Use of a Fluorescent Brightener to Demonstrate Cellulose in the Cellular Slime Molds

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The presence and location of cellulose in different stages of the life cycles of the cellular slime molds can be demonstrated by use of the disodium salt of 4,4'-bis(4-anilino-6-bis (2-hydroxyethyl)-amino-s-triazin-2-ylamino)-2,2'-stilbene disulfonic acid, a fluorescent brightener. It may be used successfully as a direct stain at a concentration of 0.1% in half-normal saline at pH 6; and it may be incorporated into growth media as a vital stain at a concentration of 0.0025% with no inhibitory effect at any developmental stage. Vegetative myxamoebae contain no cellulose and show no fluorescence in the presence of this brightener when viewed with ultraviolet light. In later stages of the life cycle, the time and sites of cellulose formation can be demonstrated with the brightener because of its fluorescence. e.g., in the slime covering of the pseudoplasmodia, in the sorophore sheath, in the walls of stalk cells and spores, in the walls of microcysts, and in the walls and sheath material of macrocysts. The brightener appears to be a very sensitive indicator for cellulose, and it has certain advantages over other cellulose stains, since the staining reaction (fluorescence) is very intense, long-lasting, and not obscured by unstained cellulose-free myxamoebae if such are present.

The cellular slime molds have definite and characteristic life cycles, in which the first part consists of myxamoebae that feed, divide, and are completely independent of each other. This vegetative stage ends when the myxamoebae stop feeding. They aggregate soon thereafter to form the multicellular stages in which they exhibit coordination of movement and function, leading to the formation of the fruiting structures, or sorocarps. Figure 1 shows, in diagrammatic form, the main stages in the generalized life cycle of the cellular slime molds. The usual sequence is that of the outer circle. Depending upon the species, the pseudoplasmodia may migrate for some distance before fructification occurs, or sorocarp formation may be initiated at the site of cell aggregation. Microcysts, and more especially macrocysts, are found only in certain species, and sometimes only under certain conditions (1, 8).

By using techniques such as staining reactions, polarized-light microscopy, electron microscopy, X-ray diffraction, and chemical analysis, several workers (4–7) have shown that these organisms produce cellulose, particularly in the later stages of their life cycles where it has structural importance.

Darken (3) and Cole (2) described the use of the fluorescent brightener 4,4'-bis(4-anilino-6-bis(2-hydroxyethyl)-amino-s-triazin-2-ylamino)-2,2'-stilbene disulfonic acid, in the form of the disodium salt, as a vital fluorescent stain for fungi and algae. Both of these authors commented on the specificity of the brightener for cellulotic material.

The present work describes and illustrates the application of this brightener for the study of morphogenesis and cell differentiation in four cellular slime molds.

MATERIALS AND METHODS

Cellular slime molds. The following species and strains were investigated: Dictyostelium discoideum NC-4, D. purpureum WS-321, D. mucoroides S-28b, and Polysphondylium pallidum WS-320. These strains were selected because among them examples of all the known cellulose-containing stages and structures in the life cycles of the cellular slime molds can be readily obtained.

Media used. For routine maintenance, the cellular slime molds were grown in two-membered cultures with Escherichia coli strain B/r, on a medium containing 0.25% peptone (Difco), 0.025% yeast extract (Difco), 0.1% glucose, and 1.5% agar (Difco). The

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medium was buffered to pH 6 with 0.82% KH₂PO₄ and 0.18% Na₂HPO₄. Structures to be stained on microscope slides were taken from cultures growing with *E. coli* on a medium containing 0.1% peptone, 0.1% lactose, and 1.5% agar at pH 6. The media used for the vital staining of the cellular slime molds are described below.

