PROTARGOL METHODS

WILHELM FOISSNER

Universität Salzburg, Institut für Zoologie, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria

INTRODUCTION

Protargol methods are indispensable for descriptive research of ciliates. The first procedures were provided by Kirby (4), Moskowitz (7), Dragesco (2) and Tuffrau (9, 10) and many more modifications were subsequently proposed (1, 5, 6, 8, 11, 12, 13). Here, the three variations which produce good results in our laboratory are described. These procedures work well with most ciliate species (some, however, only rarely impregnate well, e. g. *Loxodes*, *Paramecium*) but require at least 20 specimens. Contrary to the silver carbonate method, a single specimen cannot usually be handled successfully. Depending on the procedure used, protargol can reveal many cortical and internal structures, such as basal bodies, cilia, various fibrillar systems, nuclear apparatus. The silverlines, however, do never impregnate. The shape of the cells is usually well preserved in permanent slides, which is an advantage for the investigation but makes photographic documentation more difficult. However, pictures as clear as those taken from wet silver carbonate impregnations can be obtained with the Wilbert modification if the cells are photographed prior to embedding in the albumen glycerol. Examples: Fig. 1 - 7.

PROTOCOL 1

Most of our preparations are done using this procedure (3). The quality of the slides is usually adequate but frequently not as good as with the Wilbert modification. The latter demands more material and experience; inexperienced workers may easily lose all the material. As in all protargol methods, the procedure is rather time consuming and complicated. Experiments with students showed that beginners have a fair chance of obtaining good slides. A centrifuge may be used for step 2; staining jars are necessary for steps 6-16.

1. Fix organisms in Bouin's or Stieve's fluid for 10-30 minutes.
   Remarks: The fixation time has little influence on the quality of the preparation within the limits given. Ratio fixative:sample fluid should be at least 2:1. Pour ciliates into fixative using a wide-necked flask in order to bring organisms in contact with the fixative as quickly as possible. Both fixatives work well but may provide different results with certain organisms. Stieve's fluid may be supplemented with some drops of 2 % osmium tetroxide for better fixation of very fragile ciliates, e. g. the hypotrich *Urosoma*. This increases the stability of the cells but usually reduces their impregnability.
2. Concentrate by centrifugation and wash organisms 3-4 times in distilled water.
   Remarks: There are now 2 choices: either to continue with step 3 or transfer the material through 30-50-70 % alcohol into 70 % alcohol (isopropanol or ethanol) where it remains stable for several years. Transfer preserved material back through the graded alcohol series into distilled water prior to continuing with the next step. Impregnation may be slightly modified in preserved material.
3. Clean 8 slides (or less if material is very scarce) per sample. The slides must be grease-free (clean with alcohol and flame). Insufficiently cleaned slides may cause the albumen to detach. Mark slides on back if several samples are prepared together. I use staining jars with 8 sections so that I can work with 16 slides simultaneously by putting them back

to back.

