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ADDITIONS TO THE GENUS *HETEROCHLAMYDOMONAS* (CHLOROPHYCOPHYTA)

MARTHA F. LANGFORD AND EDMOND R. COX
Middle Tennessee State University, Murfreesboro Tennessee 37130 and
Tennessee Wesleyan College, Athens, Tennessee 37303

ABSTRACT

Two new species of the chlorophycean genus *Heterochlamydomonas* were isolated into axenic culture from soil collected in Fall Creek Falls State Park, located in adjoining portions of Bledsoe and Van Buren Counties, Tennessee. Comparative morphological, cultural, and physiological studies were made of the two new taxa and the previously described species *Heterochlamydomonas inaequalis*.

INTRODUCTION

The genus *Heterochlamydomonas* was established by Cox and Deason (1969) based on the study of an alga isolated from soil collected aseptically in Cedars of Lebanon State Forest, Wilson County, Tennessee. Recently, two new taxa of this genus were isolated into axenic culture during an extensive investigation of soil collected in Fall Creek Falls State Park, located in adjoining portions of Bledsoe and Van Buren Counties near Pikeville, Tennessee (Langford 1968). Fall Creek Falls State Park is a game preserve and recreational area of approximately 17,000 acres situated on the west escarpment of the Cumberland Plateau in Southeastern Tennessee between 35° 40′, and 35° 50′ longitude and 85° 20′ and 85° 30′ latitude. (Caplenor 1954). The two samples which yielded the species described here were collected from surface soil along the rim of one of the deep gorges found in certain regions of the park.

The primary goal of the study was to augment our knowledge of the taxonomy of the soil algal flora of middle Tennessee. The apparent need for the adequate characterization of algae found in soil has been emphasized in previous investigations (Deason and Bold 1960, Chantanachat and Bold 1962, Mattox and Bold 1962, Bischoff and Bold 1963, Brown and Bold 1964, Hofstetter 1968, Cox and Deason, 1968, 1969).

Several auxiliary attributes (see Materials and Methods), other than the morphological characteristics usually used to demarcate algal taxa, were investigated to determine if they would provide additional useful information for species delimitation. With the expanded interest in soil algae, particularly in the last decade, and the isolation and study of numerous taxa which exhibit superficial similarity in mixed cultures, the physiological, cultural, immunochemical and ultrastructural attributes of organisms in axenic cultures have become increasingly important in the elucidation of the taxonomy of many soil algae (Parker, Bold and Deason 1961, Bold and Parker 1962, Kessler and Soeder 1962, Kessler et al 1963, Shihira and Krauss 1963, Brown and Bold 1964, Smith and Bold 1966, Kessler and Czygan 1967, McLean 1968, Nichols et al 1968, Brown and McLean 1969).

MATERIALS AND METHODS

The algae described herein were isolated by plating out surface growth (phototactic green rings) which developed within 14 days after 10 g of aseptically collected soil from each of two soil samples were added singly to 125 ml Erlenmeyer flasks containing 50 ml of sterile Bristol's solution (Deason and Bold 1960). All cultures utilized in this study were maintained under "standard conditions" at 22.0 - 25.0°C under illumination approximating 350 ft-c provided by "cool white" fluorescent bulbs on an automatically controlled 12 hr - 12 hr light-dark cycle unless indicated otherwise. Axenic cultures of each alga were obtained using the spray method of Wiedemann et al (1964). Morphologic observations were usually made on cultures which had been grown on Bristol's agar for 14 days under standard conditions.

A Wratten number 48 filter was used in the study of cellular organization as suggested by Friedmann (1966).

In addition to morphologic observations, several auxiliary attributes of each of the species described in this study and also for *Heterochlamydomonas inaequalis* (Cox and Deason 1969) were investigated. These included studies of: colony characteristics on Bristol's agar; color changes exhibited by algal growth upon aging on Bristol's agar; comparative growth in several complex media; growth in Bristol's solution enriched with selected organic compounds; ability to hydrolyze starch.

