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Rapid Methods for Obtaining Permanent Mounts of Radulae

(continued)

by

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3. Cleaning, arranging, smoothing out and flattening of the extracted radula

The isolated radula has to be cleaned and freed from certain membranes and it should be made as flat as possible. These operations are carried out on a microscope slide in a small drop of water (or an aqueous stain, cf. 5) or acetic acid. A binocular dissecting microscope is indispensable for all save the largest material.

First of all it is necessary to find out which is the side that bears the teeth, because the radula has to be mounted the right side up, i.e. teeth uppermost. As a rule the oldest (distal) part of the radula (nearest to the jaw) bends down and the nascent end bends up (see fig. 1; the side

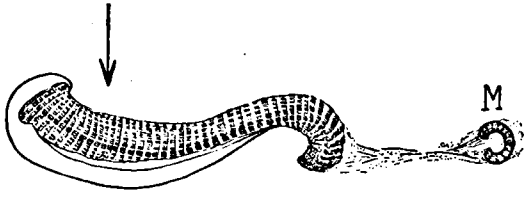


Fig. 1. Diagram of radula and jaw (M) as they appear after extraction from the macerated specimen. The side indicated by the arrow is the side that bears the teeth. The arrow also points to the place where to start the smoothing-out operation.

indicated by the arrow is the right side). The handling of the radula requires some skill — which must be acquired by experience — and the proper tools. Larger ones can be cleaned with paint brushes and dissecting needles, but the smaller ones need a more subtle treatment. Matchsticks sharpened to a very fine and flexible point are very suitable, but they have to be replaced after having been used once or twice. The author uses the finest and flexible needles provided by nature in the form of cactus spines. Those of Opuntia, Echinocactus and Cereus, among other

species, are excellent. They can be used many times and retain their rigidity in water whereas matchsticks become soft and useless after some time. A spine can be conveniently secured by means of sealing wax, cover glass cement, resin or canada balsam in a holder consisting of a length of glass tubing drawn out at one end (see fig. 2). When working with

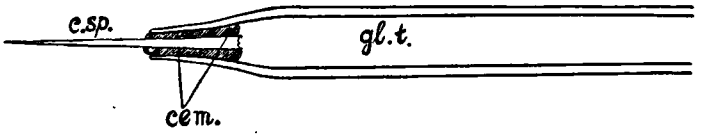


Fig. 2. A cactus spine (c. sp.) mounted in a piece of glass tubing (gl. t.) by means of a cementing substance (cem.). About natural size.

needles or cactus spines it is important to hold them in an almost horizontal position and to use them as scrapers rather than as needles so as to reduce possible damage to the specimen to a minimum (see fig. 3).

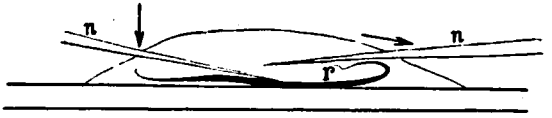


Fig. 3. Diagrammatic representation of the handling of a radula (r) in a drop of liquid on a microscope slide. The radula is pressed down and held in place by one needle (n, left) while the other needle (n, right) is used to smooth out the radula. Both needles are kept in an almost horizontal position.

Start in the area indicated by the point of the arrow in fig. 1 by disrupting the membrane which keeps the radula in its bent position with one needle, holding the radula in place with the other one. Then smooth out that part and continue with the nascent end (left in fig. 1). Separate the jaw and remove the membranes connecting it with the radula. Flatten the posterior end. Check the slide without a cover glass under a microscope, using a low-power objective, to see if the teeth are up; if not, turn the radula and smooth it out again. When handling minute radulae it is sometimes difficult to see if they are the right side up. If such a radula is the wrong side up, but properly smoothed out, it is better to leave it as it is (see below). Remove membranes and other rejects from the slide. Wash the radula if necessary by taking away the supernatant liquid with a strip of blotting paper and adding a droplet of clean water (preferably distilled water should be used). Leave only a small amount of liquid on the slide and apply a cover glass by dropping it at once horizontally on the specimen. Press the cover glass firmly down without

causing any slippage between it and the slide (a flat end of a small cork is excellent for the purpose) and check again under a microscope. If the mount is not satisfactory, add water at the side of the cover glass, remove the latter and start all over again. If the radula appears properly smoothed out and flat, put a weight (e.g. a 25 g or 1 oz. weight) on the cover glass and leave it on till all the water has evaporated and the radula is quite dry. This weighting is not always necessary, but it is essential if the radula shows a tendency to curl up again.

