A method for the preservation of bdelloid rotifers for taxonomical and anatomical studies

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Summary

Hot glutaraldehyde kills active bdelloid rotifers quickly before they can contract and produces fully extended, intact and undistorted specimens. The specimens obtained with this method are superior to those killed with hot water or hot ethanol with or without added acids, which often result in distorted and damaged specimens. Since hot glutaraldehyde preserves the external as well as the internal details of anatomy, the specimens are suitable both for identification purposes and, especially if they are stained (for example, with neutral red) for anatomical studies.

Introduction

Bdelloid rotifers are notoriously difficult to study. The identification of species requires the examination of mostly external morphology, which, in turn, for almost all species requires that the animal be fully extended with its corona–if it has one–unfolded. The stated difficulty arises primarily from the lack of a standard method to anesthetize and preserve the fully extended bodies of bdelloids without damage or distortion. The lack of such a method also makes it difficult to examine their internal anatomies. As a result, there have been relatively few anatomical studies of bdelloids done with the light microscope since the early decades of the twentieth century.

Before it became a controlled substance, the anesthetic of choice was cocaine hydrochloride, although its proponents never stated if it was effective against all species [1]. In recent years, bupivacaine has been used to anesthetize bdelloids. However, not only may bupivacaine not be effective against all species [2], but it may also be difficult to obtain by amateur microscopists. Edmondson [3] and Pennak [4] recommended killing bdelloids with boiling water. Although this is easy to carry out, boiling water literally cooks the animals often with unintended results. Tests I conducted with boiling water yielded specimens that were extended, but had distorted and damaged body parts and coagulated internal organs that could not be examined further. I present here a method that instead uses hot glutaraldehyde that preserves bdelloids in a superior condition suitable for both identification purposes and anatomical studies.

Methods and Materials

Glutaraldehyde was used in the form of a commercial disinfecting solution Wavicide-01 (Medical Chemical Corp., California, U.S.A.) that contained 2.65% glutaraldehyde along with sodium nitrite at an undisclosed concentration and other proprietary ingredients. To ascertain that the results obtained with Wavicide were due to glutaraldehyde only, two tests were carried out using an EM grade 8% glutaraldehyde solution (Ted Pella, Inc., California, U.S.A.) diluted to 2.6% with water. Since the results obtained with Wavicide and EM grade glutaraldehyde were identical, throughout the rest of this paper I will refer to the subject substance simply as glutaraldehyde. The killing and preservation method was as follows. Active bdelloids were transferred to a small volume (50-100 µl) of water or a culture medium placed on the bottom of a small glass jar preferably wider than tall (~25 mm high with an internal diameter of ~40 mm). The rest of the procedure was performed before the drop containing the bdelloids evaporated. Three to four ml of glutaraldehyde solution was heated in a small beaker. (A hot plate, an alcohol burner, or even a candle may be used for this purpose. Caution: carry out this operation in a well-ventilated location and avoid prolonged exposure to glutaraldehyde fumes.) The temperature of the glutaraldehyde solution was monitored with a thermometer that was also used to stir it to produce an even heating. When the temperature reached about 80 °C, all of the solution was poured quickly on the drop containing the bdelloids. The initial placement of bdelloids in a small volume of liquid at room temperature ensured that after the addition of glutaraldehyde

the temperature of the mixture would remain high enough to kill the animals quickly before they could contract. After about 30 minutes in glutaraldehyde, bdelloids were located under a stereomicroscope and transferred with either a fine-tipped pipette or a micro-spatula to ~200 µl of ~5% aqueous solution of glycerol in a suitable receptacle, such as a cavity slide or a watch glass. This receptacle was left exposed to low humidity (~50%) ambient air for several days to allow the glycerol solution to lose its water while infiltrating the bodies of bdelloids gradually without causing excessive shrinkage. Finally, to remove any remaining water the receptacle was placed in a desiccator for a day or two. Bdelloids fixed with glutaraldehyde may be kept in glycerol indefinitely or mounted in glycerine jelly. To stain bdelloids, neutral red was dissolved either in the glutaraldehyde solution used to kill them or in the 5% glycerol solution. The amount of neutral red added was determined by trial and error to obtain a solution neither too light, which did not stain enough, nor too dark, which made it hard to see the rotifers in it.

Tests were also done by substituting in place of glutaraldehyde plain water, 10% lactic acid, 38% ethanol and 38% ethanol with 5% acetic acid. Higher alcohol and acid concentrations were avoided to prevent osmotic shrinkage of bdelloids. These liquids were also heated to 80 °C before being poured on bdelloids. Afterwards, the specimens killed with these fluids were placed in Wavicide for about 30 minutes, followed by transfer to glycerol.