Colony characteristics were studied both macroscopically and at a magnification of 20X. A loopful of algal cells from an axenic culture (Bristol's solution) was streaked in a zigzag pattern across the surface of sterile Bristol's agar in Petri dishes using a transfer loop. After incubation for two weeks under standard conditions, isolated colonies, presumably developing from single cells, were described according to the brief glossary of descriptive terms suggested by Bischoff and Bold (1963).

For comparative studies of growth, certain complex organic media were utilized: Bacto-Nutrient Agar, Bacto-Nutrient Broth, Bacto-Thioglycollate Medium, and Yeast Extract Agar. The media were dispensed in cotton-plugged test tubes in 15 ml aliquots and sterilized in the autoclave at 121.5°C for 15 minutes. Each medium was innoculated with 3 drops from a homogeneous algal suspension using sterile Pasteur capillary pipettes. After two weeks incubation under standard conditions, estimation of the amount of growth in each tube of medium was made using the descriptive terms "excellent," "good," "fair," "trace," or "none." Comparisons were made with predetermined standards.

Another series of attributes investigated concerned the differential responses of the algal species to certain reagent graded organic compounds when grown under standard conditions as well as in total darkness. The following organic substances were added in concentrations of 0.75% to Bristol's solution: L-arabinose, D-glucose, D-fructose, D-xylose, D-galactose, and sodium acetate. The media were dispensed in 15 ml aliquots in test tubes plugged with cotton and sterilized in an autoclave for 15 minutes at 121.5°C. Three drops were removed from homogeneous algal suspensions using sterile capillary pipettes and inoculated in quadruplicate into culture tubes containing sterile enriched medium. One set of duplicate tubes was placed under standard conditions of light and temperature and the other set was stored in complete darkness at the same temperature. After 14 days, both sets of cultures were observed for amount of growth and evaluated on the basis of comparison with predetermined standards as "excellent," "good," "fair," "trace," or "none."