When dry, turn the slide upside down and tap it with one of the short sides gently on the table. Almost invariably the cover glass will drop off, leaving the radula so firmly attached to the slide that it stays in place during subsequent oxidizing, staining, rinsing and mounting. A minute radula accidentally mounted upside down (see above) will usually come off with the cover glass. In this case treat it on the cover glass and mount it finally by placing the cover glass „upside down" (the radula up) on a small drop of canada balsam on a slide. Put a drop of the selected permanent mounting medium on top of the specimen and cover it with a second (clean) cover glass. The mounted radula will be the right side up.

The pressing action on the cover glass is essential for two reasons. Firstly for obtaining a perfectly flat mount which can be used for photomicrographic purposes, because a considerable area will be in focus even at fairly high magnifications (see e.g. Plate 4), and secondly to make the radula adhere tightly to the slide which enables convenient handling of the specimen during oxidizing, staining, etc.

4. Modification of the chitin

The usual method is a treatment with a boiling and slightly acidified N/10 (or stronger) solution of potassium permanganate (one drop of glacial acetic acid is added to a few ml of the solution of $KMnO_4$ immediately before use). The treatment is continued until the radula has turned almost black. The formed MnO_2 is dissolved by rinsing with a solution of oxalic acid and after complete decolouration the radula is washed in water. Almost any basic dye may now be employed.

The author has found that chlorine dioxide (ClO_2), which has been used by entomologists for a considerable time to bleach and soften the chitinaceous parts of insects previous to microtome sectioning (see e.g. Schulze, 1922), is quite satisfactory for treating radulae. This reagent does not only bleach and modify the chitin, but also softens the radula and makes it pliable. This is of great advantage when one has extracted the radula from old dried-out specimens, because they become as easy to handle again as if they were quite fresh.

The entomologists use a product which is essentially a solution of ClO_2

in acetic acid and sold under the name of „Diaphanol". The same results are obtained by using the author's method: a small amount of a solution of sodium chlorite ($NaClO_2$, „Textone") is placed in a small tube and the specimen is inserted. One or more drops of glacial acetic acid are added till the first gas bubbles are formed and the liquid turns yellow. Close the tube immediately with a cork and shake gently. Leave the specimens in it for 4 to 24 hours at room temperature. The concentration of the chlorite is not critical, but 2 to 10% is suitable.

ClO_2 is very agressive and poisonous and should be carefully handled. One should not use anything else but matchsticks, or glass, china or platinum instruments to take the specimens out of the reagent. Transfer the specimens to a drop of sodium sulphite or bisulphite (Na_2SO_3 or $NaHSO_3$) or hypo ($N_2S_2O_3$) solution, then wash. The treatment of the specimen can be accelerated by heating the chlorite reagent, but this cannot be recommended because of the escaping fumes of ClO_2.

5. Staining

As a rule staining and subsequent rinsing, etc. are carried out on the slide. The solutions of the dyes should be diluted to prevent overstaining, except chrysoidin which is used as a saturated aqueous solution. A drop of the stain is applied to the specimen and after sufficient staining the drop is removed by tilting the slide or by means of a strip of blotting paper. For rinsing, a drop of water is placed on the specimen and poured off; this is repeated several times, then as much water as possible is removed with a strip of blotting paper.

It is often of great advantage to stain during the cleaning and smoothing-out treatments. The extracted radula is placed on a slide in a solution of the dye and the various manipulations are carried out in the drop of dye solution. By the time the radula is smoothed out it will be sufficiently stained in most cases. It is subsequently rinsed in water and flattened under a cover glass (see 3). This method gives excellent results with chrysoidin and with carbol-fuchsin without any previous modification of the enamel and with dahlia, gentian violet or other basic dyes after modification of the chitin by means of chlorine dioxide. In the latter case, the extracted radula is treated with the chlorite reagent immediately after maceration and the cleaning and smoothing-out treatments are postponed till after staining.