**Brightener.** Initially, the brightener was obtained from Calbiochem, Los Angeles, Calif., in the form of powder under the trade name Biofluor. A further supply of the brightener was obtained from American Cyanamid Co., Bound Brook, N.J., as a powder under the trade name Calcofluor White ST. Aqueous and saline solutions showed only slight fluorescence, and in such solutions the brightener was markedly more specific for cellulose than it was when placed in aqueous-organic or completely organic solvents. As a direct stain, the brightener was used with a 0.1% solution in half-normal saline at pH 6. As a vital stain, it was incorporated into growth media at a concentration of 0.0025%. It withstood autoclaving for 15 min at 121 C without apparent reduction in activity.

Intact slime mold structures to be stained were picked off the surface of the growth media and placed in the brightener solution on microscope slides; the cover glasses were supported on other cover glasses to avoid crushing the specimens. The brightener stained the structures best if these were not fixed. The use of half-normal saline as the solvent produced the least distortion of the specimens; i.e., it did not cause them to swell or to become disrupted as readily as did distilled water. Structures to be sectioned were fixed with Carnoy's fixative, taken through an ethyl alcohol series to xylene and to paraffin wax, and sectioned at 5-μ thickness. Sections on slides were brought back to water and washed for 36 to 48 hr with running tap water to remove all traces of the organic solvents. This is essential; otherwise all the tissue fluoresces and not just the cellulose. When the washing was complete, the brightener solution was applied to the slide and a cover glass was placed over the preparation.

For vital, in situ staining and examination of microcysts around an aggregation, *Polysphondylium pallidum* WS-320 was grown on the phosphate buffer medium containing the brightener. For vital, in situ staining of macrocysts, *D. mucoroides* S-28b was

**FIG. 1. Main stages in a generalized life cycle of the cellular slime molds, showing where cellulose is present.**
grown on the 0.1% peptone-0.1% lactose medium containing the brightener. These media were poured as thin layers in plastic petri dishes to reduce the distortion usually encountered in glass vessels and to preclude absorption of the ultraviolet light when the specimens were examined.

Examination and photography of the specimens. For studies with bright-field ultraviolet and bright-field white light, specimens were examined by use of a Leitz fluorescent microscope equipped with a Leitz H-130 mercury lamp as the ultraviolet source, a Leitz UG1 4-mm ultraviolet filter, and an OG1 barrier filter in the barrel of the microscope. Examinations under polarized light were made using an AO-Spencer polarizing-microscope. Photographs were taken on 35-mm Kodak Plus-X film. Exposure times ranged from 0.04 to 0.1 sec for white light, and from 15 to 60 sec for ultraviolet and polarized light, depending upon the specimens and the magnifications employed.

RESULTS

The affinity of the brightener for cellulose in aqueous and saline solutions has been determined in several ways:

(i) By visually comparing the fluorescence of the brightener in aqueous and saline solutions in the presence of glucose, starch, glycogen, cellulose, cellulose (paper, cotton fibers), cellulose acetate (safety film base, dialysis tubing), and chitin. With glucose, glycogen, starch and cellulose, the fluorescence was of the same low intensity as the control containing the brightener alone. Cellulose and cellulose acetate fluoresced very intensely. Chitin, present in the cell walls of many fungi, also fluoresced, but this substance has not been reported as present in the cellular slime molds.

(ii) By examining known materials such as the above with added brightener by use of a fluorescent microscope.

(iii) By comparing brightener-stained structures of the cellular slime molds illuminated with ultraviolet and with polarized light (see Fig. 4).

(iv) By comparing the fluorescence of brightener-stained structures with similar ones in which the cellulose was stained with chloridiole of zinc and with congo red under alkaline and acid conditions (7).

All of these tests indicated that in aqueous and saline solutions, the brightener has an affinity for cellulose in all structures where this structural component has been shown to be present by other tests (4, 7).

The brightener reacts with very small amounts of cellulose, and the staining reaction, namely, the fluorescence in ultraviolet light, is very intense and long-lasting. The fluorescence is not easily obscured by unstained material, as Fig. 5 illustrates very clearly. The penetration of the brightener into intact cell masses, and into individual cells, is very rapid, and slide preparations can be examined for fluorescence immediately after they have been prepared.