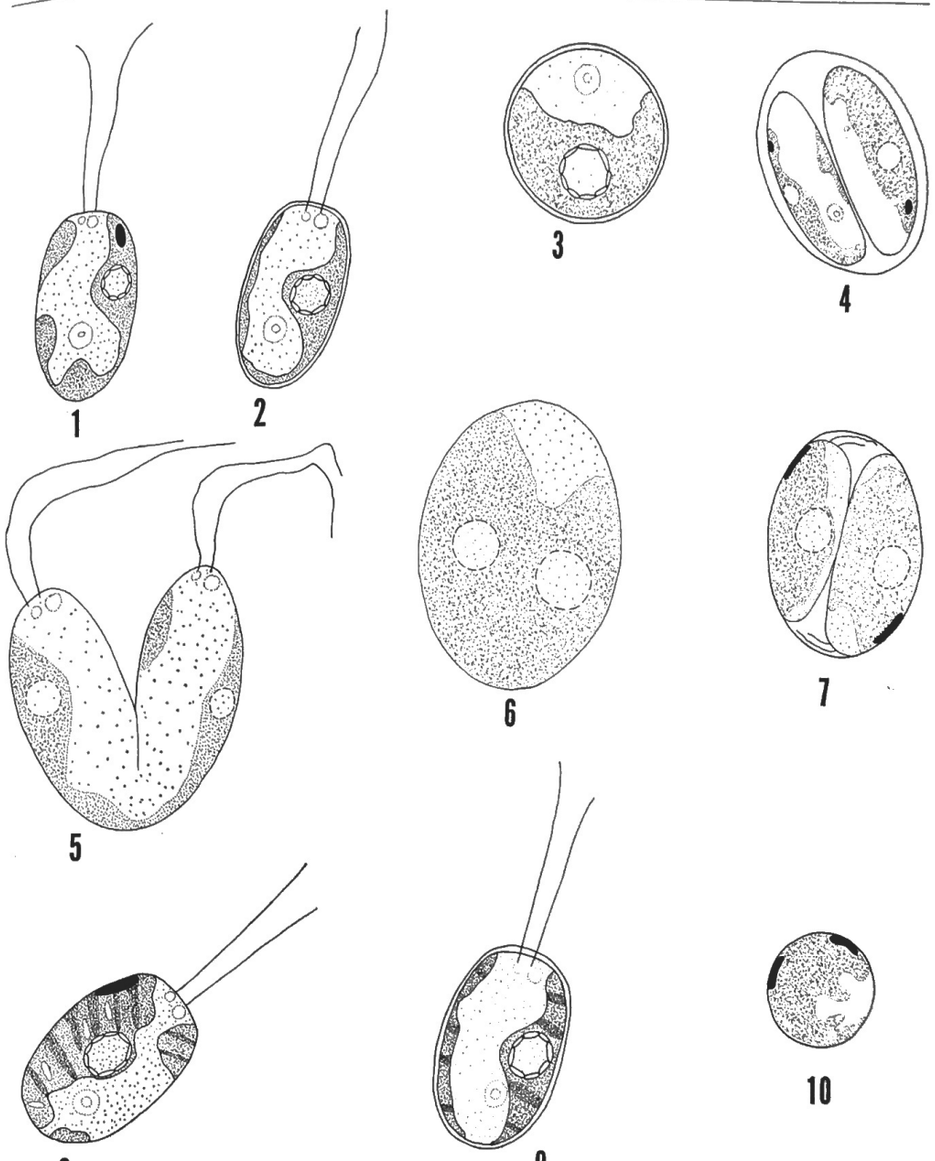

Figure 1-6. *Heterochlamydomonas lobata*. Fig. 1. Vegetative cell in surface view showing lobed chloroplast, oval stigma, and unequal flagella. X3000. Fig. 2. Vegetative cell in median optical section. X3000. Fig. 3. Large immobile cell prior to bipartition. X3000. Fig. 4. The formation of daughter cells by endogenous bipartition. X3000. Fig. 5. Fusion of gametes, X4000 Fig. 6. Zygote beginning to assume spherical form. X4000.

Figure 7-10. *Heterochlamydomonas rugosa*. Fig. 7. Two daughter cells resulting from endogenous bipartition. X3000. Fig. 8. Vegetative cell in surface view showing rugose chloroplast with perforations, large oval stigma, and unequal flagella. X3000. Fig. 9. Vegetative cell in median optical section. X3000. Fig. 10. Spherical zygote following motile period. Note that two stigmata are still visible at this stage. X3000.

Extracellular amylasic activity was determined using a starch agar medium prepared by adding ACS-grade soluble starch in a concentration of 0.2% to Bristol's solution solidified with 1.6% agar. After sterilization in the autoclave, the medium was cooled and dispensed in sterile Petri dishes. Following solidification, algal cells were streaked across the surface of the medium. After 14 days incubation under standard conditions, aqueous I_2KI solution was used to detect the presence of starch. Amylasic activity could be detected by the presence of a clear (starch-digested) zone adjoining the algal growth.

All experiments concerned with auxiliary attributes were performed in duplicate and repeated at least twice.

Axenic cultures of each newly described alga have been deposited in the Culture Collection of Algae, Indiana University, Bloomington, Indiana.

Observations
Diagnosis

Heterochlamydomonas lobata sp. nov.

Cellulae libere natantes 6.0-9.0 x 3.0-6.0 microns anguste aut late ellipsoideae, nonnullae antice truncatae aut aliquantulum asymmetricae. Cellulae sphaericae immobiles ad 10.5 microns diam. accrescunt. Papilla nulla; membrana cellularum mobilium levis teruisque, immobilium senescens non spissescens. Cellulae mobiles praebentes 2 flagella inaequa antice inserta, quasi eadem longitudine acs corpus cellulae, chloroplastum parietalem, cellulam partim circumdantem, solidium, margine perspicue lobato, pyrenoidem aequatoriam, stigma ovale anterius, 2 vacuolas contractiles anteriores et nucleum posteriorem. Cellulae in matrice gelatinosa communi inclusae. Matrices singulae nullae.