4. Put 1 drop each of albumen-glycerol and concentrated organisms in the centre of a slide. Mix drops with a mounted needle and spread over the middle third.
   Remarks: Use about equally sized drops of albumen-glycerol and suspended (in distilled water) organisms to facilitate spreading. The size of the drops should be adjusted so that the middle third of the slide is covered after spreading. Now remove sand, grains, etc. The thickness of the albumen layer should be equal to that of the organisms. Some thicker and thinner slides should however also be prepared because the thickness of the albumen layer greatly influences the quality of the preparation. Cells may dry out and/or shrink if the albumen layer is too thin; if it is too thick it may detach or the cells become impossible to study with the oil immersion objective.
5. Allow slides to dry for at least 12 hours (overnight) at room temperature.
   Remarks: Slides may be allowed to dry for up to 48 hours but no longer if quality is to be maintained. Oven-dried (2 hours at 60 °C) slides are usually also of poorer quality.
6. Place slides in a staining jar filled with 95 % alcohol (isopropanol or ethanol) for 20-30 minutes. Place a staining jar with protargol solution into an oven (60 °C).
   Remarks: Slides should not be transferred through an alcohol series into concentrated alcohol as this causes the albumen layer to detach! Decrease hardening time to 20 minutes if albumen is already rather old and/or not very sticky.
7. Rehydrate slides through 70 % alcohol and 2 distilled water steps for 5 minutes each.
8. Place slides in 0.2 % sodium permanganate solution. Remove first slide (or pair of slides) after 60 seconds and the others at 15 second intervals. Collect slides in a staining jar filled with distilled water.
   Remarks: Bleaching is by permanganate and oxalic acid (step 9). The procedure described above is necessary because each species has its optimum bleaching time. The sequence in which slides are treated should be recorded as the immersion time in oxalic acid must be proportional to that in the permanganate solution. The albumen layer containing the organisms should swell slightly in the permanganate solution and the surface should become uneven. If it remains smooth, the albumen is too sticky and this could decrease the quality of the impregnation. If the albumen swells strongly, it is possibly too weak (old) and liable to detach. Use fresh $KMnO_4$ solution for each series.
9. Quickly transfer slides to 2.5 % oxalic acid. Remove first slide (or pair of slides) after 160 seconds, the others at 20 second intervals. Collect slides in a staining jar filled with distilled water.
   Remarks: Same as for step 8! Albumen layer becomes smooth in oxalic acid.
10. Wash slides 3 times in distilled water for 3 minutes each.
11. Place slides in warm (60 °C) protargol solution and impregnate for 10-15 minutes at 60 °C.
    Remarks: Protargol solution can be used only once.
12. Remove staining jar with the slides from the oven and allow to cool for 10 minutes at room temperature.
    Remarks: In the meantime organize 6 staining jars for developing the slides - distilled water - distilled water - fixative (sodium thiosulfate) - distilled water - 70 % alcohol - 100 % alcohol (isopropanol or ethanol).
13. Remove the first slide from the protargol solution and drop some developer on the layer of albumen. Move slide gently to spread developer evenly. As soon as the albumen turns yellowish, pour off the developer, dip slide into the first 2 distilled water steps for about 2 seconds each and stop development by submerging the slide in the fixative (sodium thiosulfate), where it can be left for 5-10 minutes.
    Remarks: Now control impregnation with the compound microscope. The impregnation intensity is sufficient if the infraciliature is just recognizable. The permanent slide will be too dark if the infraciliature is distinct at this stage of the procedure! The intensity of the impregnation can be controlled by the concentration of the developer and the time of

development. 5-10 seconds usually suffice for the diluted developer! Some species (e. g., most microthoracids) must be treated with undiluted developer. Development time increases with bleaching time. Therefore commence developing with those slides which were in the bleaching solutions for 60 and 120 seconds, respectively. The thinner the albumen layer, the quicker the development.

14. Collect slides in the fixative (sodium thiosulfate) and transfer to distilled water for about 5 minutes.
    Remarks: Do not wash too long; the albumen layer is very fragile and detaches easily!
15. Transfer slides to 70 % - 100 % - 100 % alcohol for 5 minutes each.
16. Clear by two 10 minute transfers through xylene.
17. Mount in synthetic neutral mounting medium.
    Remarks: Do not dry slides between steps 16 and 17! Mounting medium should be rather viscous to avoid air-bubbles being formed when solvent evaporates during drying. If air-bubbles develop in the mounted and hardened slide, re-immerse in xylene for some days until the coverslip drops off. Remount using a more viscous medium and remove possible sand grains protruding from the albumen. Usually, some air-bubbles are found immediately after mounting; these can be pushed to the edge of the coverslip with a finger or mounted needle. The preparation is stable.

REAGENTS

a) Bouin's fluid (prepare immediately before use; components can be stored)
   15 parts saturated, aqueous picric acid ($C_6H_3N_3O_7$; preparation: add an excess of picric crystals to, e. g. 1 litre of distilled water; shake solution several times within a week; some undissolved crystals should remain; filter before use).
   5 parts formalin (HCHO; commercial concentration, about 37 %)
   1 part glacial acetic acid (=concentrated acetic acid; $C_2H_4O_2$)

b) Stieve's fluid (slightly modified; prepare immediately before use; components can be stored)
   38 ml saturated, aqueous mercuric chloride (dissolve 60 g $HgCl_2$ in 1 litre of boiling water)
   10 ml formalin (HCHO; commercial concentration, about 37 %)
   3 ml glacial acetic acid (=concentrated acetic acid; $C_2H_4O_2$)

