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ORIGINAL ARTICLE

Protargol Synthesis: An In-house ProtocolXuming Pan^a, William A. Bourland^b & Weibo Song^a^a Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, Ocean University of China, Qingdao, 266003, China^b Department of Biological Sciences, Boise State University, Boise, Idaho, 83725-1515, USA**Keywords**

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IN 1897, chemist Arthur Eichengrün submitted a U.S. patent application for “silver albumose” on behalf of the German chemical company, IG Farben. The compound, marketed under the trademark “Protargol”, was introduced as a more effective and less irritating alternative to the noxious silver nitrate solutions then in use for the treatment of gonococcal infections. Today, the compound is almost exclusively referred to in the generic (i.e. protargol). Protargol was a huge commercial success and, together with his other inventions, conferred great wealth and social status on Eichengrün. Sadly, he fell victim to the virulent anti-Semitism of the National Socialist regime during World War II. Miraculously, he survived deportation to the Theresienstadt concentration camp and died in 1949 at the age of 82 (Vaupel 2005).

The medical usefulness of protargol declined rapidly with the advent of the antibiotic era. The first use of protargol as a neurohistological reagent is usually attributed to Bodian (1936), but Regaud and Dubreuil (1903) used protargol together with osmium tetroxide for silver impregnation of epithelial tissues preceding him by more than 30 yr. In this report, we use the terms “staining” and “impregnation” interchangeably although the latter is technically more correct (Uchihara 2007). Cole and Day (1940) first reported the use of protargol for the microscopic

ABSTRACT

The protargol staining method has proved to be indispensable for revealing the cellular structures of a variety of protozoa, especially the flagellates and ciliates. Protargol provides permanent stains of a variety of cellular structures: nuclei, extrusomes, basal bodies, and microfilamentous constituents of cells. Together with the older silver nitrate methods, protargol impregnations have provided the basis for the detailed descriptions of nearly all ciliates to date. The performance of commercially available preparations has varied widely. Recently, suppliers have stopped stocking the effective forms of protargol resulting in a worldwide shortage. Thus, it has become necessary for protistologists to explore on-site synthesis of this critically important agent. An optimum protocol for synthesis of protargol should be rapid, relatively inexpensive, simple enough to be done by non-chemists, and achievable without specialized equipment. In this article, the authors briefly review the interesting history of protargol and describe a protocol, based on the early studies of neuroanatomists, that yields a protargol producing impregnations of ciliates comparable to those obtained with previously available commercial preparations.

study of flagellates and ciliated protozoa (*Paramecium*) and were followed by many others (Deroux and Tuffrau 1965; Kirby 1950; Kozloff 1960; Tuffrau 1967; Wilbert 1975). Dragesco and Dragesco-Kernéis (1986), Foissner (1991), Montagnes and Lynn (1993), and Vd'ačný and Foissner (2012) provide detailed descriptions of the most useful protocols for protargol staining of ciliates.

Although used in the silver impregnation of biological specimens for more than a century, the exact mechanism of protargol impregnation is still incompletely understood. Briefly, the silver proteinate provides both silver ions and silver salts that, usually after a bleaching step, then differentially deposit on subcellular structures including nuclei, basal bodies, and microfibrils. Visualization of the deposits requires their reduction to metallic silver either directly in situ (i.e. the argentaffin reaction) or by means of a developer (i.e. the argentophil reaction). This simplistic explanation belies the well-known and often frustrating capriciousness of protargol impregnation methods, the results of which are subject to seemingly countless variables (Davenport et al. 1952; Foissner 1991; Uchihara 2007). The most important factor is the protargol itself (Peters 1959). Small et al. (1980) and Zagon (1970) studied the sites of silver deposition in ciliates impregnated with protargol. Protargol for use in microscopy is sold in two forms (i.e. “strong” and