The preserved specimens were photographed with a Motic BA410T microscope to which a Sony 6000 mirrorless camera body was coupled. All photographs were taken with oblique illumination obtained by placing a cardboard stop with a slightly concave edge immediately below the fully open condenser diaphragm. This stop blocked 50% of the full diaphragm opening. The height of the condenser was adjusted so that the image of the field diaphragm was either focused (Köhler) or slightly below or above the former point. Afterwards, the field diaphragm was opened almost fully. For some photographs, the Motic condenser was replaced by a two-lens Abbe condenser removed from an old Olympus EH Microscope. The iris diaphragm of this condenser was dispensed with and the stop for oblique illumination was placed immediately below the bottom lens covering 70% of the total lens area. The placement of the stop as close to the bottom lens of the condenser as possible and the wide opening of the field diaphragm provided an even lighting with the desired pseudo-relief effect [5] even with a 100x objective (Fig. 3). Stacking of images was done with Helicon Focus. All photographs were sharpened slightly and adjusted for increased contrast in Adobe Photoshop Elements.

Results and Discussion

Specimens killed with hot water, hot 10% lactic acid, hot 38% ethanol and hot 38% ethanol with 5% acetic acid often had gross body parts, especially the corona, distorted or damaged (Fig. 1A). Moreover, their internal organs acquired an unnatural, coagulated appearance. In comparison, hot glutaraldehyde killed and preserved bdelloids without any distortion or damage and the specimens retained the finest external and internal details (Fig. 1B-C). For example, a small ligula seen on the edge of the upper lip of live *Macrotrachela quadricornifera* (Fig. 1D) was also seen in specimens killed with hot glutaraldehyde (Fig. 1B). This anatomical detail helped identify the animals as *M. quadricornifera ligulata* [6]. Likewise, the details of the internal anatomy, often difficult to examine in active bdelloids, because they are almost never stationary for more than a few seconds, remain visible in specimens killed and preserved with hot glutaraldehyde. These include the various glandular organs in the neck and the head, especially if they are stained (Fig. 2), as well as the ovaries and the sphincter between the stomach and the intestine (Fig. 3), the latter seen by previous authors in histological sections [7].

There are two drawbacks of the method. First, the eye spots of the species in the genera *Philodina* and *Rotaria* fade gradually after they are killed with glutaraldehyde (Figs. 1C, 2C). Since the normally dark red eyes of *Rotaria* are visible in freshly killed specimens, the fading may not be blamed on glutaraldehyde or the elevated temperature and its cause is not clear at the moment. Second, the corona of bdelloids killed with hot glutaraldehyde usually appear slightly narrower and more inclined inward than do the coronas of their live conspecifics (compare Fig. 1B and 1D). I suspect this happens because during a fleeting moment before they die, the animals react to the hot glutaraldehyde

