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Nile Blue A as a Fluorescent Stain for Poly-β-Hydroxybutyrate

ANTHONY G. OSTLE* AND J. G. HOLT

Department of Microbiology, Iowa State University, Ames, Iowa 50011

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Poly-β-hydroxybutyrate granules exhibited a strong orange fluorescence when stained with Nile blue A. Heat-fixed cells were treated with 1% Nile blue A for 10 min and were observed at an excitation wavelength of 460 nm. Glycogen and polyphosphate did not stain. Nile blue A appears to be a more specific stain for poly-β-hydroxybutyrate than Sudan black B.

Poly-β-hydroxybutyrate (PHB) is a polymeric ester which functions as an energy and carbon reserve in procaryotic cells. PHB exists as discrete inclusions or granules in the cell (3, 4, 10). PHB granules have been recognized by their affinity for the dye Sudan black B (1, 8, 17), which is a presumptive test for the presence of PHB (12).

The presence of PHB has been used as a taxonomic criterion for the classification of bacteria for some time, since this characteristic appears to be stable (6). The amount of PHB produced is highly variable and dependent upon culture conditions (14).

Nile blue A (Nile blue sulfate, Basic blue 12) is a basic oxazine dye which is soluble in water and ethyl alcohol (9). The oxazone form of the dye (Nile pink) is formed by the spontaneous oxidation of Nile blue A in aqueous solution or by refluxing Nile blue A with dilute sulfuric acid. Nile pink is soluble in neutral lipids which are liquid at the staining temperature (15). The Nile blue A-Nile pink mixture has been used as a histological fat stain in prepared tissue sections (2, 13; V. C. Ferrans, Ph.D. dissertation, Tulane University, New Orleans, La., 1963). In this paper we describe a method for selectively staining PHB granules with Nile blue A.

A 1% aqueous solution of Nile blue A (Matheson, Coleman & Bell stock NX0395) was prepared and filtered before use. Mild heating may be necessary to fully dissolve the stain, but acid

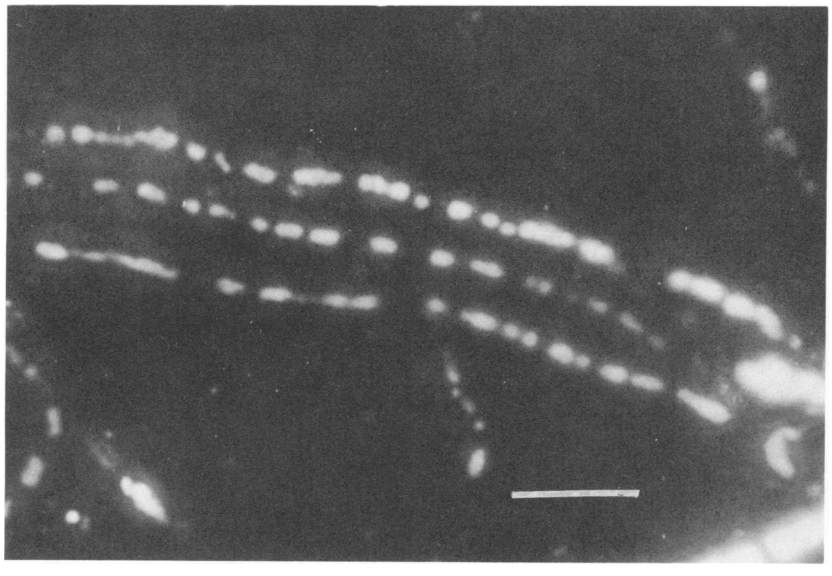

FIG. 1. PHB granules in *Bacillus megaterium* KM stained with Nile blue A and observed under fluorescent light. Note the individual granules within the cells. Bar, 3.0 μm.