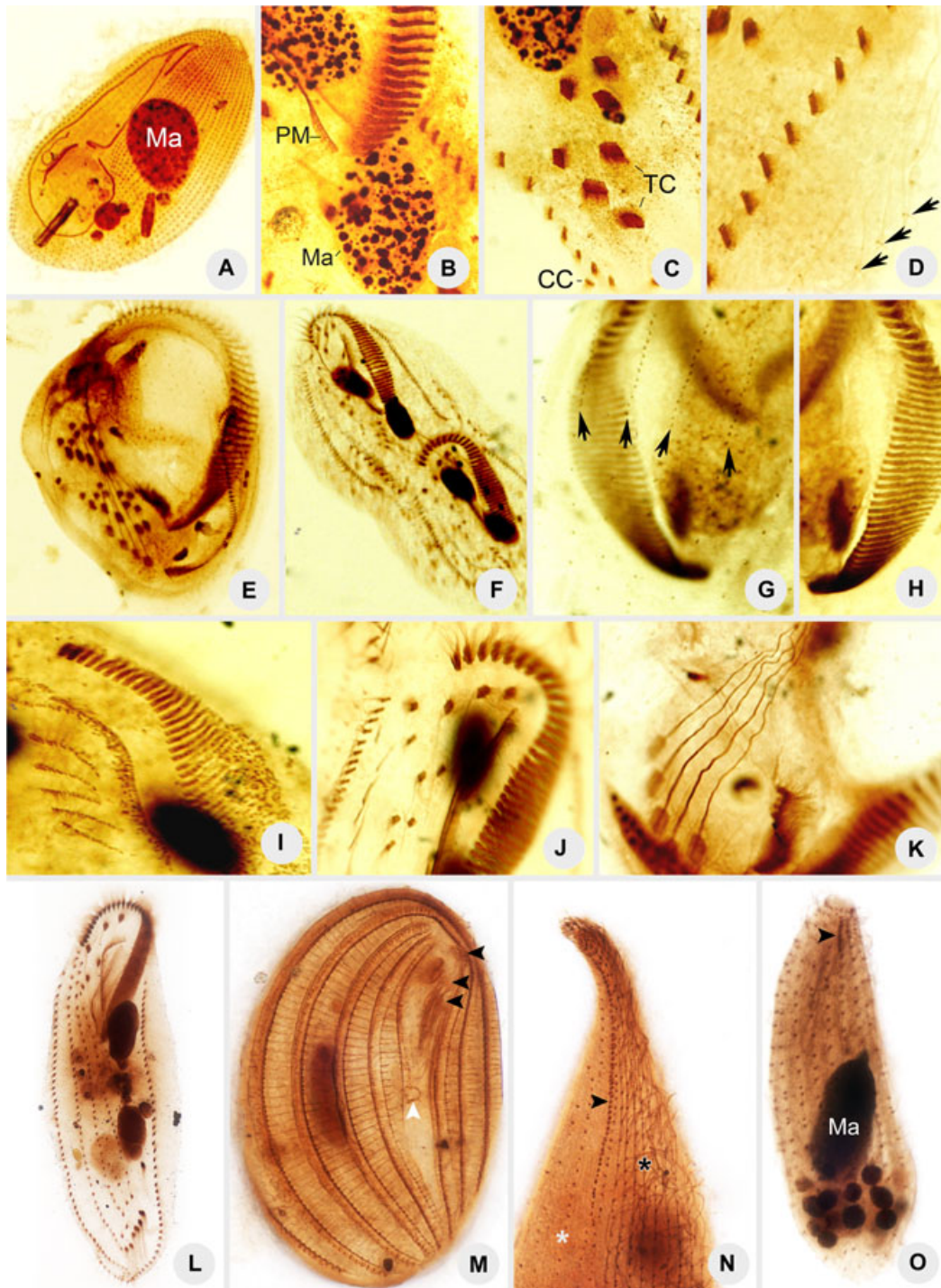


Figure 1 Photomicrographs of ciliates after protargol impregnation (Wilbert method, **A–K**; Foissner protocol **A, L–O**) using agents produced by the current protocol. **A**. Ventral view of *Pleuronema coronatum*. **B–D**. Ventral views of *Stylyonychia mytilus*, arrows in **D** show dorsal cilia. **E, G, H, K**. Ventral (**E, H**) and dorsal (**G, K**) views of *Euplotes woodruffi*, arrows in **G** show newly formed dorsal kineties while **H** shows the adoral zone of membranelles. **F, I, J**. Different morphogenetic stages in *Oxytricha* sp. (**I**, Anlagen in the opisthe; **J**, Details in the opisthe). **L**. Ventral view of *Paraurostyla weissei*. **M**. Ventral view of *Pseudomicrothorax dubius*, black arrowheads mark the three adoral membranelles and the white arrowhead marks the excretory pore of the contractile vacuole. **N**. Dorsal view of *Amphileptus* sp., the black arrowhead marks the dorsal brush row separating the sparse left (white asterisk) and more closely spaced right (black asterisk) somatic kineties. **O**. Dorsal view of *Fuscheria terricola*, the black arrowhead marks the dorsal brush. TC = transverse cirri; CC = caudal cirri; Ma = macronucleus; PM = paroral membrane.

“mild”). The two forms differ in their physical appearance: “strong” forms are light to medium brown (i.e. cocoa or cinnamon colored) very fine powders and the “mild” forms are coarser, shiny, very dark brown to black crystals. Paradoxically, “strong” protargol has a lower total silver concentration (7.5–08.5%) than “mild” (19–23%) protargol whereas silver ion concentrations are higher in “strong” ($\sim 10^{-4}$ M) than in “mild” (10^{-6} – 10^{-8} M). The “mild” protargols are generally ineffective for neurohistologic silver impregnation (Peters 1959). Thus, the higher silver ion concentrations of “strong” protargol appear crucial to their efficacy in silver impregnation methods.