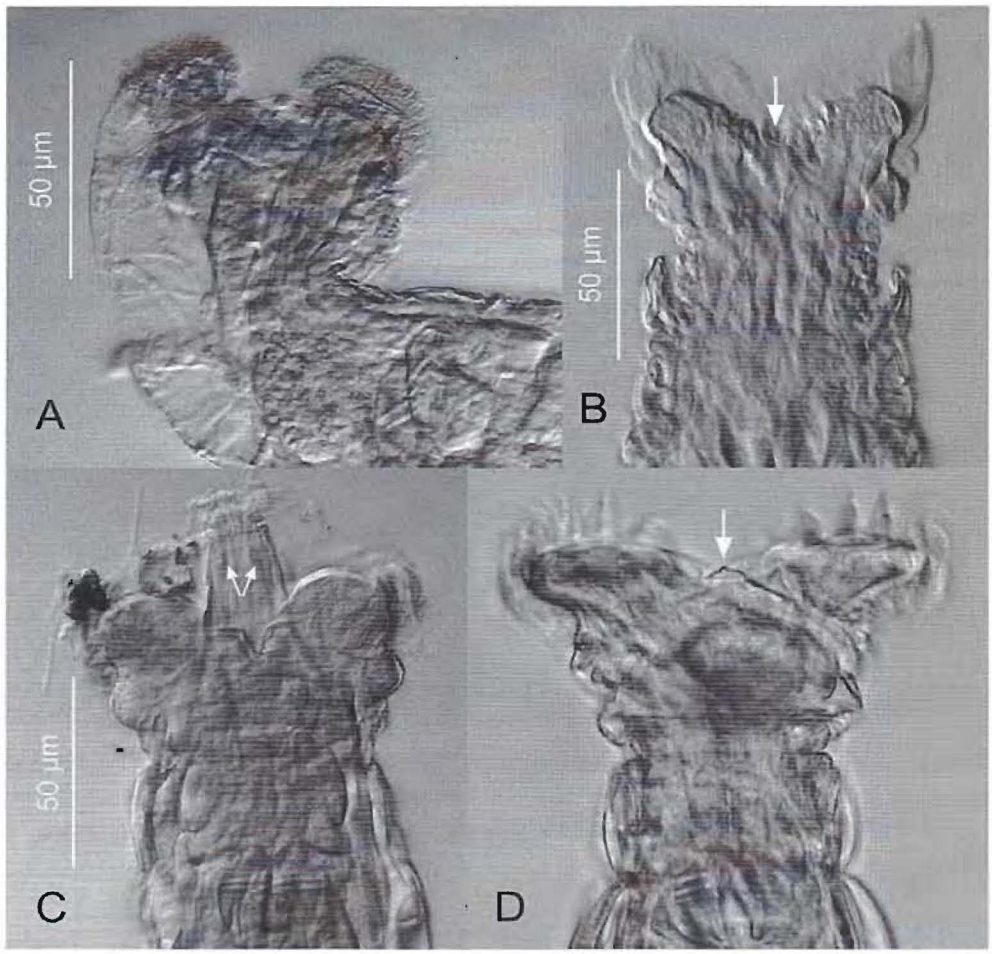

FIG. 1. **A. The head of *Macrotrachela quadricornifera ligulata*** Bērziņš, 1950 killed with hot water. Stack of two images. **B. Ventral view of the head of *M. quadricornifera ligulata*** killed with hot glutaraldehyde. The ligula on the dorsal upper lip is visible (arrow). Stack of two images. **C. Dorsal view of the head of *Rotaria neptunoida*** Harring 1913 killed with hot glutaraldehyde. Note the faded eye spots on the rostrum (arrows) and the bits of debris stuck to the left lobe of the corona. Stack of 3 images. **D. Dorsal view of the head of live *M. quadricornifera ligulata*** with the ligula on the upper lip visible (arrow). Unstacked image (no scale available).

