The sensitivity of growing bacterial cells to destructive agents or influences is many times that of bacterial spores (Curran, 1952). Some of these agents act on sulfhydryl groups of proteins; on the other hand, sulfhydryl groups belong to the most sensitive groups in proteins and are vital for the function of many enzymes (Barron, 1951). The question may be raised whether the degree of resistance during the various phases of bacterial development could not be correlated with the appearance of sulfhydryl groups in the bacterial proteins. Experiments which bear on this hypothesis are the subject of the present paper.

MATERIALS AND METHODS

**Cultures.** A culture of *Bacillus globigii* (356 Sc-4 NR Smith strain) was obtained from Dr. McCoy, University of Wisconsin. The following medium was found optimal for obtaining the orange mutant in good yield: NaCl, 3 g; beef extract, 3 g; glucose, 10 g; and peptone, 20 g treated with norite, per liter of distilled water. Fifty ml of this medium in a 500 ml flask were inoculated with a 2 per cent inoculum of 12 hr cells, 7 day cells, or a spore suspension from 8 flasks which had been washed several times and finally treated for 10 min at 85 C. Three sets of twenty flasks each were inoculated with the three inocula above and placed on a Brunswick rotary shaker at 30 C. Flasks from each set were removed at varying times, the cells were collected by centrifugation at 3,000 × G, washed with M/15 phosphate buffer of pH 7.0, centrifuged, and finally resuspended in 30 to 40 ml of the same buffer.

**Preparation of spores.** The initial steps in the preparation of spores of *B. globigii* were identical with those of the cell preparations. The flasks containing the inoculated medium were removed from the shaker after 7 days; and the spores, remaining whole cells, and cellular debris were collected by centrifugation, washed with M/15 phosphate buffer, recentrifuged, and finally resuspended in 30 ml of M/15 phosphate buffer. The 30 ml of suspension were treated with sonic irradiation for 3 to 5 min; this time was sufficient to strip the cellular debris from the spores. Then by differential centrifugation the spores could be collected from the solubilized cell material. To assure the complete destruction of all viable cells the resuspended spores were heated for 10 min at 85 to 90 C. Additional spores were obtained from the Chemical Corps Biological Laboratories, Camp Detrick. These were used for independent sulfhydryl determinations of spores.

**Disintegration of cells.** The solubilisation of the cell protein of *B. globigii* was accomplished by sonic vibration. The 30 to 40 ml of suspension containing about 1 g protein were poured into the chamber of a 10 kc Raytheon magnetostriction oscillator and gassed with helium. The oscillation rate was adjusted to a maximum and allowed to operate for 5 min at 15 C when breaking cells and for 30 min when breaking spores. Shorter periods can be used to strip the vegetative debris from the spores.

**Determination of sulfhydryl groups.** Three methods were used: (a) Titration with p-chloromercuribenzoate with nitroprusside as an external indicator (Benech and Benech, 1948): About 20 mg of protein in 2 ml of 0.5 M phosphate buffer of pH 7 were titrated with 0.001 M p-chloromercuribenzoate. Samples (0.05 ml) were drawn and checked for color development with nitroprusside. When denaturation was carried out (Hellerman et al., 1943), either 0.5 g of guanidine hydrochloride or 2 g of urea were added for 100 mg of protein. The samples were kept under H₂ for 30 min and then titrated as described. (b) Reaction with o-iodosobenzoate and potassium iodide and back titration with sodium thiosulfate (Hellerman et al., 1941): A solution of 20 mg protein in 5 ml of 0.5 M phosphate buffer of pH 7 was mixed with 5 ml of 0.001 M o-iodosobenzoate. After 2 min, 4 ml of 0.5 N HCl containing 0.5 g

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**APPEARANCE OF SULFHYDRYL GROUPS DURING GROWTH OF BACILLUS GLOBIGII**

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KI (freshly prepared) were added. The liberated iodine was titrated with 0.002 N Na₂S₂O₃ using starch as indicator. Denaturation with guanidine hydrochloride was not satisfactory because precipitation of proteins occurred on addition of HCl. (c) Amperometric microtitration with silver nitrate in ammoniacal medium (Rosenberg et al., 1950): Either glutathione (Schwarz) or dodecyl mercaptan (Matheson) was used as a standard. These substances were used without further purification, and recoveries reported are based on an assumed 100 per cent purity of these materials. Two mg of protein were used in a total volume of 0.5 ml. Increments of 0.001 ml of 0.002 N AgNO₃ were added, and the end point was found from a plot of $\mu$amp against volume of titrating reagent.

* Determination of protein. When feasible, the biuret method was used. A ratio of $\frac{\text{mg } N}{\text{mg protein}} = 0.16$ was found. With cultures of the orange mutant the dichromate method of Johnson (1949) for total organic solids was used. The values obtained by this method were correlated with the biuret method using protein from a colorless culture.

**RESULTS**

If the cells of *B. globigii* were grown on the usual nutrient broth, they varied in color, shape, and quantity, but only orange cells are obtained and in larger quantity (about 2.5-fold) when the peptone used was pretreated with activated norite. The compound or compounds removed from the peptone have not been determined. Other variations in the medium which were tried (tap water, Mg+++, yeast extract, proteose) did not show much advantage (table 1).

With the p-chloromercuribenzoate method for determination of sulphydryl groups, recoveries of 94 ± 2 per cent of the glutathione standard were obtained. The protein of 48 hr cells contained 0.1 per cent sulphydryl.¹ With the o-iodosobenzoate method 100 ± 5 per cent of the glutathione standard was recovered. In one culture 0.26, 0.18, and 0.13 per cent sulphydryl was found in samples drawn at 16, 24, and 42 hr, respectively, after sonic disintegration. With either method, previous denaturation of the protein did not change the results.