Unfortunately, effective forms of “strong” protargol suitable for silver impregnations of ciliated protozoa are no longer commercially available (Bourland, pers. observ.). The reason for the disappearance of protargol from the market is unknown and inquiries to vendors have been uninformative (Bourland, pers. commun.). Author WB used the commercial product, Protargol-S (Polysciences Inc., Warrington, PA), prior to its discontinuation. Authors XP and WS used Protargol-S from another vendor (Sigma-Aldrich, St. Louis, MO, cat. no. P0086-5G). Both of these products were likely made by the same manufacturer, and both vendors confirm that neither one is now available (Bourland, pers. commun.). Some vendors continue to sell products as “strong” protargol or Protargol-S that have the physical characteristics of the “mild” (i.e. black or dark brown crystals) rather than “strong” form (fine cocoa to cinnamon colored powder). Bourland (pers. observ.) and Foissner et al. (1999) have noted the unsuitability of all dark crystalline forms of protargol for impregnation of ciliates even if the product is erroneously labeled as “strong”. Complicating matters further, two vendors sell “protargol”, one designated as “strong” and the other as “mild”, both with the same Chemical Abstract Service registry number (9008-42-8). Both products are ineffective for silver impregnation of protists (Bourland, pers. observ.). Appeals to the Biological Stain Commission have not yet resolved the problem (Bourland, pers. commun.).

In view of the abrupt disappearance of effective products from the world marketplace, and because protargol impregnation is an essential tool in the armamentarium of protistologists, our two laboratories have independently explored methods of “in-house” synthesis of silver proteinate that will provide impregnations of ciliated protozoa comparable to those obtained with previously available commercial products. Much of the work on the mechanisms of silver impregnation and the synthesis of silver proteinates for this purpose was performed in the 1950s (Davenport et al. 1952; Myhre 1952; Porter and Davenport 1951). This report represents an integration of our experiences into a practical method for protargol synthesis. The synthetic protocol described herein is modified from that of Davenport et al. (1952).