c) Albumen-glycerol (2-4 month stability)
   15 ml egg albumen
   15 ml concentrated (98%-100%) glycerol ($C_3H_8O_3$)
   Pre-treatment of the egg albumen and preparation of the albumen-glycerol: Separate the white carefully from the yolk and embryo of 3 eggs (free range eggs are preferable to those from battery chickens, whose egg white is less stable and sticky). Shake the white, by hand (do not use a mixer!) for some minutes in a narrow-mouthed 250 ml Erlenmeyer flask until a stiff white foam is formed. Allow the flask to stand for about 1 minute. Pour the viscous rest of the egg white in a second Erlenmeyer flask and shake again until it is stiff. Repeat until most of the egg white is either stiff or becomes watery; usually 4-6 Erlenmeyer flasks of foam are obtained. Leave all flasks undisturbed for about 10 minutes. During this time a glycerol-like fluid percolates from the foam. This fluid and the white from the last flask are collected and used. Add an equal volume of concentrated glycerol and a small thymol crystal ($C_{10}H_{14}O$) for preservation to the mixture. Mix by shaking gently and pour mixture into a small flask. Leave undisturbed for 2 weeks. A whitish slime settles at the bottom of the flask. Decant the clear portion, discard slime and thymol crystal. A "good" albumen-glycerol drags a short thread when touched with a needle. The albumen is too thin (not sticky enough) or too old if this thread is not formed. Fresh albumen which is too thin may be concentrated by leaving it open for some weeks so that water can evaporate. If the albumen is too sticky, which may cause only one side of the organisms to impregnate well, it is diluted with distilled water or old,

less sticky albumen to the appropriate consistency. The preparation of the albumen-glycerol must be undertaken with great care because much depends on its quality. Unfortunately, all commercial products which I have tried detach during impregnation.

d) 0.2 % potassium permanganate solution (stable for about 1 day)
   0.2 g potassium permanganate ($KMnO_4$)
   ad 100 ml distilled water

e) 2.5 % oxalic acid solution (stable for about 1 day)
   2.5 g oxalic acid ($C_2H_2O_4 \cdot 2H_2O$)
   ad 100 ml distilled water

f) 0.4 % protargol solution (stable for about 1 day)
   100 ml distilled water
   ad 0.4 g protargol
   Remarks: Use light-brown "protargol for microscopy" (e. g., Merck's "Albumosesilber für die Mikroskopie" or "Proteinate d'Argent", Roques, Paris, France). Some dark-brown, cheaper products do not work! Sprinkle powder on the surface of the water and allow to dissolve without stirring; use a wide-mouthed bottle for solving the protargol.

g) Developer (mix in sequence indicated; sodium sulfite must be dissolved before hydroquinone is added)
   95 ml distilled water
   5 g sodium sulfite (Na_2SO_3)
   1 g hydroquinone ($C_6H_6O_2$)
   Remarks: This recipe yields the stock solution which is stable for some weeks and should be used undiluted for certain ciliates (step 13). Usually, however, it must be diluted with tap water in a ratio of 1:20 to 1:50 to avoid too rapid development and one-sided impregnation of the organisms. Freshly prepared developer is usually inadequate (the albumen turns greenish instead of brownish). The developer should thus be prepared from equal parts of fresh and old (brown) stock solutions. Take great care with the developer as its quality contributes highly to that of the slides. If the developer has lost its activity (which is not always indicated by a brown colour!) the silver is not or only insufficiently reduced and the slides stain too faintly. A fresh developer should therefore be prepared for each "impregnation week" and some old developer kept. Fresh developer can be artificially aged by adding some sodium carbonate (Na_2CO_3). However, better results are obtained with air-aged solutions, i. e. by a developer which has been kept uncovered for some days in a wide-mouthed bottle. It first turns yellowish, then light brown (most effective) and later dark brown and viscous (at this stage the developer has lost most of its activity but is still suitable for artificial aging of fresh developer = 1:1 mixture mentioned above).

h) Fixative for impregnation (stable for several years)
   25 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)
   ad 100 ml distilled water

PROTOCOL 2

This modification produces excellent results but demands much experience. I manipulate large cells with micropipettes in a watch-glass, whereas the centrifuge is used for steps 1-4, 7, 8 if cells are smaller than about 150 µm. The watch-glass method is used when there are only a few specimens of larger cells; thus an attempt is worthwhile even if only 20 cells are available. The organisms are very soft after development and fixation and are thus easily be compressed between slide and coverslip, which greatly facilitates photographic documentation.

