ISOLATION AND IDENTIFICATION OF THE SUDANOPHILIC GRANULES OF SPHAEROTILUS NATANS

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ABSTRACT

Rouf, M. A. (Washington State University, Pullman) and J. L. Stokes. Isolation and identification of the sudanophilic granules of Sphaerotilus natans. J. Bacteriol. 83:343-347. 1962.—The characteristic and prominent sudanophilic granules in five strains of Sphaerotilus natans were shown to be composed of poly-β-hydroxybutyric acid by the method of isolation, melting point determinations, and infrared absorption spectrum analyses. The polymer may account for 11.0 to 22.5% of the dry weight of the cells. It accumulates during active growth of S. natans and decreases sharply in amount after maximal growth. It accumulates to an equal extent in cultures with and without sheaths.

The cells of the filamentous, sheathed bacterium, Sphaerotilus natans, usually contain prominent and numerous refractile granules of variable size and shape. Büsgen (1894) concluded that the granules are composed of fatty materials since they are soluble in ether. By means of stains, also, Linde (1913) indicated that the granules contain lipids. In addition, he found volutin but not glycogen in the cells. According to Zikes (1915) and other investigators, young filaments are not granulated, but sudanophilic inclusion bodies appear in the cells as the filaments age. Zikes could not detect glycogen and, in contrast to Linde, he found little or no volutin. More recently, Stokes (1954) and Höhn (1955) reported the common and abundant occurrence of sudanophilic granules in many strains of S. natans. According to Pringsheim (1949), the granules are of diagnostic value for this species.

S. natans will also deposit intracellular globules of sulfur when exposed to H₂S (Skerman, Dementjeva, and Carey, 1957).

It is now known that the sudanophilic granules of many genera and species of bacteria are composed mainly of poly-β-hydroxybutyric acid in association with a small amount of lipid material (Lemoigne, Delaporte, and Croson, 1944; Williamson and Wilkinson, 1958; Haynes et al., 1958; Forsyth, Hayward, and Roberts, 1958). It was of interest, therefore, to determine whether the sudanophilic granules of S. natans are also composed of poly-β-hydroxybutyric acid.

MATERIALS AND METHODS

Five strains of S. natans were used. Strains 13338, 13339, and B1493 are part of the group of nine strains previously investigated (Stokes, 1954). Strain 11021, isolated by Pringsheim, was obtained from the American Type Culture Collection. Strain P is one which we recently isolated from river water.

The cultures were grown at 25°C in shallow, stationary layers of broth. The medium contained 0.2% each of peptone and glucose, 0.02% MgSO₄·7H₂O, 0.005% CaCl₂, 0.001% FeCl₃·6H₂O, 0.01 m phosphate buffer (pH 7.1), and distilled water. The phosphate buffer was sterilized separately. The medium was distributed in 200-ml amounts into 2-liter Erlenmeyer flasks. One liter of each culture was grown for analysis.

After growth, the cells and filaments were harvested by centrifugation, washed twice with distilled water, and suspended in a known volume of water ranging from 60 to 100 ml; 2-ml samples were removed for the determination of the dry weight of the cells.

To isolate the sudanophilic granules, the cells from the remainder of the above aqueous suspension were collected by centrifugation, resuspended in 100 ml of 2 N H₂SO₄ and incubated at 100°C for 1 to 2 hr to hydrolyze and remove water-insoluble polysaccharides. The residue was collected, washed twice with distilled water in the centrifuge, and digested with the alkaline hypochlorite reagent of Williamson and Wilkinson (1958) for 12 to 18 hr at 35°C. This reagent digests and dissolves virtually all cellular com-
ponents except poly-β-hydroxybutyric acid. The residue from the hypochlorite treatment was collected and washed three times in the centrifuge. To remove lipids, the residue was placed in about 100 ml of a mixture of one part of acetone and two parts of ether for several hours; this was followed by extraction with 30 ml of ether alone for several hours. The ether-extracted residue was treated with 30 ml of hot CHCl₃ to dissolve poly-β-hydroxybutyric acid, and the suspension was then centrifuged to remove CHCl₃-insoluble material. Finally, the supernatant fluid was dried to constant weight at 65 C. This procedure is essentially that used by Stanier et al. (1959) to isolate poly-β-hydroxybutyric acid from Rhodospirillum rubrum.

The infrared spectra of the isolated substances were determined with a Beckman IR-5 spectro-

FIG. 1. Sudanophilic granules in Sphaerotilus natans; living, unstained material photographed with the phase microscope (dark contrast), X 5,900.
FIG. 2. Infrared spectra of poly-$\beta$-hydroxybutyric acid isolated from Bacillus megaterium (A) and Sphaerotilus natans strains 11021 (B), 13838 (C), and B1493 (D).

photometer. The CHCl$_3$ solutions used contained approximately 2.5 mg of material per ml.