MATERIALS AND METHODS

Equipment: One 500-ml beaker, one 100-ml beaker, one 100-ml graduated cylinder, one 150-ml ceramic or glass

(not metal) mortar and pestle, one 2-ml plastic pipette, several large glass stirring rods, one (6 × 6 cm) glass funnel, grade 2 (> 8 μm particle retention) 15 cm diam. qualitative filter paper, one glass Petri dish (15–20 cm), a standard laboratory fume hood or well-ventilated work space, standard 3-ply surgical mask, latex gloves, pH meter (preferred) or pH paper (pH range 6–9).

Reagents: 350 ml distilled water, 300 ml 100% (anhydrous) ethanol, 20 g silver nitrate (reagent grade), 500 ml acetone, 5 ml concentrated (29%) ammonium hydroxide, and 50 g of one of the following peptones (referred to by number in the text):

- (1) Oxoid Tryptone (Fisher Scientific, Pittsburgh, PA, cat. no. OXLP0042B).
- (2) Proteose Peptone (US Biological, Salem, MA, cat. no. P9113-20).
- (3) Peptone from gelatin (Sigma-Aldrich, St. Louis, MO, cat. no. 70951-1KG-F).
- (4) Gelysate Peptone BBL (BD, Franklin Lakes, NJ, cat. no. 211870).
- (5) Peptone (Beijing Aoboxing Bio-Tech Co., Beijing, China).

All equipment and reagents can be purchased from major scientific supply companies (e.g. Fisher Scientific or Sigma-Aldrich, St. Louis, MO, or their respective international subsidiaries) except as noted. Peptones other than those listed may be ineffective.

PROTOCOL

The procedure can be completed within 48 h as follows: purify the peptone (Steps 1–6) on the first morning and pulverize the silver precipitate on the second morning (Steps 7–11).

- (1) Add 50 g of peptone to a 500-ml beaker containing 150 ml absolute ethanol. Add 50 ml distilled water while shaking the mixture. Allow the brownish, gummy precipitate to settle and carefully pour off the cloudy supernatant reserving the precipitate. Allow to stand 5 min to evaporate residual fluid from the precipitate.
- (2) Dissolve the precipitate in 40 ml of distilled water, warming on a heating plate at no more than 60 °C while stirring constantly (do not burn). When completely dissolved (10–20 min), add 120 ml absolute ethanol to the solution while stirring constantly. The solution will become milky.
- (3) Cover the beaker and cool it with running water for 30 min to ensure complete precipitation. When precipitation is complete, carefully pour off the supernatant retaining the gummy precipitate and allow this to stand for 10 min. Dissolve the precipitate by adding 40 ml distilled water while gently swirling the beaker. This solution is the “purified peptone”.
- (4) Measure the purified peptone in a graduated cylinder. Pour half of this solution back into the beaker (500 ml), and the other half into the smaller beaker