Use of the brightener as a vital stain is illustrated by Fig. 2, where A and B, and C and D, show the same clusters of macrocysts of D. mucoroides S-28b, but photographed with white light and with ultraviolet. The location of cellulose in the walls, and in the material covering the macrocysts, is clearly shown by the fluorescence in B and D. The mass of still amoeboid cells to the left in A and B is just starting to segment preparatory to macrocyst formation, as indicated by the faintly fluorescent material seen in B. In contrast, the material at the right has already formed macrocysts and the cellulose in their walls fluoresces intensely. Figures 2E and F show macrocysts and part of an aggregation of P. pallidum WS-320, as seen with white light and fluorescent microscopy, respectively. The cellulose walls of the microcysts fluoresce strongly, whereas there is no fluorescence from the aggregating myxamoebae. The slime material covering the thick streams near the aggregation center accounts for the bright fluorescence seen at the top in F. The background is completely dark, since there is no fluorescence from the medium.

The application of the brightener as a direct stain to intact slime mold structures is shown in Fig. 3 with developing sorocarps of D. discoideum NC-4. Figures 3A and B illustrate an early stage in the culmination process. The sorophore sheathing the basal disk, the slime sheath covering the culminating mass, and a remnant of the slime track left behind as a trail by the migrating pseudoplasmodium contain cellulose, and hence accounts for the fluorescence seen in B. Figures 2C and D show a later stage in culmination. The lower portion of the stalk, seen in D, is strongly fluorescent owing to the cellulose in the sorophore sheath and in the walls of the fully differentiated cells contained within it. Near the apex, fluorescence is confined to the lengthening sorophore sheath and to the slime envelope that covers the mass of still-undifferentiated myxamoebae. The rather diffuse fluorescence exhibited by the rising sorogen indicates that spores, with cellulose walls, are differentiating in a circular band near the surface of the rounded mass of prespore cells. The different intensities of fluorescence resulting from different amounts of cellulose in the walls of maturing and mature spores is shown in Fig. 3E and F. These were obtained from a developing sorocarp of an age similar to the one in Fig. 3C and D. Figures 3B and D again show that the myxamoebae do not fluoresce; here too, the back-
ground is dark since the saline solution of the brightener does not fluoresce.

A slightly oblique, longitudinal section through a developing sorocarp of *D. discoideum* is illustrated in Fig. 4, the same structure being shown as photographed with polarized light (A) and with ultraviolet light (B). Note that highlights seen in A correspond to those in B. In A these are due to the bi-refringence of the cellulose of the sorophore sheath, and in B they are due to the fluorescence of cellulose in the presence of the brightener. At the lower part of the section

**Fig. 2.** Structures of cellular slime molds grown on media containing the brightener. *A, B, C, and D*: macrocysts of *Dictyostelium mucoroides* S-28b. *A* and *C* illuminated with white light, *B* and *D* with ultraviolet light. *E* and *F*, an aggregation of *Polysphondylium pallidum* WS-320 developing in the presence of microcysts. *E*, photographed with white light; *F*, with ultraviolet light.
Fig. 3. Developing sorocarps of Dictyostelium discoideum NC-4 mounted in the brightener solution. A and B show an early stage; C and D, a later stage. E and F show maturing and mature spores from a sorocarp such as that in C and D. A, C, and E, illuminated with white light; B, D, and F, with ultraviolet light.
Fig. 4. Sections through developing sorocarps of Dictyostelium discoideum NC-4. A and B, oblique sections mounted in the brightener solution after rehydration: A illuminated with polarized light, B with ultraviolet light. C, a transverse section, similarly rehydrated, mounted in the brightener solution, and illuminated with ultraviolet light.

through the stalk, the torn sheath can be clearly seen in B. Figure 4C illustrates a transverse section through the prespore mass of a culminating sorocarp of *D. discoideum*, mounted in the brightener and illuminated with ultraviolet light. The strongly fluorescent walls of the stalk cells and the sheath around the stalk are surrounded by nonfluorescent prespore myxamoebae in the process of differentiating into spores. Towards the edge of the section, the prespore cells are more mature and have some cellulose in their walls, which are fluorescing with the brightener.