**TABLE 1**

<table>
<thead>
<tr>
<th>FLASK</th>
<th>SOURCE OF AMINO ACIDS</th>
<th>COLOR OF CELLS</th>
<th>TURBIDITY*</th>
<th>INOCULATED WITH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peptone</td>
<td>White</td>
<td>0.246</td>
<td>White mutant</td>
</tr>
<tr>
<td>2</td>
<td>Peptone + proteose</td>
<td>Slight orange</td>
<td>0.465</td>
<td>White mutant</td>
</tr>
<tr>
<td>3</td>
<td>Peptone + yeast extract</td>
<td>Slight orange</td>
<td>0.398</td>
<td>White mutant</td>
</tr>
<tr>
<td>4</td>
<td>Peptone + MgSO₄</td>
<td>White</td>
<td>0.246</td>
<td>White mutant</td>
</tr>
<tr>
<td>5</td>
<td>Peptone + tap water</td>
<td>Slight orange</td>
<td>0.505</td>
<td>White mutant</td>
</tr>
<tr>
<td>6</td>
<td>Norite treated peptone</td>
<td>Orange</td>
<td>0.832</td>
<td>White mutant</td>
</tr>
<tr>
<td>7</td>
<td>Yeast extract</td>
<td>Slight orange</td>
<td>0.478</td>
<td>White mutant</td>
</tr>
<tr>
<td>8</td>
<td>Peptone</td>
<td>White</td>
<td>0.390</td>
<td>Orange mutant</td>
</tr>
<tr>
<td>9</td>
<td>Norite treated peptone</td>
<td>Orange</td>
<td>1.022</td>
<td>Orange mutant</td>
</tr>
</tbody>
</table>

* 2 ml of culture diluted to 10 ml with distilled water.

$0 = \text{no turbidity.}$
been inoculated initially with a 12 hr culture was determined (figure 1), the initial sulfhydryl content was found to be high (0.15 per cent). There was an immediate drop during the lag phase followed by a rise during the early log phase. The usual constancy during the remaining log phase was observed, followed by a slow decline at its termination.

Other independent sulfhydryl determinations on spores gave the following results: untreated spores, 0.02 per cent; spores after sonic disintegration, 0.035 per cent; spores after grinding with alundum, 0.035 per cent. The mean percentage of sulfhydryl groups of different spore suspensions was 0.035 ± 0.005 per cent. The sulfhydryl content of the disintegrated spore preparations before centrifugation was 0.035 per cent; after centrifugation the same value was obtained.

The values found by titration of spore preparations are at the lower limit of sensitivity of the titration method. These values therefore have to be accepted with some reserve. Even when titrating distilled water, a certain amount of AgNO₃ is required (range corresponding to 0.005 to 0.01 per cent sulfhydryl in spores), and it may be questioned whether the additional small AgNO₃ consumption of the spore preparations is exclusively due to sulfhydryl groups. These uncertainties become of course negligible at higher sulfhydryl titers. The values given for the spore preparations therefore may be considered as an upper limit.

**DISCUSSION**

Spore germination and the shortening of the lag period are favored by a low OR potential (Mitchell, 1951), and a great increase in physiological activity occurs during the late lag and early log period (Winslow and Walker, 1939). Brachet (1944) showed that an abundance of sulfhydryl compounds is required by cells in mitosis and in cellular division and growth. The function of many individual enzymes is dependent on the presence of intact sulfhydryl groups in the protein (Barron, 1951), and it has been shown recently that coenzyme A, which holds a key position in intermediary metabolism, functions through its free sulfhydryl group (Lynen et al., 1951). In view of these facts it may be expected that during the so-called "physiological youth" period the sulfhydryl content of a bacterial culture should rise sharply.
Heat, unfavorable pH, oxidizing agents, and disinfectants greatly affect cells during the late lag and early log phase (Sherman and Albus, 1923). Sulfhydryl groups are known to belong to the most labile groups in proteins and also are affected by such influences. In view of the correlation between sulfhydryl group appearance with growth as communicated here, it seems likely that the sensitivity of cells in the late lag and early log phase is related to the high sulfhydryl group requirement during these periods if appearance of sulfhydryl groups may be taken to mean requirement of sulfhydryl groups. On the same basis, spores which are greatly resistant to the mentioned deleterious influences and which have a very low sulfhydryl content may owe this resistance to a very low sulfhydryl requirement, independent of the protection by the spore wall.

The immediate drop in sulfhydryl when the medium is inoculated with a 12 hr culture is interesting. Possibly the cells continue to age before initiating a new growth cycle (Chesney, 1916). The short lag of about 5 hr could be a result of the high initial content of sulfhydryl groups in these cells since sulfhydryl groups are necessary for cell growth. With the 7 day inoculum the lag was extended to 10 hr.

SUMMARY

Good yields of the orange mutant of Bacillus globigii have been obtained when peptone treated with norite was used in the medium.

The sulfhydryl content of spores of B. globigii was found to be about 20 per cent of that of the cells during the log phase.

The total increase in sulfhydryl content from about 0.03 to 0.15 per cent occurred during the late lag and early log phases of growth. The sulfhydryl content during the remaining log phase remained constant with a slow decline occurring after the termination of the log phase.

When the inoculum of the medium was high in sulfhydryl groups, there was an initial drop followed by the increase already mentioned.

Denaturation of the solubilized protein with alcohol, guanidine HCl, or urea did not increase the sulfhydryl titer.

REFERENCES