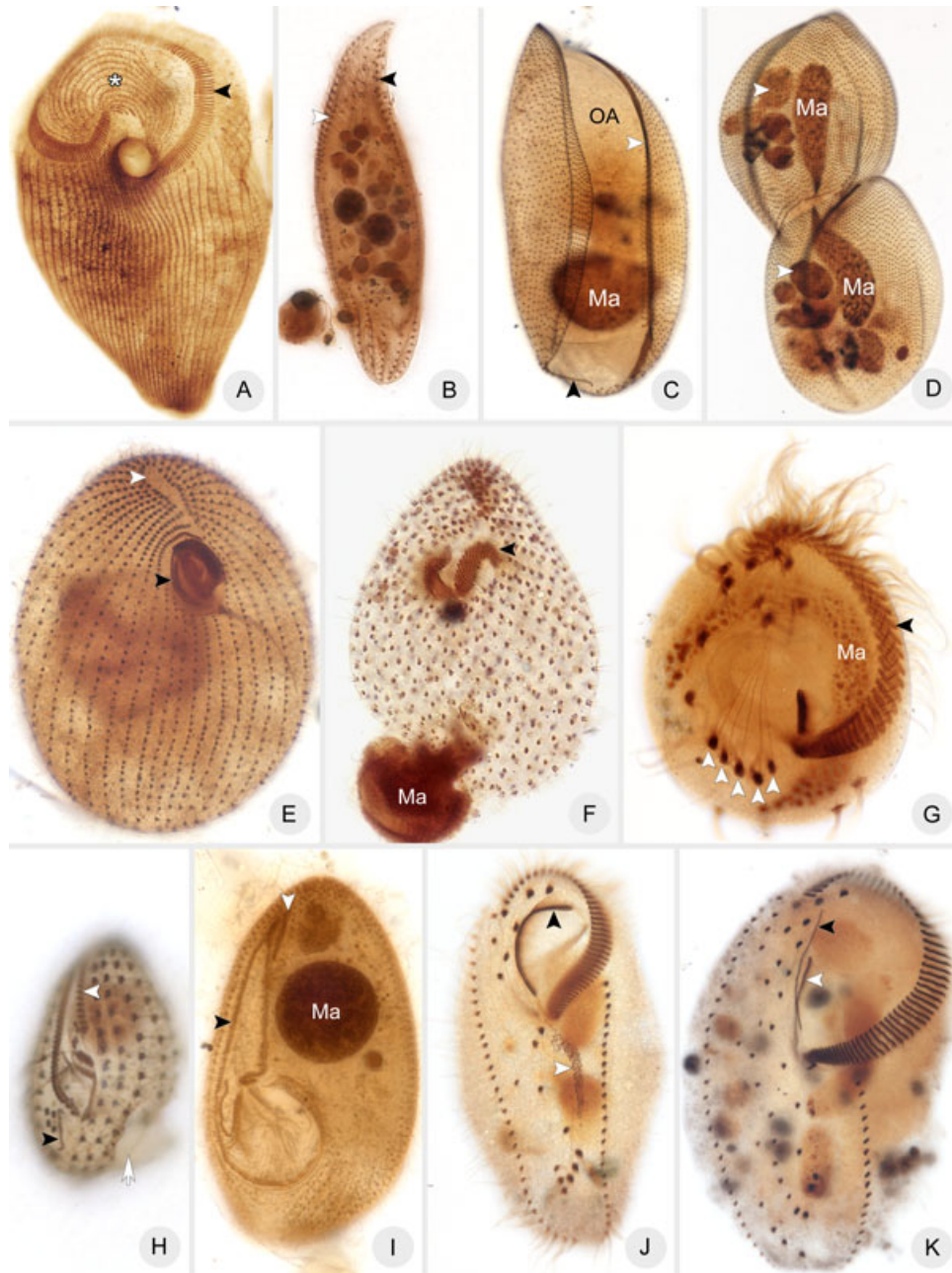


Figure 2 Photomicrographs of ciliates after protargol impregnation (Foissner protocol A, **A–I**; Wilbert method, **J, K**) using now discontinued commercial protargol (Protargol-S, Polysciences Inc.). A. *Stentor multififormis*, the black arrowhead marks the adoral membranelles that enclose the peristomial bottom (asterisk). B. Left lateral view of *Litonotus* sp., white arrowhead marks a perioral kinety and the black arrowhead marks the dorsal brush row. C, D. Ventral view of a trophont (C) and dorsal view of a late divider (D) of *Lembadion magnum*, the white and black arrowheads (C) mark the adoral and paroral membranes respectively. The white arrowheads (D) mark prey within food vacuoles. E. Ventral view of *Glaucoma scintillans*, the black arrowhead marks the paroral membrane and the white arrowhead marks the bare frontal field. F. Ventral view of *Exocolpoda augustini*, the black arrowhead marks the distinctive boomerang-shaped left oral polykinetid. The macronucleus (Ma) has extruded through a rupture in the posterior of the cell. G. Ventral view of *Euplotes* sp., the black arrowhead marks the adoral zone of membranelles and the white arrowheads mark the transverse cirri. H. Ventral view of *Ctedoctema acanthocryptum*, the white arrowhead marks adoral membranelle 1, the black arrowhead marks the cytoppyge and the white arrow marks a ruptured left posterolateral bleb often seen during observation of this species. I. Ventral view of *Pleuronema coronatum*, the black arrowhead marks the paroral membrane and the white arrowhead marks adoral membranelle 1. J. Ventral view of an early divider of a *Cyrtohymena* sp., the black arrowhead marks the distinctive curved paroral membrane and the white arrowhead marks the opisthe oral primordium. K. Ventral view of *Laurentiella strenua*, the black and white arrow heads mark the paroral and endoral membranes, respectively. Ma = macronucleus; OA = oral apparatus.