1. Fix organisms as described in the first protargol procedure (Foissner's modification).
2. Wash and, if so desired, preserve organisms as described in the first protargol method (Foissner's modification).

Remarks: Wash cells either in the centrifuge (small species) or in a watch-glass. To change fluids allow cells to settle on bottom of watch-glass and remove supernatant with a micropipette under the dissecting microscope; concentrate cells in the centre of watch-glass by gentle swirling.

3. Transfer organisms with a small amount of distilled water to an at least tenfold quantity of sodium hypochlorite (NaClO) and bleach for about 3 minutes.
   Remarks: This is the critical step in this modification. If bleaching is too strong or too weak all is lost: cells either dissolve or do not impregnate well. Systematic investigations showed that not the bleaching time but the amount of active chloride in the sodium hypochlorite and the pre-treatment of the cells (fixation method, fresh or preserved material) are decisive for the quality of the preparation. Different species need different concentrations. Unfortunately, the concentration of active chloride in the commercial products varies (10-13 %) and is dependent on the age of the fluid. It is thus impossible to provide more than only a few guidelines: 100 ml distilled water + 0.2-0.4 ml NaClO (if product is fresh and cells were not stored in alcohol) or 100 ml distilled water + 0.5-1.6 ml NaClO (if product is older and cells were stored in alcohol). The transparency of the cells under the dissecting microscope may serve as a further indicator: fixed, unbleached cells appear dark and opaque, whereas accurately bleached cells are almost colourless and rather transparent (depends, however, also on size and thickness of the cell). Thus, increase the concentration of sodium hypochlorite stepwise if cells appear too dark with the recommended concentrations. We routinely start with 3 different hypochlorite concentrations if enough material is available.
4. Wash organisms at least 3 times with distilled water and finally once in the protargol solution.
   Remarks: Wash thoroughly, especially when fluids are changed with micropipettes, because even the slightest traces of the sodium hypochlorite disturb the impregnation.
5. Transfer to 1 % protargol solution and impregnate for 10-20 minutes at 60 °C.
   Remarks: This and the next step should be carried out in a watch-glass even for material which is otherwise manipulated with the centrifuge. The impregnation time depends on the kind of material and the degree of bleaching. Check the progress of impregnation every 3-4 minutes under the compound microscope by picking out a few cells with the micropipette under the dissecting microscope; add these to 1 drop of developer. Dilute developer and/or interrupt development by adding a little fixative (sodium thiosulfate) if impregnation is strong enough.
6. Remove most of the protargol solution with a micropipette and add some drops of developer to the remainder containing the organisms.
   Remarks: Fresh, undiluted developer is usually used (but see step 5). Control development in compound or dissecting microscope. As soon as the infraciliature becomes faintly visible, development must be stopped by adding a few drops of sodium thiosulfate. Judging the right moment is a question of experience; the permanent slide will be too dark if the infraciliature is very distinct at this stage of the procedure!
7. Stabilize the impregnation by 2 approximately 5 minute transfers through sodium thiosulfate.
   Remarks: The developer need not be removed before fixation. For small species this and the next step can be carried out in a centrifuge. Larger species must be manipulated with micropipettes because cells become very fragile and would be damaged in a centrifuge. Cells are very soft at this stage and can thus be easily compressed and photographed. Transfer some of the more darkly impregnated specimens with a very small amount of the fixative onto a clean slide using a micropipette and cover with a coverslip. Organisms are usually flattened by the weight of the coverslip; excess fluid may be removed from the edge of the coverslip with a piece of filter paper.
8. Wash very thoroughly in distilled water (3 times with the centrifuge; 7-10 times in watch-glass with micropipettes). Finally remove as much of the water as possible.

Remarks: Even the slightest traces of the fixative destroy the impregnation within a few days or weeks.

