THE ENZYMIC CONTENT OF BACTERIAL SPORES

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That bacterial spores are lacking in enzymic activity is a natural deduction from the fact that the spore is a resting stage in the life history of the organism. However, the work of the Kopeloffs on the demonstration of active enzymes in mold spores suggests the possibility of active enzymes in bacterial spores. It is the purpose of this paper to record some preliminary experiments on the possibility of demonstrating active enzymes in bacterial spores. After our work was begun the following statement was found in Effront's "Biochemical Catalysts in Life and Industry" (p. 312):

In this connection we must also take into consideration the observation of Effront, according to which the bacterial spores, attenuated either by an antiseptic or by heat, show themselves the more productive of enzymes the more difficult their germination. Under certain conditions and in the presence of antiseptics the spores may produce an intense secretion of enzyme in a liquid without, however, arriving at germination.

Up to the present time we have been unable to obtain Effront's original paper and so we do not know what data he had to support his statement.

1 Published by the Permission of the Director of the Michigan Agricultural College Experiment Station.
Read at the Detroit meeting of the Society of American Bacteriologists, December 29, 1922.
2 "Do mold spores contain enzymes?" M. Kopeloff and L. Kopeloff, Jour. Agric. Research, 1919, 18, 195-209.
4 Between the time of submitting this paper for publication and the reading of the proof Effront's original article ("Sur l'Action Chimique des Spores," Jean Effront. Le Moniteur Scientifique, Queensville, Feb., 1907, Liv. 782, pp. 81-87) has been found and studied; his data agree in every respect with ours.
In the present work suspensions of washed spores were added to certain substrates in the presence of antiseptics and observations and chemical determinations were made for the purpose of detecting chemical changes in the substrates.

Twelve cultures of aerobic, spore-forming bacteria were used. The names of the species will not be given at the present time because a positive identification has not been made. The cultures were purified by plating and then sown upon the surface of agar in Kolle flasks and allowed to incubate for five and one-half months at room temperature under a bell jar. The growths were then scraped off with a sterile, bent glass rod and suspended in sterile physiological salt solution. These spore suspensions were centrifuged for forty to fifty minutes, which resulted in precipitating the spores and leaving the few vegetative rods in the supernatant liquid which was pipetted off. This process of washing was repeated a second time and then the spores were made into a uniform suspension in physiological salt solution. In this connection it is worthy of note that before the washings, the growth contained small numbers of vegetative rods which had failed to sporulate, but that after washing, such vegetative rods could not be found. Apparently the spores were appreciably heavier than the rods. Possibly also the latter were dead since these cultures were several months old. From 1 to 2 cc. of these spore suspensions were used to inoculate the various substrates.

The following is a description of the substrates used with the results secured with each substrate:

**OXIDASE TESTS**

1. Two cubic centimeters of spore suspension added to 10 drops tincture of guaiac (1 gram resin in 60 cc. of 95 per cent alcohol). All negative.

2. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent alcoholic α-napthol solution. All negative.

3. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent aqueous solution of p-phenylene-diamine hydrochloride. All negative.

4. Indophenol test on 2 cc. of spore suspension. All were slightly positive, while controls were negative.
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REDUCTASE TESTS

One-half cubic centimeter of a methylene blue solution (5 cc. saturated alcoholic solution of methylene blue + 195 cc. of water) was added to 10 cc. water. Added 1 cc. of spore suspension. All tubes negative after five hours at 45 to 48°C.

CATALASE TESTS

A sterile fermentation tube was filled with 1 per cent hydrogen peroxide. Two cubic centimeters of spore suspension were added. All 12 organisms produced an evolution of gas, 4 of them being very active. Boiling of the spore suspension greatly reduced the activity, though in no case was it prevented entirely.

LIPASE TESTS

One cubic centimeter of the spore suspension was added to 100 cc. of a butterfat emulsion (made by emulsifying pure butterfat with gum acacia and water, adding 1 per cent formalin and sterilizing in flasks in the autoclave). After incubating for two weeks in a dark room, the acidity in 10 cc. of an uninoculated control was compared with that in the inoculated flasks. To 10cc. of the emulsion was added 50 cc. of neutralized water and then 50 cc. of a neutralized mixture of alcohol-ether (1 : 1). One-tenth normal alcoholic potassium hydroxide was used for titration and rosinic acid was used as the indicator. Eleven of the cultures were clearly negative, while one of them gave an increase of 0.4 cc. of tenth normal potassium hydroxide over the control (0.8 for control, 1.2 for culture).

CASEINASE TESTS

To 350 cc. of sterile skim milk was added 2 cc. of the spore suspension, enough chloroform to form a distinct layer on the bottom and enough toluene to form a ¼ inch layer on top. The flasks were then incubated at room temperature for periods varying in the different tests from two to four weeks. Suitable controls were also incubated. Examinations by the Breed method
were made at the beginning and from time to time during the periods of incubation to see if the spores would germinate.

The controls and the cultures were then examined chemically for hydrolysis of the casein by Sorensen's Formol Titration Method, after first precipitating the casein and removing ammonia by aeration. The details of the process will not be given at present since some modification will be necessary to get entirely satisfactory results. There were some indications of hydrolysis of the casein in 3 cases, in one of which the hydrolysis was marked. However, it is not certain that the hydrolysis was produced by caseinase, since the method as conducted gave some opportunity for chemical hydrolysis.

GELATINASE TESTS

Only four cultures were tested for gelatinase. Tubes of neutral, sterile, solidified 0.5 per cent phenol-gelatin were inoculated on the surface with 1 cc. of the spore suspension. Two to 3 cc. of toluene were placed on top of the suspension and the tubes incubated for seventeen days. Liquefaction of the gelatin was observed as follows:

<table>
<thead>
<tr>
<th>Culture</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>25</td>
</tr>
<tr>
<td>Culture 2</td>
<td>13</td>
</tr>
<tr>
<td>Culture 3</td>
<td>6</td>
</tr>
<tr>
<td>Culture 4</td>
<td>3</td>
</tr>
</tbody>
</table>

Smears were made of the suspensions and of the liquefied portions of the gelatin and examined carefully for spores and bacilli. No bacilli were observed but the spores were numerous. None of them showed evidences of germination. A test for amino acids by Sorensen's Formol Titration Method was also conducted as follows:

The gelatin was melted and the contents thoroughly mixed by agitation of the tube. Five cubic centimeters were added to some distilled water and the whole brought to neutrality to phenolphthalein. Ten cubic centimeters of neutralized formalin were then added and the acidity titrated with tenth normal potas-
sium hydroxide, using phenolphthalein as indicator. The following results were secured:

<table>
<thead>
<tr>
<th></th>
<th>cc. N/10 K.H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uninoculated)</td>
<td>0.0</td>
</tr>
<tr>
<td>Culture 1</td>
<td>1.5</td>
</tr>
<tr>
<td>Culture 2</td>
<td>0.7</td>
</tr>
<tr>
<td>Culture 3</td>
<td>0.4</td>
</tr>
<tr>
<td>Culture 4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Both of these tests clearly prove that gelatinase was present even though germination did not take place.

From the foregoing experiments the tentative conclusion seems to be justified that bacterial spores do exhibit some enzymic activity even when there is no evidence of germination. If further experiments substantiate the above preliminary tests we may have to modify some of our ideas of spores. Aside from the theoretical interest of the question, it has some practical bearing on the preservation of foods and possibly also on the rôle of the spores in the soil.