- (100 ml). Note: Steps 5 and 8–10 should be carried out in a laboratory fume hood or well-ventilated room to avoid concentrated ammonium hydroxide fumes and to evacuate the highly flammable acetone vapors.
- (5) Add 2 ml of concentrated ammonium hydroxide to the purified peptone in the large beaker. Mix well and add a solution of 20 g of silver nitrate dissolved in 60 ml of distilled water. A light brown precipitate forms and slowly settles to the bottom of the beaker.
 - (6) Seal both beakers with parafilm and store for 12–24 h in a cool (13–18 °C), dark place.
 - (7) Pour off and discard the supernatant from the large beaker retaining the precipitate (the precipitate should adhere firmly enough to the glass to stand reasonably tough handling). Add 100 ml of distilled water and allow this to remain on the precipitate for 10 min at room temperature (18–25 °C) without stirring. Pour off the water, and add another 100 ml of distilled water. Let this stand for 10 min then pour off the water and let the washed precipitate stand for 5 min.
 - (8) Add the reserved purified peptone from the small beaker (100 ml) to the washed precipitate and dissolve it by gently swirling. Dissolution may be hastened by heating to no more than 60 °C while stirring constantly. Allow the solution to cool to room temperature. Measure the pH of the solution. If the pH is < 8, add concentrated ammonium hydroxide drop by drop to adjust the pH of the solution (pH 8.0–9.0).
 - (9) Add 100 ml of acetone to this solution while stirring slowly with a glass rod (about 5 min). The solution will become milky. Allow it to stand for 5 min, then carefully pour off and discard the milky acetone supernatant retaining the precipitate. Let it stand for 10 min (make sure no fluid is left). Add another 100 ml of acetone, stirring slowly, and let this stand for 5 min. Pour off the acetone and allow the precipitate to stand for 10 min. Repeat this process until the acetone remains clear and not milky. As it is gently stirred, the precipitate will gradually achieve the consistency of a stiff paste that adheres to the walls of the beaker. Scrape this paste off the sides of the beaker with a glass rod or ceramic (not metal) spatula and transfer to the mortar containing 30 ml acetone to cover the product.
 - (10) Steadily pulverize the paste to powder under acetone with the pestle. This is a rather tedious part of the procedure, usually requiring 30 min and sometimes as long as 2 h. While grinding, the paste becomes more brittle and flaky, and very fine particles begin to cloud the acetone. The acetone with the suspended particles is poured into a glass funnel lined with #2 filter paper retaining the very fine precipitate. Add another 30 ml aliquot of acetone and repeat the process until all the gummy precipitate in the mortar has been pulverized and transferred to the funnel. Usually ~10 or more aliquots are required before all the paste is pulverized.
 - (11) Let the filter paper with the acetone-moist material stand 30 min at the room temperature then transfer it to a completely dry glass Petri dish. Spread the

product in the dish with a ceramic spatula or the end of a clean dry glass microscope slide, scraping constantly to evaporate the acetone (20–30 min). A completely dry, very fine, light brown powder should result. Store the powder in a brown glass or opaque polyethylene bottle in a cool dry place. The addition of a small silica desiccant sachet to the bottle may prevent clumping of the very hygroscopic powder.

