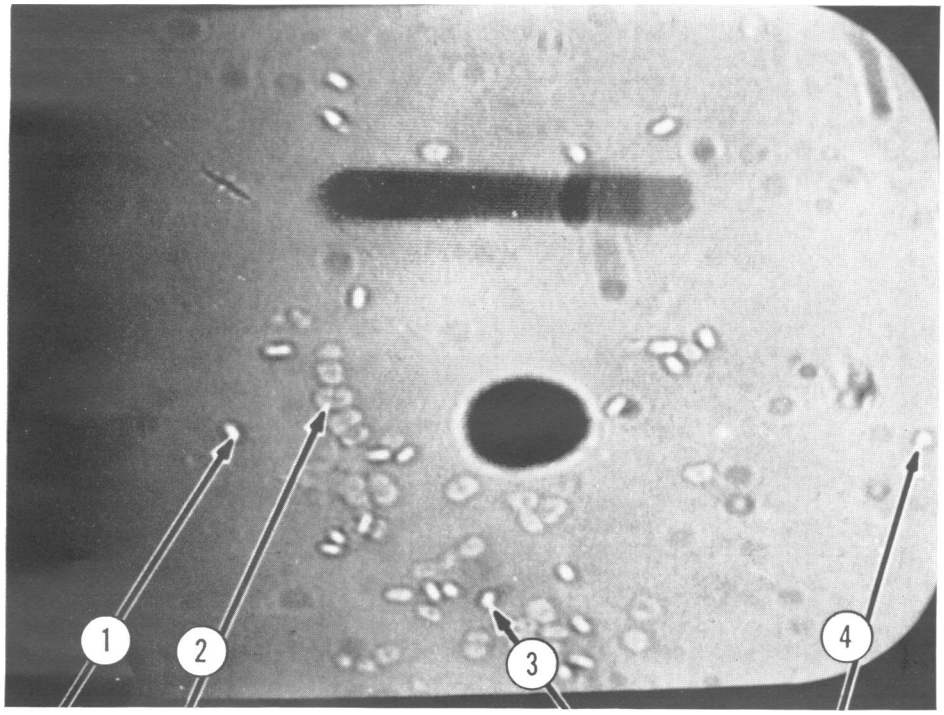

FIG. 4. *Mixture of unstained Escherichia coli (arrow 1), unstained Sarcina lutea (arrow 2), and stained Bacillus globigii spores (arrows 3 and 4). Immunofluorescence of B. globigii spores was taken in this background.*

cross-staining (so far undetected) may be discernible immediately, especially if morphology and UV-absorption profiles do not conform.

In larger organisms or cells, subdivisions into various subcellular antigen-antibody studies appear to be feasible in a quantitative manner.

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IMPROVED STAIN FOR VISUALIZATION OF *AZOTOBACTER* ENCYSTMENT

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Electron microscopy of the encysting azotobacter reveals a sequence of morphogenic stages resulting in a mature cyst of characteristic and well defined cytology (Wyss, Neumann, and Socolofsky, J. Biophys. Biochem. Cytol. **10**:555, 1961). The usual stains employed with light microscopy give no clue to the encystment process, and often give a dubious picture of the mature cyst. We devised a new staining procedure which shows cytological structures that correlate very well with electron micrographs of the encystment process; this stain has been used in these laboratories for the study and detection of encysting azotobacter for 1 year with very satisfactory results.

The stain, based on that proposed by Horkin and Horkin (Am. J. Clin. Pathol. **25**:466, 1955) for the detection of intestinal parasites, con-

tained: water, 100 ml; glacial acetic acid, 8.5 ml; Na_2SO_4, 3.25 g; Neutral Red (Difco), 200 mg; Light Green SF Yellowish (Allied Chemical Corp., New York, N.Y.), 200 mg; and ethyl alcohol, 50 ml; each ingredient was added to the water with continuous mixing on a magnetic stirrer. Stirring was continued for an additional 15 min; an amorphous precipitate which formed was removed by filtering the solution through a 0.5-μ filter membrane. Bacterial growth from solid or liquid media was suspended in the stain and could be observed at once as a "wet mount." Such suspensions, kept in closed containers, could be preserved for months with no apparent loss of cellular detail or change of color. Cysts that had aged on the surface of agar plates did not stain well unless the suspension was heated; this was done by passing the prepared wet mount over a flame in exactly the same way as a smear is "fixed" for staining.