As stains can be mentioned Chrysoidin (di-amino-azobenzene-hydrochloride), carbol-fuchsin, Dahlia (Hofmann's Violet), Gentian Violet or Crystal Violet or Methyl Violet, Haematoxylin or Haematein, Congo Red, and Chlorazol Azurine G 200 (I.C.I.). Apart from the last, the origin and purity of the dyes is immaterial, but the best are to be preferred.

Chrysoidin is used as a saturated aqueous solution. Staining is carried out without previous modification of the radula, because this stain is specific for enamel. Stain in the cold for at least 5 minutes; the best results are obtained if the drop of the stain is allowed to evaporate almost completely on the slide. Rinse in water. Specimens that appear over-stained in water are cleared later in phenol-xylene and often give excellent results if mounted in canada balsam.

Fuchsin, in the form of carbol fuchsin as used in bacteriology, e.g. Ziehl's formula (Strasburger, 1923, p. 463): dissolve 10 parts of alcohol and 1 part of fuchsin in 100 parts of 5 per cent. phenol solution. For staining radulae, this mixture is diluted about ten times with water. Staining is carried out by gentle heating over a micro-burner for about 10 seconds. This stain can also be applied to the radula without previous modification of the chitin. There is always danger of overstaining, but if properly carried out, the cusps stain a bright cherry-red, the basements of the teeth pink and the basal membrane takes up hardly any colour. If chrysoidin does not stain well, the fuchsin stain can be applied to the radula stained in chrysoidin first. The best results are often obtained by staining lightly with carbol fuchsin over chrysoidin (not the other way round).

Slight over-staining with fuchsin can be corrected by clearing in phenol-xylene just before mounting.

Dahlia, gentian violet and other basic dyes are used after modification of the chitin (Bowell's oxidation-dahlia technique). The author uses gentian violet with excellent results. The concentration of the dye should be about 0.01 per cent. Rinse in water.

Haematoxylin, or the oxidized form, haematein, is quite satisfactory in some cases with or without previous modification of the chitin, especially with radulae which contain little enamel. It often gives better results with Prosobranchia than any other method. Various formulae can be used, e.g. Ehrlich's or Delafield's. Over-staining is easily corrected by a differentiation in water slightly acidified with hydrochloric acid.

The author uses either congo red or chlorazol azurine G 200 for a rapid diagnosis by boiling the specimen in caustic soda to which a few drops of a 1% solution of dye has been added. The boiling is continued till the radula has become quite isolated. The radula takes up enough dye to produce a better contrast when the specimen is examined in water or glycerol, but as a rule only the youngest teeth at the nascent end are properly stained. This is, however, sufficient for diagnostic purposes. Occasionally, especially in radulae of aquatic forms, the staining is throughout and allows permanent mounting. The upper figure on Plate 6 shows a photograph of the radula of **Theodoxus fluviatilis** stained with

chlorazol azurine during maceration. A comparison with the lower figure, representing a radula of the same species stained by means of Bowell's oxidation-dahlia technique, shows that the first contains as many details as the second. The radulae of several Opisthobranchia, e.g. of Aeolidia papillosa, can also be treated in this way.