Yields vary, but 7–12 g of silver proteinate powder should be recovered. We list five peptones (one from China and four available world-wide) that have yielded effective products but, consistent with the findings of others, many peptones fail to produce useable silver proteinate and the characteristics of the “optimal” peptone are still unknown (Bourland 2013 unpubl. observ.; Davenport et al. 1952; Porter and Davenport 1951).

Additional tips, variations and cautions:

Step 1: As soon as the precipitate has settled, pour off the cloudy supernatant promptly. There should be as little water as possible remaining on the precipitate before proceeding to Step 2.

Step 2: Dissolve the precipitate completely in distilled water. If any insoluble material remains, carefully pour off the solution into another flask to which the alcohol is added (Step 2).

Step 3: Avoid stirring or agitation to maximize precipitation.

Step 8: Ensure a pH of 8.0–9.0, otherwise the nucleus may not stain.

Step 9: After pouring off each aliquot of acetone from the precipitate, allow it to dry as completely as possible before adding the next aliquot. Author WB prefers to carry out Step 9 in the mortar instead of the beaker. The solution from Step 8 is added directly to the mortar containing 30 ml of acetone. The milky acetone supernatant is poured off from the mortar.

Step 10: We have no experience with the automatic mortar and pestle but this might prove useful.

Step 11: Spread and stir the product immediately and continuously to avoid rehydration from ambient humidity, otherwise a gummy mass may result. Poor solubility may indicate excessive protein denaturation at some point in the process. When dissolving in distilled water for the impregnation procedure, any small amounts of residual precipitate may be removed by filtration or centrifugation prior to use and do not necessarily preclude effective staining.

All Steps: Use only glass or ceramic (not metal) implements. Avoid any loss of peptone and silver precipitate at each step. Silver waste must be disposed of in accordance with local, state, and country requirements. All laboratory personnel must use appropriate laboratory safety equipment, gloves (latex has better acetone resistance than nitrile), and eye protection.

The protargol produced by this protocol may be so strong that a more dilute solution (0.25–0.33% rather than 1%)

may be necessary for the Wilbert method (Foissner 1991). Author WB has successfully used the amounts of all reagents described in the original protocol which calls for a slightly lower ratio of silver nitrate (10 g) to peptone (40 g) but otherwise follows the sequence of steps described above (Davenport et al. 1952). Yields of ~9–10 g have been achieved with this approach using peptones 3 or 4. If the pH of the protargol solution at the time of impregnation is < 8.0, adjust it by adding a 1:10 aqueous solution of concentrated ammonia drop by drop to obtain a pH of 8.0–9.0.

RESULTS AND DISCUSSION

Results of our impregnations of different groups of ciliates using protargol synthesized by the protocol described (Fig. 1) are comparable to those we obtained previously with commercial products (Fig. 2). Authors XP and WS have used the homemade protargols successfully in the Wilbert (1975) method and author WB has had good results with Vd'ačný and Foissner (2012) modification (Fig. 2, A–K).

Between our two laboratories, the method has been carried out approximately 50 times (XP and WS, 30–40 times using peptones 1 and 5; WB, 15 times using peptones 1–4 and several ineffective peptones not listed). Peptones 1–5 have yielded effective products. However, some of the many other peptones sold do not produce useable silver proteinates (i.e. a precipitate fails to form at appropriate points in the protocol or the final product is insoluble in water). A large number of available peptones remain untested and may prove useable. The characteristics of the “optimal” peptone for protargol synthesis are still unknown (Bourland, pers. observ.; Davenport et al. 1952; Porter and Davenport 1951). As has been true for commercial products, the staining characteristics of “homemade” protargol may differ between batches and many other variables affect the results (e.g. type of material, type of fixative, bleaching method and time, type and duration of development, etc.).

It is unclear whether an effective commercial product will reappear in the future. In the meantime, the protocol described here allows protistologists to continue their work by securing an uninterrupted supply of protargol that should give results comparable to those obtained with previously available commercial products.

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