Reproductio asexualis per bipartitionem cellularum immobilium, ad 2, 4 vel 8 cellulasfiliales formandas, effecta.

Reproductio sexualis isogamica. Membrana zygotorum maturorum non ornata.

Culturae duarum hebdomadum aetate in agaro Bristolii dilute virides, viscides, post tres menses dilute virides manentes.

Origo: loco Fall Creek Falls State Park, Bledsoe and Van Buren Counties, Tennessee dicto; m. Aug. 1966.

Latin diagnosis prepared by Dr. Hannah Croasdale of Dartmouth College.

Mature vegetative cells of this alga are free-swimming, narrowly to broadly ellipsoidal (usually truncate anteriorly and rounded posteriorly), or sub-globose (Fig. 1, 2, 11). Occasional motile cells are slightly asymmetrical, being more rounded along one cell margin. They range from 6.0 microns to 9.0 microns in length and from 3.0 microns to 6.0 microns in width. Vegetative cells from 14-day-old Bristol's agar cultures are embedded within a common gelatinous matrix, demonstrable by India ink (Fig. 11) and methylene blue stains. Individual matrices are absent. These cells may have or lack flagella. Those with flagella become immediately motile upon transfer to distilled water; those without flagella produce them within a short time and also become actively motile. A papilla is absent. Two flagella of distinctly unequal length are inserted close together anteriorly and approximate the cell body in length (Fig. 1, 2). Each cell contains 2 anterior contractile vacuoles and a single, posterior nucleus. The chloroplast is massive, parietal, has a smooth surface, and partially encircles the cell. The margin of the chloroplast is usually conspiculously lobed. A single pyrenoid is always equatorial in position and ranges from 1.5-3.0 microns in size. An oval stigma is located anteriorly (Fig. 1). The protoplast is closely adpressed to a thin but distinct cell wall.

Motility is of relatively long duration, lasting 24 hours or more. When motile cells become quiescent, the flagella disappear, and the cells begin to enlarge. The subsequent immobile cells are ellipsoidal, subglobose or globose in shape (Fig. 3), and may attain a maximum diameter of 10.5 microns. The cell wall, however, remains smooth and thin during enlargement.

Asexual reproduction is accomplished by the endogenous bipartition of the protoplast of immobile cells to form 2, 4, or 8 daughter cells (Fig. 4) which are liberated by gradual gelatinization of the parent cell wall.

Sexual reproduction is isogamous and is usually observed within several hours after cells are transferred from Bristol's agar slants to distilled water. Zoogametes (facultative isogametes) fuse laterally (Fig. 5). The ensuing planozygotes remain motile for several

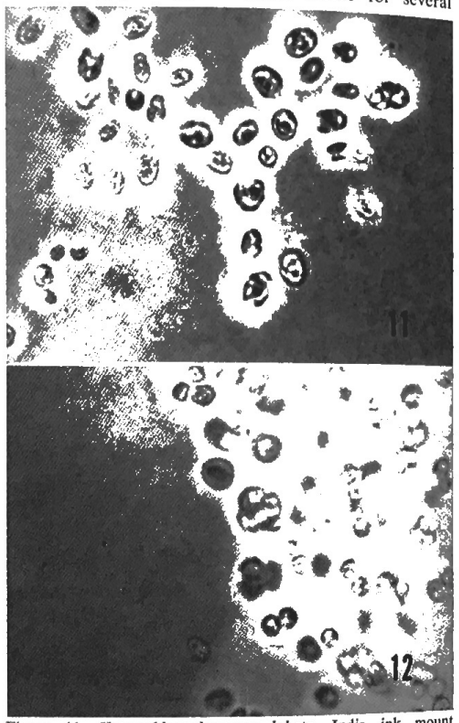

Figure 11. *Heterochlamydomonas lobata.* India ink mount showing vegetative cells embedded within a common gelatinous matrix. X970.