The vegetative form of azotobacter, grown on Burk's medium with *n*-butanol as carbon source, on potato infusion agar, or in Augier's (Ann. Inst. Pasteur **91**:760, 1956) liquid medium, stained a light yellowish-green. As the cells proceeded toward encystment, the cytoplasm condensed to a smaller volume, receded from the outer cell covering, and took on a deeper greenish coloration. During this sequence of events, the outer cell covering thickened and assumed a brownish-red hue. As the cysts approached maturity, a clear unstained area, the intine, was revealed. Mature cysts showed a distinct "central body" that stained deep green with lighter staining areas in it, an unstained intine comprising a large part of the cyst volume, and a thick and compacted brownish-red exine (Fig. 1).

This stain was superior to Anthony's capsule stain (Socolofsky and Wyss, J. Bacteriol. **81**:946, 1961) and the stain of Callao and Hernandez (Microbiol. Espan. **14**:129, 1961) for the detection and study of the encystment process in azotobacter. The major advantages are: (i) no cellular distortion due to drying and fixing procedure, (ii) rendition of cellular constituents in contrasting colors, and (iii) close agreement with structural features seen by phase-contrast and electron microscopy. It is especially useful in distinguishing between mature cysts and the precyst forms of azotobacter. Unpublished data from our laboratories indicate that stains inadequate for this purpose include: Gram stain, Ziehl-Neelsen acid-fast stain, spore stain of Schaeffer and Fulton, negative capsule stain of Schuhardt and Young, India ink negative stain, Benians' negative stain, Sudan Black B fat stain, Wright's blood stain, and Giemsa stain.

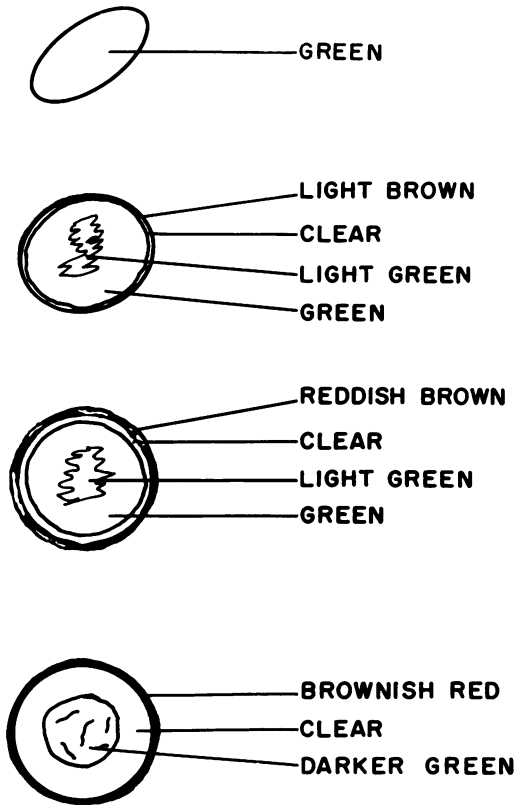

FIG. 1. *Artist's schematic rendition of color photomicrographs obtained with the stain described. From top to bottom: 2-day-old vegetative cell; 3-day-old precyst, 5-day-old young cyst, and 10-day-old mature cyst of Azotobacter chroococcum grown on Burk's medium with n-butanol as the carbon source.*

The cysts of Azotobacteraceae, as revealed by this new stain, agree in appearance with the descriptions and illustrations of azotobacter cysts given by Winogradsky (Ann. Inst. Pasteur **60**:351, 1938), Wyss, Neumann, and Socolofsky (J. Biophys. Biochem. Cytol. **10**:555, 1961), and Tchan, Birch-Andersen, and Jensen (Arch. Mikrobiol. **43**:50, 1962).