9. Smear a moderately thick layer of albumen-glycerol on a clean slide with a finger. Drop impregnated, washed cells on the albumized slide with a large-bore pipette (opening ~ 1 mm) and dry preparation for at least 2 hours.
   Remarks: The cells are too fragile to be spread with a needle. With much care it is possible to orientate cells using a mounted eyelash. Commercial albumen-glycerol can be used.
10. Harden albumen by two 10 minute transfers through concentrated alcohol (isopropanol or ethanol).
    Remarks: This and the next step are best carried out in staining jars. The albumen layer turns milky and opaque.
11. Clear by two 5 minute transfers through xylene.
    Remarks: The albumen layer turns transparent.
12. Mount in synthetic neutral mounting medium.
    Remarks: Same as for step 17 of the first protocol!

REAGENTS

If not stated otherwise, the same reagents like in the first protargol procedure (Foissner's modification) are to be used.

PROTOCOL FOR FEW SPECIMENS

I learned this simple modification in Dr P. Didier's laboratory (Clermont-Ferrand University). It sometimes produces excellent impregnations, especially with species having a firm pellicle (e. g. microthoracids). It also demands little material because the specimens are mounted on the slide without washing.

1. Collect specimens with a micropipette and place them at the centre of a grease-free slide. Remove excess fluid as far as possible.
2. Fumigate cells with 4 % aqueous osmium tetroxide for about 2 minutes.
   Remarks: Hold inverted slide close to the osmium tetroxide. Carry out procedure in a fume hood as osmic acid fumes are highly toxic.
3. Add an equal sized drop of albumen-glycerol, mix thoroughly but gently with a mounted needle and spread mixture in a moderately thin layer over the middle third of the slide.
   Remarks: Albumen-glycerol must be prepared as described in the first protargol procedure (Foissner's modification). Cells are very fragile and frequently break or dissolve.
4. Allow to dry for about 4 hours.
5. Proceed with steps 6 (coagulation of albumen in concentrated alcohol) to 17 of the first protargol procedure (Foissner's modification). Bleaching times are usually about 50 % shorter than with my modification.

LITERATURE CITED

1. Aufderheide, K J. 1982. An improvement in the protargol technique of Ng and Nelsen. *Trans. Am. microsc. Soc.* **101**:100-104.
2. Dragesco, J. 1962. L'orientation actuelle de la systématique des ciliés et la technique d'imprégnation au protéinate d'argent. *Bull. Micr. appl.* **11**:49-58.
3. Foissner, W. 1991. Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. *Europ. J. Protistol.* **27**:313-330.

4. Kirby, H. 1945. The structure of the common intestinal trichomonad of man. *J. Parasit.* **31:**163-174.
5. McCoy, J W. 1974. New features of the tetrahymenid cortex revealed by protargol staining. *Acta Protozool.* **13:**155-160.
6. Montagnes, D J S & Lynn, D H. 1987. A quantitative protargol stain (QPS) for ciliates: method description and test of its quantitative nature. *Mar. Microb. Food Webs* **2:**83-93.
7. Moskowitz, N. 1950. The use of protein silver for staining protozoa. *Stain Technol.* **25:**17-20.
8. Ng, S F & Nelsen, E M. 1977. The protargol staining technique: an improved version for *Tetrahymena pyriformis. Trans. Am. microsc. Soc.* **96:**369-376.
9. Tuffrau, M. 1964. Quelques variantes techniques de l'imprégnation des ciliés par le protéinate d'argent. *Archs Zool. exp. gén.* **104:**186-190.
10. Tuffrau, M. 1967. Perfectionnements et pratique de la technique d'imprégnation au protargol des infusoires ciliés. *Protistologica* **3:**91-98.
11. Wilbert, N. 1975. Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos* year 1975, Heft 6:171-179.
12. Wilbert, N. 1976. A standardized method for identifying and counting the vagile and sessile periphyton. *Oecologia (Berlin)* **24:**343-347.
13. Zagon, I S. 1970. *Carchesium polypinum*: cytostructure after protargol silver deposition. *Trans. Am. microsc. Soc.* **89:**450-468.