Figure 12. *Heterochlamydomonas rugosa.* India ink mount showing vegetative cells embedded within a common gelatinous matrix. X970.

hours then settle down and round up gradually (Fig. 6) to form spherical zygotes with unornamented walls. Although sexual reproduction is of frequent occurrence, zygote germination was never observed. Attempts to induce germination were not successful.

TABLE I. COMPARATIVE GROWTH OF HETEROCHLAMYDOMONAS SPP. IN/ON CERTAIN COMPLEX MEDIA*

	H. inaequalis	H. lobata	H. rugosa
Nutrient Agar	Excellent	None	Trace
Yeast Extract Agar	Excellent	Fair	Trace
Nutrient Broth	Fair	None	None
Thioglycollate Medium	None	None	None

* Grown under standard conditions of temperature and illumination for 14 days

Colonies on Bristol's agar are light-green after 2-weeks growth, remaining light-green after 3 months. The surface of 2-week-old colonies when observed at 20X magnification is shiny, smooth, and of viscid consistency.

TABLE II. GROWTH OF HETEROCHLAMYDOMONAS SPP. UNDER STANDARD CONDITIONS AFTER 14 DAYS IN BRISTOL'S SOLUTION ENRICHED WITH VARIOUS ORGANIC CARBON SOURCES*

	H. inequalis	H. lobata	H. rugosa
Glucose			
in light	Fair	Good	Trace
in darkness	None	None	None
Galactose			
in light	Trace	Fair	Trace
in darkness	None	None	None
Fructose			
in light	Trace	Fair	Trace
in darkness	None	None	None
Xylose			
in light	Trace	Fair	Trace
in darkness	None	None	None
Ribose			
in light	Trace	Excellent	Trace
in darkness	None	None	None
Arabinose			
in light	Trace	Fair	Trace
in darkness	None	None	None
Sodium Acetate			
in light	Trace	Trace	Trace
in darkness	None	None	None

*Grown in light (l) as well in total darkness (d)

See Tables 1, 2, and 3 for comparative results of the physiological tests for the 3 *Heterochlamydomonas* spp.

TABLE III. AMYLASTIC ACTIVITY OF HETEROCHLAMYDOMONAS SPP. AFTER 14 DAYS ON BRISTOL'S AGAR SUPPLEMENTED WITH 0.2% SOLUBLE STARCH AND GROWN UNDER STANDARD CONDITIONS

	Amylasic Activity
H. inaequalis	None
H. lobata	None
H. rugosa	None

Heterochlamydomonas rugosa sp. nov.

Celulae libere natantes 7.5-10.5 X 3.0-6.0 microns sphericae, ellipsoideae (nonnullae antice truncatae, postice rotundatae) aut paululum asymmetricae. Cellulae immobiles sphericae usque ad 13.5 microns diam. accrescunt. Papilla nulla; membrana cellularum mobilium immobiliumque levis tenuisque. Cellulae mobiles habentes duo flagella inaequa, antice inserta, ½ long. ac cellula aut paululo breviora, chloroplastum parietalem, cellulam partim circumdantem, superficie rugosa atque aliquot ad plures perforationes praebentes, pyrenoidem aequatoriam, interdum paululo anteriorem, stigma magnum ovale anterius, duas vacuolas contractiles anteriores et nucleum posteriorem. Cellulae in matrice gelatinosa communi inclusae. Cellulae singulae sine matricibus.

Reproductio asexualis per bipartitionem endogenam cellularum immobilium, ad 2, 4, 8 vel interdum 16 cellulas-filiales formandas, effecta.

Reproducto sexualis isogamica. Membrana zygoti levis tenuisque.

Culturae duarum hebdomadum aetate in agaro Bristolii dilute virides, viscides, post 3 menses dilute virides manentes.

Origo: loco Fall Creek Falls State Park, Bledsoe and Van Buren Counties, Tennessee: m. Aug., 1966.

Latin diagnosis prepared by Dr. Hannah Croasdale of Dartmouth College.