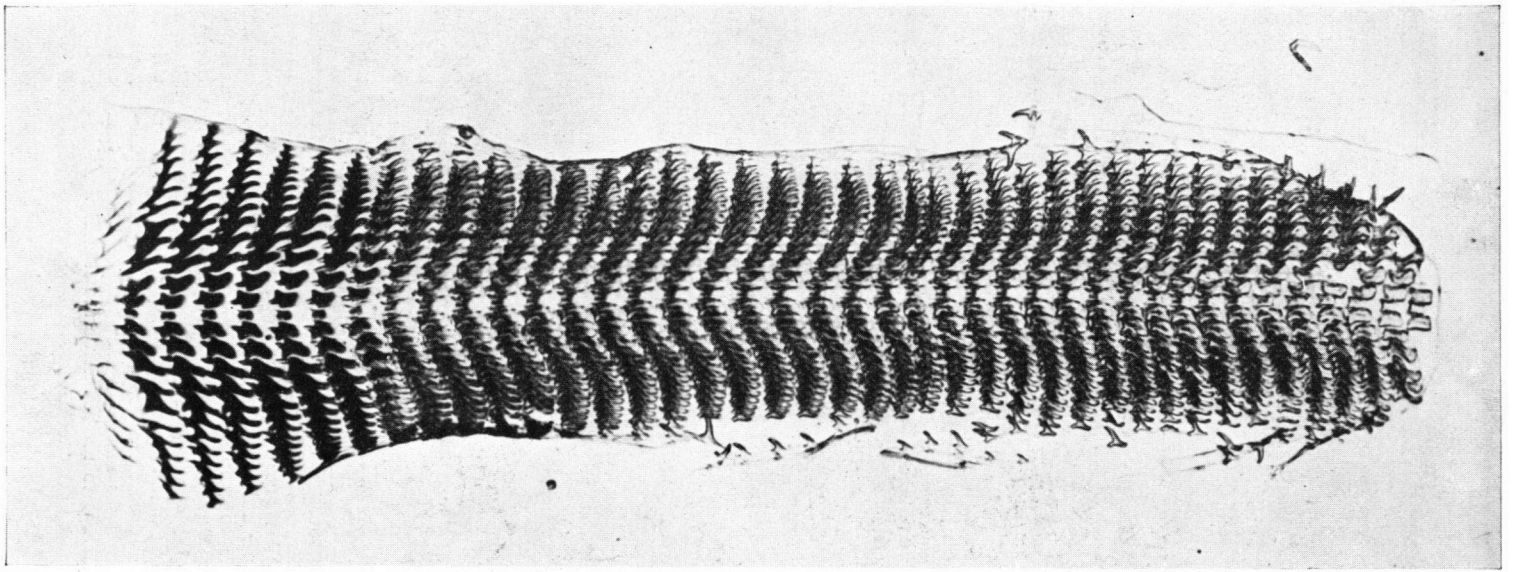
The author uses the last stain almost invariably, even if the radula is to be oxidized at a later stage, because the stained radula is much more easily recognisable after the disintegration of the animal in the caustic. Both congo red and chlorazol azurine can stand a treatment in glacial acetic acid and the radula is transferred to this medium from the caustic and smoothed out. After rinsing in water the specimen can be studied in water or in glycerol. However, this treatment by no means precludes subsequent staining with some other dye, because it can always be oxidized, stained, dehydrated and mounted at a later stage if desired. Excellent counterstaining is obtained by a treatment with chlorazol azurine which stains the young teeth a deep blue, followed by staining in chrysoidin or carbol fuchsin which stain the enamel of the older teeth yellow or red, respectively (see Plate 5).

6. Rinsing, dehydrating and mounting

As was mentioned before, rinsing is carried out on the slide. In most cases one change of the rinsing water suffices, because the stains are highly diluted, but chrysoidin staining may require more changes.

All authorities on the mounting of radulae mention a dehydrating process by means of a passage of the specimen through a series of increasing concentrations of alcohol, followed by clearing in clove oil, xylene or other suitable agents. Apart from being a tedious job, this treatment often causes shrinkage of the radula in alcohol and troublesome bending and twisting of the specimen in xylene, so that a perfectly flat specimen becomes distorted and is badly spoilt, while the alcohol range often extracts some dye from the stained specimen, which necessitates slight overstaining to anticipate this. It is very difficult, however, to judge the proper degree of over-staining correctly, so that the intensity of the staining is critical.