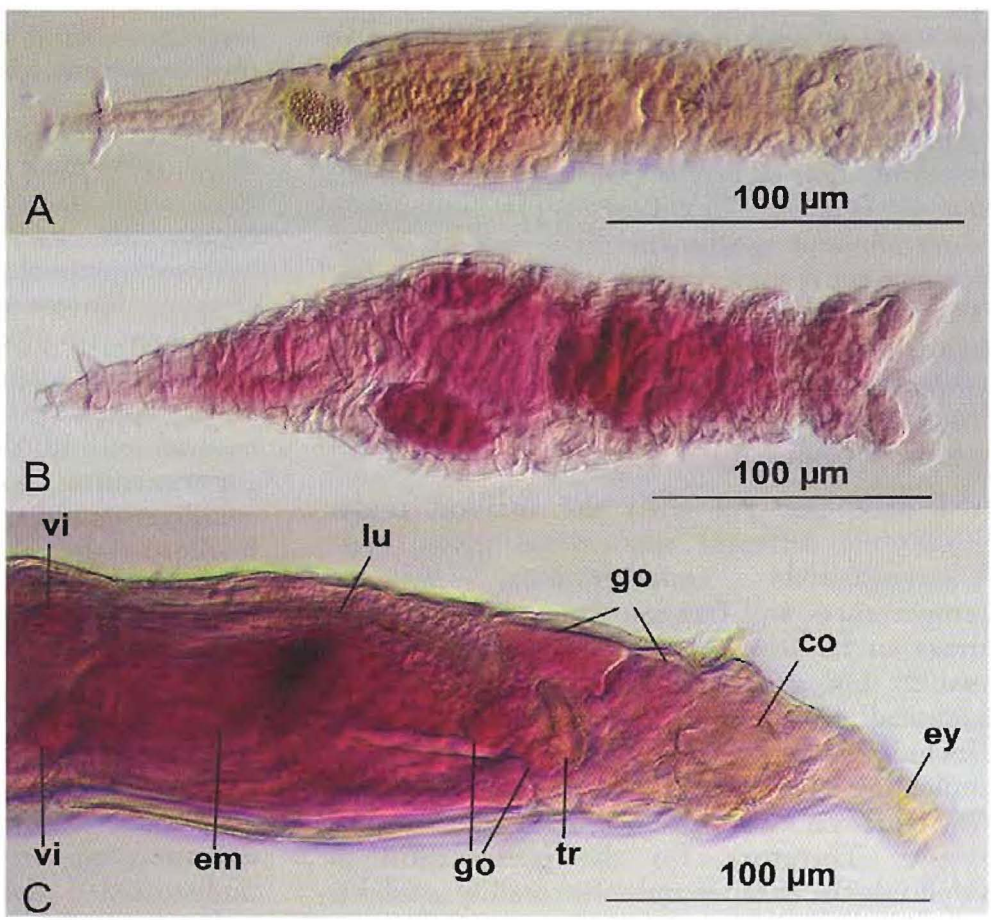

FIG. 2. **Bdelloids** killed with hot glutaraldehyde. **A. *Adineta* sp.** with faded stain. Stack of 4 images (ventral). **B. *M. quadricornifera ligulata*** freshly stained. Stack of 5 images (dorsal). **C. Lateral view of the anterior half of *R. neptunoida*** freshly stained. Unstacked single image. A large embryo (em) displaced the stomach and its lumen (lu) dorsally and caused the two vitellaria (vi) to shrink. Also visible are the trophi (tr), several glandular organs (go) in the neck, the withdrawn corona (co) and the faded eye spots (ey) on the rostrum. The dark spot obscuring parts of the embryo was a bit of debris on the outside and the lighter longitudinal band behind the trophi was a fold of the integument.