Most free-swimming cells of this alga are ellipsoidal, truncate anteriorly and rounded posteriorly (Fig. 8), but spherical cells are also of frequent occurrence. A small number of asymmetric cells were encountered. Motile cells range from 7.5 to 10.5 microns in length and from 3.0 to 6.0 microns in width. A common gelatinous matrix is evident when cells are transferred from Bristol's agar to distilled water, but as the cells become motile and swim out of the matrix, no individual sheaths are discernible. The presence of the common matrix was confirmed by staining with dilute India ink (Fig. 12). Many cells from 14-day-old cultures have flagella and stigmata and become immediately motile upon transfer to distilled water. Those without flagella produce them within a short time and also become motile. No papilla is present. Two flagella of distinctly unequal length are inserted close together anteriorly and are approximately ½ to slightly less than the cell body in length (Fig. 9). Each cell contains two anterior contractile vacuoles and a single posterior nucleus. The chloroplast is parietal, partially encircles the cell, and has a very rugose surface with several to many perforations (Fig. 8). A single pyrenoid is typically equatorial in position, but was occasionally observed to be slightly anterior, ranging from 1.5 to 2.5 microns in diameter. The stigma is large, anterior, and somewhat oval to slightly asymmetric in shape (Fig. 8). The protoplast is adpressed to a smooth, thin cell wall.

Motility is usually of short duration, lasting for 18 hours or less. As cells become quiescent, the flagella disappear and enlargement ensues. During enlargement, ellipsoidal cells may become spherical or retain their

original shape. Some large spherical cells may attain a diameter as great as 13.5 microns; however, the wall remains smooth and thin regardless of cell size.

Asexual reproduction is by endogneous bipartition of the protoplast of immobile cells to form 2, 4, 8, or occasionally 16 daughter cells (Fig. 7) which are liberated by gradual gelatinization of the parent cell wall.

Sexual reproduction is accomplished by the fusion of facultative isogametes. Cells fuse laterally. The planozygotes remain motile for several hours usually, then become quiescent. The flagella disappear and the zygotes gradually become spherical (Fig. 10). Sexual reproduction is of common occurrence for this species within a short time after cells are transferred from 2-week-old Bristol's agar cultures to distilled water.

On Bristol's agar, colonies are light-green after 2 weeks growth, and remain light-green after 3 months. The surface of colonies examined at a magnification of 20X is shiny and smooth at 2 weeks. Colonies are of viscid consistency.

See Tables 1, 2, and 3 for comparative results of the physiological tests.

For the convenience of future investigators who concern themselves with the study of taxa in this group, the morphologic attributes of the 3 species described previously or in this investigation are summarived in the following tabulation.

Heterochlamydomonas inaequalis Cox and Deason

(a) Free-swimming cells ellipsoidal (usually truncate or rounded anteriorly), or ovoidal; 5.6-9.8 x 2.8-5.6 microns. Immobile cells spherical to sub-spherical, attaining a maximum size of 14.0 microns.

(b) Motile vegetative cells with two large anterior contractile vacuoles and a posterior nucleus. No papilla present.

(c) Cell walls of motile cells thin, smooth and distinct; walls of immobile cells thicken up to 3.0 microns. Motile cells may exhibit gelatinous sheaths up to 3.0 microns in thickness. Sheaths confluent in cell aggregates.

(d) Chloroplast in motile cells parietal, partially encircling the protoplast, with a very irregular surface dissected by numerous fissures, or even segmented; with a single equatorial to slightly anterior pyrenoid; with a large convexo-concave anterior stigma.

(e) Three-month-old cultures are dark-green. Two-week-old colonies are shiny, smooth, and of viscid consistency.

(f) Immobile cells divide by endogenous bipartition.

(g) Sexual reproduction not observed.

Source: Soil (Cedars of Lebanon State Forest, Wilson County, Tennessee)

Heterochlamydomonas lobata sp. nov.

(a) Free-swimming cells broadly to narrowly ellipsoidal, sub-globose, or slightly asymmetric; 6.0-9.0 x 3.0-6.0 microns. Immobile cells ellipsoidal, sub-globose, globose, attaining a maximum size of 10.5 microns.