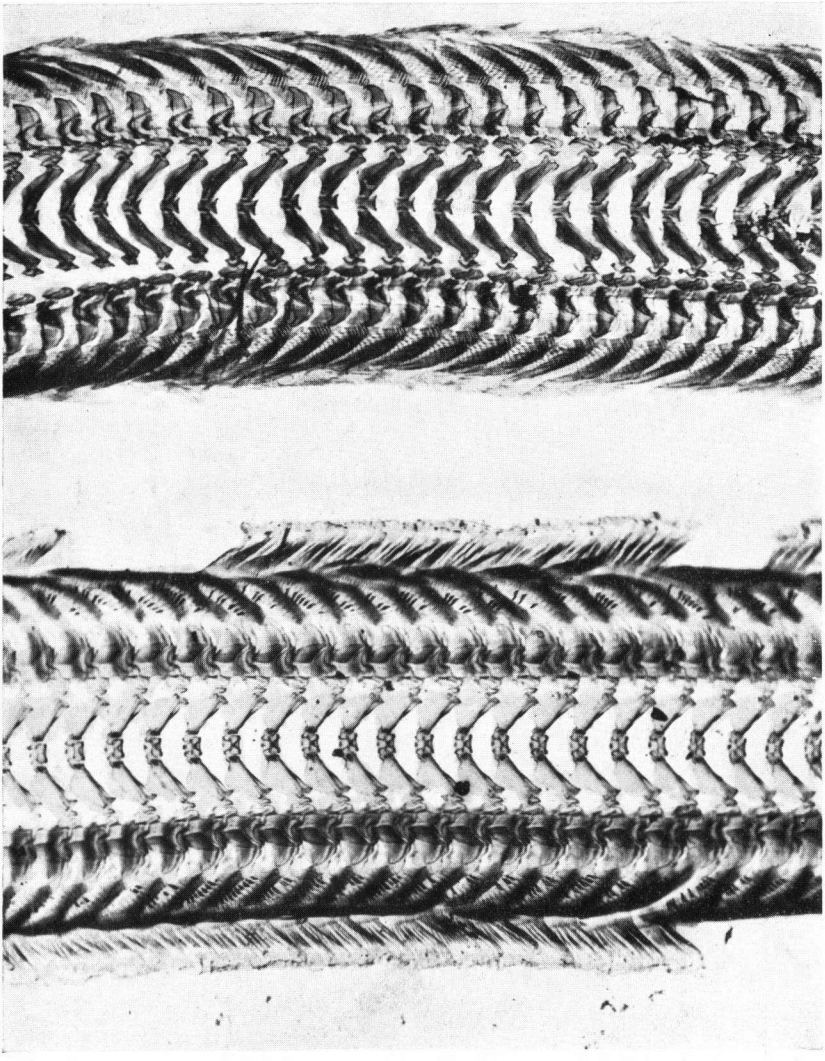
The radula is a rigid structure and can be dried without distortion or appreciable shrinkage, so that the best and most convenient method of dehydration is simply drying. The best way to do it is under a cover glass that has been weighted, as described on page 35. If the radula has been oxidized and stained after the smoothing-out treatment, it adheres so tightly to the slide after the first drying under a cover glass, that the second time it can be dried without a cover glass in a dust-free place, e.g. in a cardboard box. The advantages of this desiccation process are its simplicity, the greatly reduced chance of damage to or distortion



Oxychilus alliarius (O. F. Müll.), whole radula. Young teeth stained during maceration with Chlorazol Azurine G 200, the rest counterstained with carbol-fuchsin after smoothing-out operation. Canada balsam. Gevaert „Panchromosa" panchromatic plate 32° Sch., glycine developer. Initial magnification about 40, enlarged twice from negative to about 80. The deep blue of the stained young teeth appears black on account of the use of the red filter. Cf. text on p. 38.

PLAAT 5
(PLATE 5)

PLAAT 6
(PLATE 6)



Theodoxus fluviatilis (L.), radula. Above: stained during maceration with Chlorazol Azurine G 200. Medium: Broadfoot and Schwarz formula I. Gevaert "Replica" process plate 19° Sch. Magnification about 70. Below: treated with chlorine dioxide before the smoothing out operation, stained in Gentian Violet. Canada balsam. Same photographic process and same magnification as used for the other one. Note that this stained radula does not show more details than the one stained directly during maceration (top figure). The mount is slightly better than the other, because the marginals are spread out, whereas they are still folded back in the first, but this was a matter of luck during the smoothing-out treatment and is irrelevant to the results of the staining.

of the specimen — in fact, this chance is almost negligible — and, finally, that the intensity of the staining is not altered during dehydration which enables a proper judgment of the desired degree of staining.

The drying process must take place without heating. The specimen is left in a dust-free place till quite dry, but the process can be accelerated by placing the slide in a desiccator or any other suitable closed jar containing some calcium chloride, phosphorous pentoxide or silica gel.