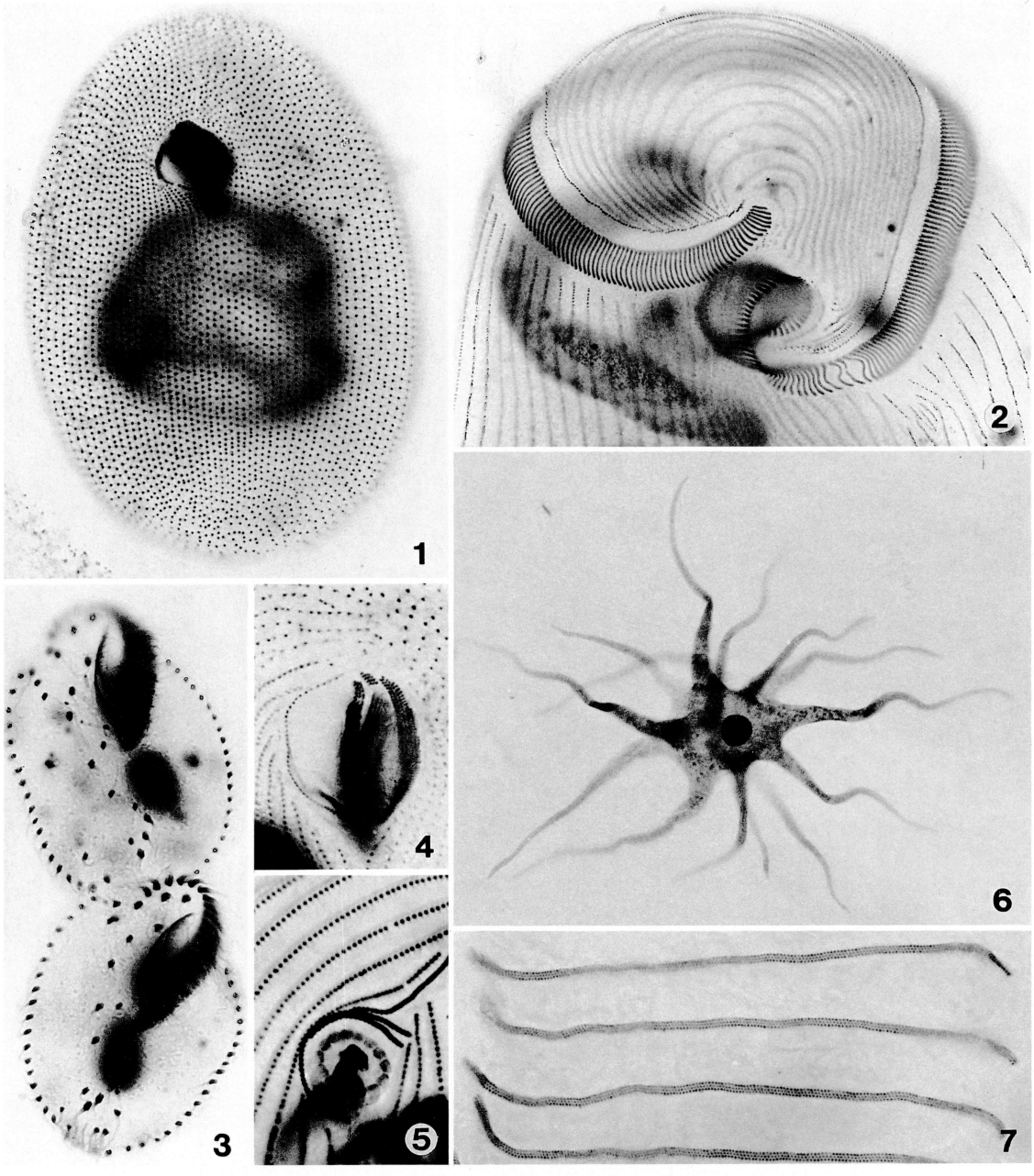

Fig. 1 - 7. Protists prepared with protargol protocols 1 (figure 6) and 2 (figure 1 - 5, 7). **1, 4.** *Epenardia myriophylli*, a tetrahymenid ciliate; ventral view and detail of oral apparatus, length about 100 µm. **2.** *Stentor roeselii*, a heterotrich ciliate; detail showing oral apparatus. **3.** *Sterkiella histriomuscorum*, a hypotrich ciliate; ventral view, length about 100 µm. **5.** *Trithigmostoma steini*, a cyrtophorid ciliate; detail showing oral structures. **6.** A naked soil amoeba, diameter about 50 µm. **7.** *Bursaria truncatella*, a colpodid ciliate; detail of adoral zone of organelles.