*D. purpureum* is a species which produces a continuous stalk as the sorogen moves across the agar in response to light. Figures 5A and B show such a sorogen. The photograph (B) made with
FIG. 5. Prostrate pseudoplasmodium (sorogen) of Dictyostelium purpureum WS-321, in which sorophore formation is in progress. A and B show an entire pseudoplasmodium mounted in the brightener. C and D show a tip of a pseudoplasmodium similar to that in A and B, slightly crushed. E and F show the site of the formation of the stalk, again slightly crushed and substantially enlarged. A, C, and E, illuminated with white light; B, D, and F, with ultraviolet light.
ultraviolet light clearly shows the stalk as strongly fluorescent, in contrast to the nonfluorescent, undifferentiated myxamoebae of the sorogen. The cellulose that accounts for the fluorescence of the apex is contained in the slime envelope that is generated in this region. The sensitivity of the brightener is well illustrated in Fig. 5C and D. These show, at a high magnification, the apex of a stalk in a sorogen comparable to that shown above. Although little cellular structure can be seen in C, except for the general alignment of the cells, the sorophore sheath and the stalk that it molds are formed in essentially the same way as in D. discoideum, described in detail by Raper and Fennell (7). The cells are elongated and positioned radially within a circular zone where the sorophore sheath is being laid down. These radially-arranged cells, suggestive of a secretory epithelium, produce the cellulose at their interior ends before being pushed forward and into the newly formed tubular sheath where they then produce cellulose walls, vacuolate, and become the compacted nonviable stalk cells. In Fig. 5D and F, the fluorescent spots with the fluorescent strands are thought to be the cellulose-producing interior ends of these prestalk cells as they form the cellulose that constitutes the sorophore sheath.

**DISCUSSION**

Because of the importance of cellulose during the morphogenesis of the cellular slime molds, it is most helpful to have a technique with which the production and location of cellulose can be closely followed, both in living material in situ on the culture substrate and in structures that have been removed for staining and examination. The staining technique described and illustrated here provides such a technique. While it cannot be said with complete certainty that in the cellular slime molds the brightener is reacting only with cellulose, the evidence for the specificity of the brightener for cellulose is strong. Compared with other reagents which have been used to stain cellulose selectively, such as chloroide of zinc, congo red, and Post and Laudermilk's stain, this reagent provides a much more sensitive test. This sensitivity, together with the very intense and long-lasting fluorescence and the rapid penetration into a cell mass as well as individual cells, makes it a very useful stain.

Although Darken (3) found that bacteria would fluoresce when stained with this brightener, this was probably due to the fact that she used the stain as a solution in aqueous cellosolve. It was found here that the brightener loses its specificity for cellulose if any organic solvents are present, a fact that makes it necessary to wash very thoroughly sections of tissues that are to be stained for cellulose with nonorganic solutions of the brightener.

At least up to the highest concentration used here, the brightener is nontoxic to the cellular slime molds. Spores suspended in the 0.1% solution remain viable and germinate normally. The brightener can be incorporated into growth media at a concentration that is high enough to stain the cellulose-containing structures formed on such media, and still cause no inhibition at any stage of the life cycle. Darken (3) found that brightener-stained mycelia of some fungi and actinomycetes would produce new growth which was fluorescent in the absence of the brightener, and that stained and washed spores of Penicillium spp. produced fluorescent germ tubes and young mycelial strands in media with no brightener present. Vegetative myxamoebae retain the brightener in similar fashion. Stained with the brightener and then washed and allowed to develop further, such myxamoebae produce fruiting structures that fluoresce, showing that the brightener enters and remains within the cells, although they themselves do not fluoresce.

The brightener should be of considerable use in morphogenetical studies with the cellular slime molds, and, because of its retention in the myxamoebae and its lack of toxicity, we are hopeful that it may be of significant value as a means of marking cells in studies with mixed populations. Such studies are contemplated for the near future.

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**LITERATURE CITED**