniques employed by GRANDJEAN are available in thicker preparations. Minute structures (Chelae, pretarsal structures) are best dissected out for clearer interpretation. Mountants with refractive indices between 1.50 and 1.55 are valuable for making thick preparations.

The type of microscope employed may influence the type of medium preferred, with respect to the refractive index of the medium. Phase-contrast microscopy, as is well known, accentuates minor variations between adjacent structures with slightly different refractive indices. This is done by allowing only that light which is refracted or absorbed by the mounted object to be focused at the eyepoint. Background light is dispersed by the joint action of an annular diaphragm and a diffraction plate (see BENNETT et al., 1951). Transmitted light microscopy, on the other hand, requires a somewhat greater contrast between the refractive index of the specimen and the surrounding medium in order to accentuate minute cuticular details, since both refracted, reflected, and background light are focused at the eyepoint.

The effect of differences in refractive index of the mounting medium, thickness of the preparation, and type of microscope used, all subtly influence the degree of clarity by which given cuticular structures can be interpreted. Also, the degree of clarity varies with different structures viewed, so that the clarity of one structure viewed under one preparation would not guarantee that structures of slightly different refractive index on the same specimen would be viewed with equal facility.

The following generalizations are noteworthy : all the preparations studied, as well as the two types of microscopes used, were found to be adequate for general acarological work. Specimens mounted in media approaching the refractive index of cuticle (1.50), such as Hoyer's, produced less distortion of structures viewed, and are best viewed under phase-contrast microscopy. If phase-contrast microscopy is not available, then it is found advantageous to use mounting media with refractive indices lower than 1.50 or greater than about 1.55.

3. — *Convenience and Practicality.*

It is generally accepted that cleared acarine material should not be subjected to excessive handling, since taxonomically important structures may be destroyed or damaged. Aqueous media are preferred since specimens may be mounted directly

from alcohol, water or clearing fluids. Specimens remain pliable in aqueous media and may be more safely handled. The technique of preparing specimens for microscopical analysis should take as little time and equipment as possible, since, after all, the mounting technique is a means to an end and not an end in itself. Finally, the specimen should be mounted in such a way that will allow the researcher to remount the specimen in order to study all the important details of its morphology. This has been found to be important when new taxonomic characters are discovered and old specimens require remounting and dissection in order to best view all necessary structures.

The technique preferred by GRANDJEAN and EVANS et *al* is of considerable value in obtaining an undistorted view of the specimen. A great deal of information is available through such a technique. Regrettably, this method is tedious, requiring excessive specimen handling.

Mounting in Hyrax requires far more time and equipment than appears necessary, especially since the preparations obtained do not show any superiority over the results obtained through less tedious means, in glycerine-jelly or C-M medium. Balsam and synthetic mounting resins are also included in this category.

The following mounting media are listed in *increasing* order of ease of use and practicality : Lactic acid, glycerine, glycerine-jelly, PVA-LP, C-M fluid and gum-chloral media. Lactic acid and glycerine are at best temporary mounting media, requiring props and a sealing compound to keep the coverslip in place. Better results can be expected with other media.

Glycerine-jelly requires a certain degree of experience and manual dexerity in order to obtain good results. With diligence, very fine preparations can be expected. Glycerine-infiltrated specimens give the best results. Preparations must be sealed immediately, preferably employing the double-mounting technique (MITCHELL and COOK, 1952).

PVA-LP, although being relatively easy to use, is notably unpredictable (see LIPOVSKY, 1953). In addition, specimens are often ruined through excessive flattening during drying. Thick-mounted preparations of delicate specimens often result in a shrivelling of appendages during curing. Old preparations are often impossible to remount, since the dry PVA-LP will not re-dissolve.

Hoyer's and C-M media tend to be the most practical, from the stand-point of ease of use and practicality. Consistently fine preparations can be expected with a wide range of uses.

The technique of mounting specimens on two coverslips, one round and the other square, and then placing the mounted specimen into an aluminum holder, has been used to some advantage with dark and thick preparations. This technique originated in Nematology, although it is being used by some acarologists.

4. — *Permanence.*

Balsam, synthetic resins, Hyrax, and PVA-LP seem to show the greatest degree of permanence, requiring no further care of the specimen. Hyrax, however, has a

tendency to shatter when the slide is dropped. PVA-LP flattens the specimen, in some cases with disasterous results and the coverslip may detach from old PVA-LP preparations should the slide be dropped.

Some of the other media suffer from problems of crystallization and evaporation. Often, the effect of minute air incursions under the coverslip in Hoyer's preparations is erroneously labeled as crystallization.

Glycerine-jelly mounts are rendered permanent by the double-mounting technique discussed by MITCHELL and COOK. Crystallization has not been a problem.