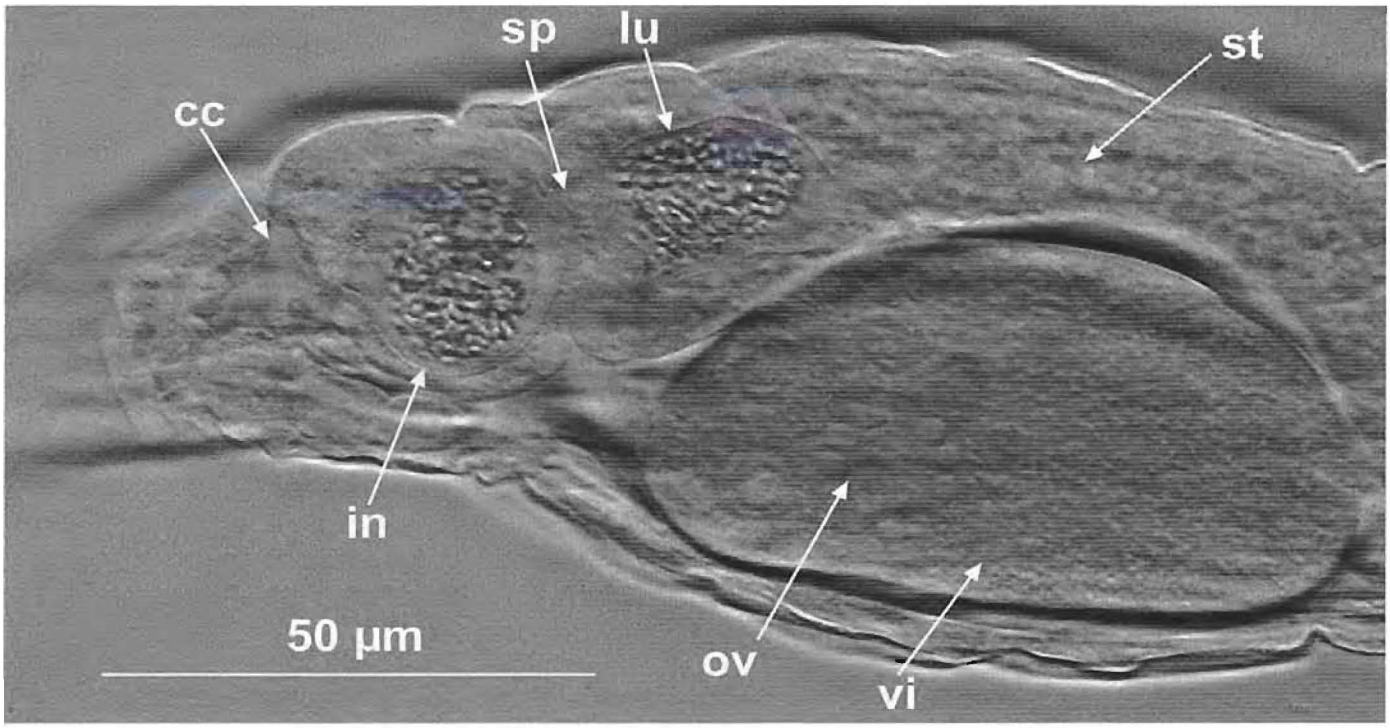

FIG. 3. **The posterior end of the trunk of *Adineta* sp.** killed with hot glutaraldehyde. Visible are the stomach (st), stomach lumen (lu), sphincter (sp) between stomach and intestine (in), contractile cloaca (cc), vitellarium (vi) and ovary (ov) the nuclei of which forming a ring. Photographed with a 100x oil objective. Stack of three images.

and begin to close their coronas. Therefore, whenever it is necessary to measure the width of the open corona, it should be done so in live animals. Another problem, perhaps not specific to the present procedure, is the attachment of microscopic debris to the bodies of dead bdelloids that sometimes obscures the parts of interest (Fig. 1C, 2C). This happens because in most bdelloid species the outer surface of the integument is sticky and remains so after death. Therefore, cleanliness of solutions, containers and tools is necessary to obtain specimens free of debris, but this is sometimes not attainable, especially if the bdelloids are killed in drops of culture medium.

I have not yet tried the method under conditions different than those given here. Glutaraldehyde concentrations, solution temperatures and fixation times different than those so far used may give better (or worse) results. EM grade glutaraldehyde gave results identical to those obtained with Wavicide (including the gradual fading of eye spots) indicating that the additional ingredients of Wavicide were not responsible for the observed results. Therefore, for the preservation of bdelloids the cheaper and more readily available disinfecting products containing glutaraldehyde at a concentration of about 2.6% (perhaps even less) should be suitable substitutes for purer and more expensive glutaraldehyde products.

Neutral red stains bdelloid organs selectively; those in the neck and the head and the vitellaria in the trunk become especially dark. The stained organs are easy to distinguish from the surrounding unstained or lighter stained tissues (Fig. 2). However, upon mounting of stained specimens in glycerol or glycerine jelly the initially dark magenta color starts to fade. This fading appears to result from the diffusion of neutral red into the mountant. I am currently experimenting with different procedures to stabilize neutral red in the bodies of preserved bdelloids and also trying out other stains. Preliminary results suggest that the addition of excess neutral red to the mountant may prevent the fading of stained specimens.

Glutaraldehyde crosslinks proteins [8], whereas hot water, hot alcohol and hot acids simply denature them. It appears that the crosslinking of proteins, as opposed to denaturation, maintains the body in an undistorted and natural-looking state.

Glutaraldehyde also renders the bodies of bdelloids quite stiff protecting them against tears and making it easy to pick them up with a probe for transfer. Unlike anesthetics, which may depend for their action on phylogeny-specific metabolism, and therefore may be ineffective in some species, the protein-crosslinking activity of glutaraldehyde would be expected to be general and effective regardless of phylogeny. I have so far preserved successfully with hot glutaraldehyde bdelloid species in the genera *Adineta*, *Macrotrachela*, *Mniobia*, *Philodina* and *Rotaria*. Treatment of bdelloids with hot formaldehyde, also a crosslinking agent, may give results similar to those obtained with hot glutaraldehyde. However, formaldehyde is classified as a human carcinogen. Although glutaraldehyde is not considered carcinogenic, it should still be handled with care, because it is a skin and lung irritant [9].

The successful preservation of bdelloids directly with glutaraldehyde now makes anesthetization almost unnecessary. It should be obvious that if bdelloids are not already extended at the moment a killing agent is added, the resulting contracted specimens will be useless for most purposes regardless of how well they may be preserved. Some species are frustratingly reluctant to unfold their coronas and there is no known procedure one can use to coax them into doing so. This is an inherent problem with bdelloids themselves that cannot be overcome by the present method. Nevertheless, I hope that the present method will fuel further research on these fascinating animals and especially encourage studies of their internal anatomies that have been mostly ignored.

Acknowledgements

The use of glutaraldehyde as a potential preservative for bdelloids (following anesthetization) was suggested to me by the late microscopist Howard L. Taylor during a visit to his house in 1999. Fifteen years passed before I decided to test his suggestion; then, after many failed attempts at anesthetizing bdelloids, I thought of killing them directly with hot glutaraldehyde. I thank Ken Hayes for the EM grade glutaraldehyde and Michael Plewka for our continuing e-mail exchanges on every aspect of bdelloids.

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