RESULTS

All of the strains grew abundantly in the peptone-glucose-mineral salts medium, mainly as white, gelatinous surface pellicles. Maximal growth was reached usually within 2 to 3 days. Microscopically, the cultures consisted of characteristic Sphaerotilus filaments, i.e., long chains of rod-shaped cells enclosed in transparent, tightly-fitting tubes or sheaths. It was not possible, however, to be certain that the cells of strain 11021 were encased in a sheath. The individual cells of mature cultures of all of the strains contained, typically, numerous refractile gran-
ules. These appeared as dark inclusion bodies in the living, unstained cells when viewed with the phase microscope under dark contrast (Fig. 1). Also, they stained readily with Sudan Black in wet mounts. The granules may be small and well separated or may coalesce to form what appears to be a single, large, somewhat irregularly shaped body which may occupy one-half or more of the volume of the cell.

Direct microscopic observations of the staining of the granules in wet mounts with Sudan Black indicated that at least 90 to 95% of the granules were sudanophilic. Tests for volutin by Albert’s staining method, as modified by Laybourne (1924), were inconclusive. Volutin granules, if present as reported by Linde (1913), can account for only a very small fraction of the total inclusion bodies in *Sphaerotilus*.

In a series of experiments, each of the five strains of *Sphaerotilus* was grown in the peptone-glucose-mineral salts broth for approximately 3 days to obtain maximal growth. The cells were then harvested and analyzed for poly-β-hydroxybutyric acid by the procedure previously outlined.

We were able to isolate, from the final chloroform extract of every strain, a white, somewhat translucent, membranous material which exhibited the reported properties of poly-β-hydroxybutyric acid. It is resistant to alkaline hypochlorite. It melts in the range of 162 to 170 C (Williamson and Wilkinson, 1958). It has an infrared absorption spectrum similar to that of poly-β-hydroxybutyric acid isolated from *Bacillus megaterium* (Haynes et al., 1958). Figure 2 contains the spectra of the polybutyrate isolated from three of the strains of *S. natans* and, for comparison, that from *B. megaterium*, an organism known to form and accumulate the polymer. All four spectra are quite similar and exhibit a very prominent band at 5.7 μ, which is characteristic of poly-β-hydroxybutyric acid (Haynes et al., 1958).

The amounts of polybutyrate isolated from *S. natans* are relatively large and range from

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Incubation time*</th>
<th>Polymer content of cells mg per 100 mg dry wt</th>
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<tbody>
<tr>
<td>13338</td>
<td>70</td>
<td>11.0</td>
</tr>
<tr>
<td>13339</td>
<td>69</td>
<td>12.6</td>
</tr>
<tr>
<td>P</td>
<td>60</td>
<td>15.8</td>
</tr>
<tr>
<td>B1493</td>
<td>68</td>
<td>18.7</td>
</tr>
<tr>
<td>11021</td>
<td>72</td>
<td>22.5</td>
</tr>
</tbody>
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* At 25 C, except for 30 C for strain P.

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**TABLE 1. Amounts of poly-β-hydroxybutyric acid in strains of Sphaerotilus natans**

**FIG. 3. Comparison of growth and accumulation of poly-β-hydroxybutyric acid in Sphaerotilus natans B1493.**
11.0% of the dry weight of the cells of strain 13338 to 22.5% for strain 11021 (Table 1). These quantities are comparable to those from other high-yielding bacteria. Thus, B. cereus may contain from 10 to 43% polybutyrate (Williamson and Wilkinson, 1958); R. rubrum, 23% (Stanier et al., 1959); and Pseudomonas methanica, 29% (Kallio and Harrington, 1960).

The filamentous, sheathed form of S. natans can dissociate into one which does not produce a sheath (Pringsheim, 1949; Stokes, 1954). We found that substitution of 0.2% Bacto-Casamino acids for Bacto-peptone in the basal medium gave rise to cultures which did not form sheaths. Such cultures, however, contained as much polybutyrate as the typical sheathed cultures.

**Effect of age.** The amount of poly-β-hydroxybutyric acid in S. natans varies with the age of the culture. Growth and polybutyrate accumulation of strain B1493 as a function of days of incubation are plotted in Fig. 3. As growth proceeds and approaches the maximal value of 430 mg of dry weight of cells per liter of culture in 3 days, there is a concomitant increase in polymer from 23.6 mg per liter after 1 day to 56.4 mg after 2 days and 67 mg after 3 days. These values correspond, respectively, to 8.1, 14.1, and 15.6% of the polymer. On subsequent incubation for 5 days and 7 days, cell weights decrease only slightly, but there is a sharp drop in the polymer content to 47.2 mg (12.3%) in 5 days, and 30.5 mg (8.1%) in 7 days. Similar results were also obtained with strain 11021.

It is evident, therefore, that the amount of polymer in the cells reaches a maximum at the end of the exponential phase of growth, and then declines rapidly. These results support the current concept that poly-β-hydroxybutyric acid is a major, reserve-food product of bacterial cells. During early stages of growth, it accumulates as sudanophilic intracellular granules, and, later, is consumed by the cells as a source of carbon and energy (Macrae and Wilkinson, 1958a,b; Stanier et al., 1959).

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**LITERATURE CITED**