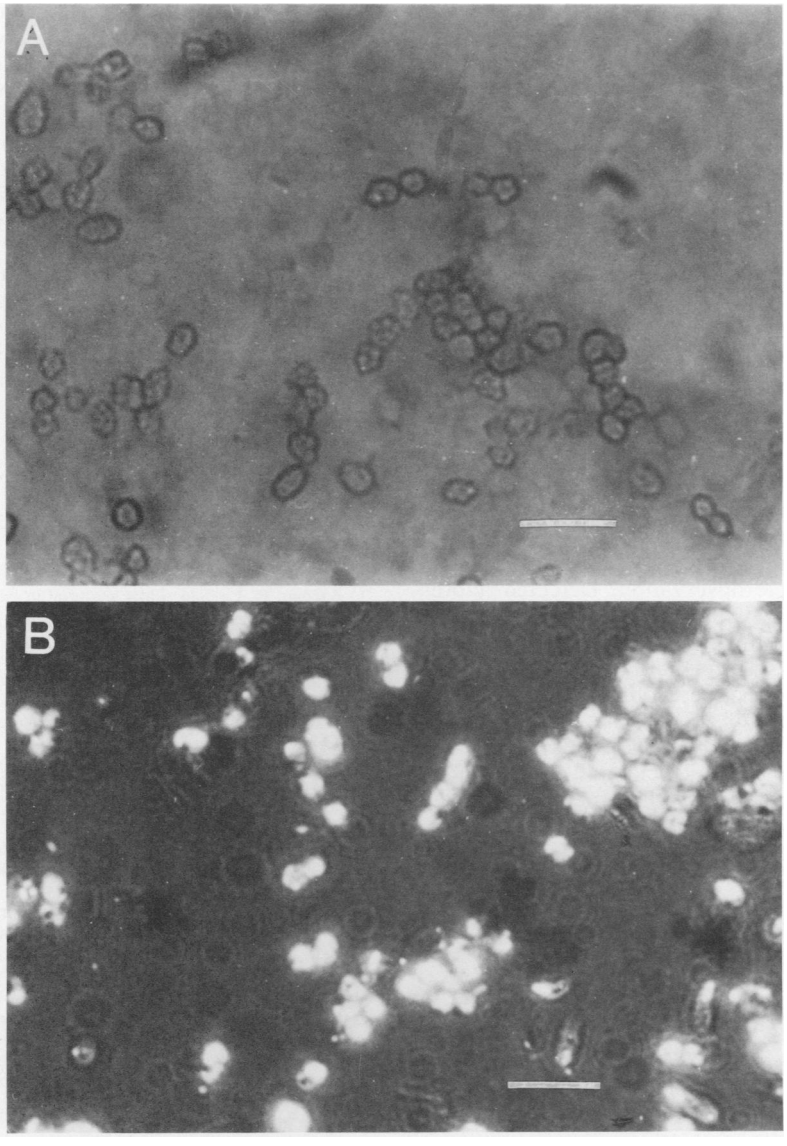

FIG. 2. PHB granules in *A. chroococcum* stained with (A) Sudan black B and (B) Nile blue A. Both preparations were from the same batch of stationary-phase cells. The Nile blue A-stained cells were photographed with simultaneous fluorescent and visible light. Bar, 2.0 μm.

reflux to produce the oxazone form was not required.

Azotobacter chroococcum and *Bacillus megaterium* KM cells were grown under conditions favorable for PHB production (5, 11, 16).

Heat-fixed smears of bacterial cells were stained with the Nile blue A solution at 55°C for 10 min in a coplin staining jar. After being stained, the slides were washed with tap water to remove excess stain and with 8% aqueous acetic acid for 1 min. The stained smear was washed and blotted dry with bibulous paper, remoistened with tap water, and covered with a no. 1 glass cover slip. The cover slip is necessary, as standard immersion oil will extract some of the fluorescent dye and obscure the field with a general yellow fluorescence. The cover slip thus protects the stained cells from immersion oil.

The preparation was examined with a Nikon Labphot microscope with an episcopic fluorescence attachment. The Nikon blue excitation

method, which provides an excitation wavelength of approximately 460 nm, was used.

PHB granules fluoresced as bright orange, with individual granules often visible within a cell. The accompanying black-and-white photomicrographs show these fluorescing orange granules as an intense bright image within the cells. (Fig 1.) Nile blue A appeared to have a greater affinity for PHB than did Sudan black B (Fig. 2). *A. chroococcum* cells grown under conditions unfavorable for PHB production (16) have only occasional PHB granules visible under phase-contrast microscopy, and cells without such granules exhibit no orange fluorescence. Cell membranes or other lipid-containing cell components apparently do not absorb enough of the dye to give detectable fluorescence.

Stained cells were examined in a fluorimeter, and the observed fluorescence was found to be strongest at an excitation wavelength of approximately 362 nm, although 460 nm was adequate for microscopic observation. Detected fluorescence increased with increasing PHB concentration, but assay of PHB by treatment with Nile blue A and measurement of fluorescence did not appear to be sensitive enough to be of use.

The presence of PHB in the bacterial strains used was confirmed by isolation of the polymer, degradation to crotonic acid, and analysis of the degradation products by UV spectra (7).

The specificity of the stain was examined by staining glycogen powder (oyster and fungal) in vitro with Nile blue A. No fluorescence was detected. Metachromatic granules in *Corynebacterium diphtheriae* did not fluoresce when stained with Nile blue A.

Heat-fixed cells of *A. chroococcum* were extracted overnight with chloroform or dichloromethane (PHB solvents). Although clear areas in the cells corresponding to the location of PHB granules could be seen, these areas did not fluoresce when stained. In cells which were unusually large or in which capsular material was present, it was often necessary to boil the chloroform and use longer extraction times to fully remove the PHB.

If heat-fixed cells were similarly treated with the lipid solvents benzene or ether (in which PHB is not soluble), the cell structure was sometimes disrupted, but PHB granules were not extracted and still fluoresced when stained. Such extractions may also be performed before heat fixation, but not after staining, as the stain may be extracted independently of the PHB.

We have concluded that Nile blue A is a satisfactory stain for PHB granules in bacteria and is in fact superior to Sudan black B for this purpose. Nile blue A appears to stain many more PHB granules than Sudan black B does and is not as easily washed from the cell by decolorization procedures. We have not found any bacterial inclusion bodies that stain in a manner similar to that of PHB when treated with Nile blue A.

It is probable that the oxazone form of the dye Nile pink is responsible for the fluorescent staining of PHB, since the characteristics of the observed fluorescence agree with the published descriptions of Nile pink (2).

When it is combined with extraction procedures, staining with Nile blue A is a satisfactory test for the presumptive identification of PHB granules in bacterial cells. Chemical analysis should still be performed to confirm the presence of PHB.

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