C-M medium allows specimens to be introduced from a lactic acid or lacto-phenol clearing solution without danger of subsequent crystallization during aging. This preparation seems to be a very versatile mounting medium if a greater refractive contrast is desired.

Hoyer's medium seems to be the most popular of the many modified Berlese fluids. Preparations made with Hoyer's are at times unreliable. Slight crystallization of the chloral hydrate component is not uncommon. Specimens cleared in lacto-phenol must be washed prior to mounting, otherwise lactic acid and phenol crystals will develop during curing of preparations. In addition, air may be drawn under the coverslip. All these undesirable qualities of Hoyer's can be prevented by following certain precautions. Reducing the amount of purified chloral hydrate to between 100 and 150 grams (refer to recipes section below) will prevent the problem of crystallization. The chloral hydrate component gives gum chloral fluids their powers of penetration and clearing. Any further reduction of this component, to less than 100 grams (deFaure's fluid uses 50 grams), will result in a tendency for the specimen to shirvel and for uncleared material to interfere with proper examination of the specimen. Specimens are best cleared in Nesbitt's if washing out of lactic acid is indesirable.

The problem of air incursion under the coverslip is a direct result of oven drying of fresh preparations. Oven drying causes the coverslip to assume a buckled, convex shape, due to an unequal drying of the medium at the edges of the coverslip. These stresses later tend to rupture ringing compounds and draw air under the coverslip. The presence of scratches on the coverslip will sometimes cause the coverslip to break under this stress. By heating the slide only long enough to set the specimen (up to 2 days at 50°C, higher temperatures will darken C-M medium) and then air drying (in a horizontal position), the preparation, if not excessively thick, will be permanent under most climatic conditions. *Thoroughly dried* preparations may be ringed with " Zut ", " Glyceel ", or other suitable ringing compounds. Ringing slides which are not suitable dry will result in air incursions if the ring becomes cracked or is punctured. The sealing compound allows for the use of immersion oil and subsequent washing of the glass surface without contamination of the mounting medium.

Conclusions.

On the strength of these findings, it is concluded that a modified Hoyer's (= André's) medium, viewed under phase microscopy, is one of the most practical media currently available to the acarologist. C-M medium is also of considerable value, especially for soft-bodied specimens and if transmitted-light microscopy is used.

The mounting technique is only partly a science, to a great extent it is also an art. As such, acarologists will continue to experiment and develop new methods.

RECIPES [1]

I. Partial list of useful clearing solutions :

1. *Nesbitt's clearing fluid.*

Distilled water	25 cc.
Chloral hydrate	40 gr.
HCL (conc.)	2 drops

(HCL may be deleted without apparently influencing the properties of this solution. Heat until fuming, but avoid boiling. Avoid prolonged exposure of specimens to this solution.)

2. *Chloral-Phenol.*

Phenol (molten)	1 part
Chloral Hydrate (saturated aqueous solution)	1 part

3. *Lactic acid* (85 %).

4. *Lacto-Chloro-Phenol.*

Lactic acid (85 %)	20 ml.
Chloral Hydrate	2 gr.
Phenol (molten)	20 ml.

5. *Lacto-Phenol.*

Lactic acid (85 %)	2 parts
Phenol (molten)	1 part
Distilled water	1 part

6. *Evans' and Browning's Fluid.*

Lactic acid (85 %)	1 part
Glycerine	1 part

1. Always employ highly purified chemical ingredients, preferably from fresh stock.

II. Partial list of useful mounting media :

1. *Modified Hoyers's (= André's) fluid.*

Distilled water	50 cc.
Gum Arabic (clear flakes)	50 gr.
Chloral Hydrate	125 gr.
Glycerine	30 cc.

((Mix in order given, heating not necessary. Filter through glass wool. If excessively dirty, then allow settling out over a period of several months. Use distilled water to thin preparations when necessary).

2. *C-M Mounting medium.*

Ethyl alcohol (95 %)	25 cc.
Methocellulose	5 gr.
Carbowax — 4,000 (Polyethylene Glycol : Union Carbide Chemicals Co.)	2 gr.
Diethylene Glycol	1 cc.
Lactic Acid (85 %)	100 cc.
Distilled water	25 cc.

(Dissolve ingredients in order given. Store in 50°C oven for several months to settle impurities, or filter through glass wool. Thin with 70 to 95 % alcohol when necessary.)

3. *Glycerine-Jelly.*

Arsenic trioxide (saturated distilled-aqueous)	150 cc.
Gelatin (Knox gelatin)	30 gr.
Glycerine	80 gr.

(Warm arsenic solution before adding gelatin, stirring constantly until dissolved, then add glycerine. Mixture hardens on cooling and must be re-liquified for use. Best results are obtained by mounting glycerine infiltrated material. Use the double-mounting technique).

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