When the specimen is perfectly dry, a drop of xylene is applied to the radula after removal of the cover glass by tapping, so as to avoid the formation of small air bubbles in the permanent mounting medium. If the presence of water is suspected or the degree of staining requires it, clear in a drop of phenol-xylene (100 g of phenol is dissolved in 100 ml of xylene; the mixture is filtered and kept in a brown bottle) before mounting.

As regards the mounting media, canada balsam has been extensively used, but recently new products have been recommended which are often better for special purposes. For a detailed discussion of the advantages of these new media over canada balsam the reader is referred to Wicks et al. (1946). The author has been using the formulae suggested by Broadfoot and Schwarz (1948) with considerable success. Canada balsam has a refractive index ($n_D = 1.528$ to 1.537) which is very near that of chitin ($n_D = 1.538$ according to Baas Becking and Chamberlin, 1925). In the author's opinion over-staining is undesirable because details may become lost and, as was clearly demonstrated by Broadfoot and Schwarz (1946), too high a transparency also obscures certain structural details, so that the best images are probably obtained by a combination of contrast and staining. The range of six refractive indices obtained by using the methacrylate-Aroclor mixtures and Canada balsam (or its substitutes like Meedol Balsam) allows for sufficient adaptation of the mounting medium to the degree of staining or to the stain used. If the colouration is on the pale side or the specimen slightly understained, use formula I, if the colour is deeper, use e.g. formula V and if slight over-staining is anticipated, use canada balsam. This is a matter of experience and proper judgment. Broadfoot and Schwarz suggested the following formulae:

Formula:	Isobutyl-methacrylate polymer (Dupont)	Aroclor 1242 (Monsanto)	xylene (solvent)	n_D between:
I	18 g	—	30 ml	1.495 and 1.500
II	18 g	3 ml	27 ml	1.495 and 1.500
III	18 g	6 ml	24 ml	1.505 and 1.510
IV	18 g	9 ml	21 ml	1.515 and 1.520
V	18 g	12 ml	18 ml	1.520 and 1.525

For mounting radulae, dilute with an equal amount of xylene. These media do not mix with phenol-xylene, so that after clearing in this agent a thorough rinse with pure xylene is necessary before mounting in one of the methacrylate-Aroclor mixtures.

Survey of rapid methods suggested

It seems worth while giving a survey of the stated evidence so as to aid future workers.

We have to distinguish between mounts intended for a rapid diagnosis and those intended for permanent preservation or for photomicrography. For a rapid diagnosis: Macerate in caustic containing either congo red or Chlorazol Azurine G 200 until radula has become quite isolated. Transfer the extracted radula to a drop of acetic acid (for fresh material about 5 per cent., for radulae extracted from dried-out specimens glacial is recommended). Clean and smooth out the radula, rinse in water, apply cover glass, press the latter down and examine, or: transfer to glycerol from water, apply cover glass, press down and examine. (If it is stained throughout and the intensity of the stain satisfactory, leave the specimen mounted in water till quite dry, remove cover glass and mount in Broadfoot and Schwarz I, cf. Plate 6, upper figure).

For permanent mounts, the following procedures are recommended:

a. chrysoidin or carbol fuchsin, or carbol fuchsin over chrysoidin;

b. modification of the chitin, followed by a basic dye;

c. haematoxylin.

a. Chrysoidin staining: Macerate, extract radula, transfer specimen to a drop of acetic acid, leave it in this drop for about 2 minutes, replace acetic acid by water, remove as much water as possible by means of a strip of blotting paper and add a drop of a saturated solution of chrysoidin in water. Clean and smooth out in this liquid. Leave the drop of stain on the radula till almost dry and rinse with water. Check specimen, adjust smoothing out if necessary, apply cover glass and press this down. Dry the slide. Remove cover glass, clear in phenol-xylene if necessary and mount. (If staining appears too deep when the specimen is examined in water, clear in phenol-xylene and mount in canada balsam; if specimen appears to be insufficiently stained, stain in carbol fuchsin, as indicated below).