(b) Motile vegetative cells with two anterior contractile vacuoles and a posterior nucleus. No papilla present.

(c) Cell walls of motile cells thin, smooth and distinct; walls of immobile cells remain smooth and thin during enlargement. Common gelatinous matrix present. Individual matrices absent.

(d) Chloroplant in motile cells is parietal, partially encircling the protoplast, massive, with a smooth surface and a conspiculously lobed margin; containing a single equatorial pyrenoid; with an oval anterior stigma.

(e) Three-month-old cultures are light-green. Two-week-old colonies are shiny, smooth, and of viscid consistency.

(f) Immobile cells divide by endogenous bipartition.

(g) Sexual reproduction by isogamy.

Source: Soil (Fall Creek Falls State Park, Bledsoe and Van Buren Counties, Tennessee)

Heterochlamydomonas rugosa sp. nov.

(a) Free-swimming cells ellipsoidal, truncate anteriorly and rounded posteriorly, spherical, or slightly asymmetric; 7.5-10.5 x 3.0-6.0 microns. Immobile cells ellipsoidal or spherical, attaining a maximum size of 13.5 microns.

(b) Motile vegetative cells have two anterior contractile vacuoles and a posterior nucleus. No papilla present.

(c) Cell walls of motile cells thin, smooth and distinct; walls of immobile cells remain smooth and thin during stages of enlargement. Common gelatinous matrix present. Individual cells lack sheaths.

(d) Chloroplast in motile cells is parietal, partially encircling the cell, with a rugose surface and several to many perforations; containing a single equatorial pyrenoid; with a large oval to irregularly shaped anterior stigma.

(e) Three-month-old cultures are light-green. Two-week-old colonies are shiny, smooth, and of viscid consistency.

(f) Immobile cells divide by endogenous bipartition.

(g) Sexual reproduction isogamous.

Source: Soil (Fall Creek Falls State Park, Bledsoe and Van Buren Counties, Tennessee)

Discussion

Cox and Deason designated the new genus *Heterochlamydomonas* as a unicellular chlorophycean alga: having motile vegetative cells with flagella of distinctly unequal length; with the protoplast adpressed to a distinct cell wall; and reproducing asexually by successive bipartition. The two taxa described in this study, *Heterochlamydomonas lobata* and *Heterochlamydomonas rugosa,* exhibit these generic attributes. Careful comparative studies of the 3 taxa in culture revealed that both *H. lobata* and *H. rugosa* differed from *H. inaequalis*: in the size and shape of motile cells; in the absence of sheaths about individual cells; in chloroplast structure; in the size of immobile cells; in cell wall thickness of immobile cells; in the color of 3-month-old agar cultures; in the occurrence of sexual reproduction; and in certain auxiliary attributes (see Tables 1, 2, and 3).

In addition, *H. lobata* also exhibits distinct differences from *H. rugosa*: in the size and shape of motile cells; in chloroplast structure; in the size of immobile cells; and in certain auxiliary attributes (see Tables 1, 2, and 3).

Since the organisms herein described demonstrate several well-defined morphologic and physiologic differences, they are designated as separate taxa of the genus *Heterochlamydomonas.*

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SPLENIC FIBRINOID NECROSIS IN MOUSE RADIATION CHIMERAS WITH SECONDARY DISEASE*

William D. Gude, C. C Congdon, and G. E. Cosgrove
Biology Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

Abstract

Fibrinoid necrosis was observed in three strains of mice following irradiation and transplantation of parental spleen or allogeneic bone marrow. Radiation alone or injection of hematopoietic cells without radiation did not induce the lesion. The presence of fibrin within areas of necrosis was confirmed with five commonly used histological stains. The morphological and staining characteristics of these lesions were identical with those generally described in fibrinoid necrosis.

Irradiated mice that have received allogeneic bone marrow, parental spleen, or parental bone marrow cells develop a clinical syndrome called "secondary disease." It is also known as "foreign spleen or bone marrow reaction" and appears within 2 weeks after cell injection. Considered to be an immunologic reaction, the disease

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