Carbol fuchsin staining: Macerate, transfer to acetic acid, clean and smooth out. Rinse in water. Add carbol fuchsin, diluted 1 : 10, heat gently for 10 seconds or less over micro-burner and rinse in water. Complete or adjust smoothing out of specimen; finish as indicated for chrysoidin staining.

If staining with chrysoidin is too faint, add diluted carbol fuchsin and

stain as indicated above, but only for a few seconds. Rinse in water and finish as indicated above.

b. Modification of the chitin, followed by a basic dye: Macerate, extract radula and transfer specimen to a small tube containing 2 to 10 per cent. sodium chlorite solution. Add a few drops of glacial acetic acid till first gas bubbles are formed and the liquid has turned yellow. Close tube with a cork and shake gently. Leave specimen for at least 4 hours in the agent, transfer it to a slide and wash in a drop of water. Replace water by a drop of a solution of $NaHSO_3$ or hypo (1 to 5 per cent. is suitable), leave specimen in it for at least two minutes, rinse thoroughly in water and add a highly diluted solution of dahlia (Hoffmann's Violet), gentian violet or another suitable basic dye. Clean and smooth out in the stain.

Examine slide under a low-power objective now and again till staining is satisfactory. **Do not over-stain!** Rinse in water and finish or adjust smoothing out if necessary. Flatten radula by squeezing it under a cover glass. Dry mount, remove cover glass, clear and mount in a suitable permanent medium.

Or, alternatively: Macerate; clean and smooth out in acetic acid or water and flatten specimen under a cover glass. Dry the mount and remove cover glass. Add a slightly acidified permanganate solution and boil gently over a micro-burner (add more of the permanganate solution if necessary) till radula appears quite black. Remove excess of liquid and add oxalic acid solution (saturated). Leave specimen till colourless and rinse well in water. Stain as indicated above. Rinse, remove as much water as possible and dry mount without a cover glass. Clear and mount dried stained radula in a suitable permanent medium.

c. Haematoxylin: Macerate, etc. and smooth out in a diluted solution of haematoxylin till staining is satisfactory, rinse in water and finish mount as indicated above.

In many cases, better results are obtained after oxidation of the radula as indicated above (see b), i.e. either by chlorine dioxide or by permanganate, before staining. Finish mount as indicated above.

Additional suggestions

When studying radulae one should not rely on one staining method, but try out several different procedures in order to find the one that gives the best results in each individual case. Moreover, one stain may show certain features whereas other structural details appear more clearly after a treatment of the radula with a different stain. Some general indications can be given as to which staining method is the best for a certain group of Mollusca, but there are always exceptions.

By adding a stain to the caustic solution used for maceration one will always be able to detect those radulae which are sufficiently and satisfactory stained by either congo red or chlorazol azurine. If neither stain is satisfactory, try another method.

Radulae with well-developed cusps (Cephalopoda, many Pulmonata such as slugs, Zonitidae etc.) stain well in chrysoidin or carbol fuchsin.

The modification-basic dye technique can be used in a great number of cases. According to the author's personal experience it may give satisfactory results with nearly any group: Cephalopoda, Opisthobranchia, Rhipidoglossa, Taenioglossa, Pulmonata, etc.

Haematoxylin staining is only recommended in case all other methods have failed, as a last resource. Good results are obtained with freshwater Taenioglossa and with certain aquatic Pulmonata.

If sufficient material is available, always try another staining method even though the first mount was a success. Different details may be prominent in the second case and this may aid substantially in interpreting the structure of the radula under examination. If the material is scarce, try to deduct the best staining method from an examination of the radula after the maceration. If still in doubt, try chrysoidin staining first, then carbol fuchsin; if the staining is not satisfactory, modify the chitin and try a basic dye, if staining is still unsatisfactory, try haematoxylin as a last resource.

From a point of view of beauty it is of course most satisfying to mount a complete radula, quite intact and with the edges properly smoothed out, but for studying its structure it is sufficient if only a part of it is perfectly mounted.

Beginners are recommended to start their experiments with radulae of medium size, the minute ones being too difficult to handle without sufficient experience and the very large ones being either far too easy to deal with, so that they present no difficulties at all, or very troublesome because they resist being smoothed out. If the first attempts are disappointing, remember per aspera ad astra.

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