CHEMICAL DETOXIFICATION OF FLEXNER DYSENTERY ANTIGEN

II. Studies of Accelerated Growth Rates

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A major problem which arises in work on the chemistry of bacteria is that of obtaining adequate amounts of organisms with the facilities of the ordinary research laboratory. For this purpose, in growing bacteria for chemical studies on Flexner dysentery organisms (Barnes, Dewey, Henry, and Lupfer, in press), a method was developed for obtaining large amounts of this organism rapidly, and in high concentration, using simple constituents and standard laboratory apparatus. The method is based on the observations that the organism flourishes in the presence of aeration (Topley and Wilson, 1936; Hoberman and Dubos, unpublished observations), and that growth is enhanced if the effect of the hydrogen ions produced during growth is buffered by suitable salts (Hoberman and Dubos). Conditions for culturing organisms in which a large surface is exposed to a gas phase have been described by several investigators (Mironova, 1941; Clifton, 1943; Johnson, Bruce, and Dutcher, 1943; Hoberman and Dubos).

By this method the virulent type III (Z) strain with which we were concerned was grown to concentrations 8 to 10 times those obtained over 18 to 24 hours by usual culture methods (table 1, figure 1). Also, the rate of growth was increased so greatly that each liter of culture medium produced this concentration in 2½ to 3 hours. In this period, therefore, the final result was a net increase of 60- to 80-fold over the usual 20-hour growth rates. If, at the end of the 3-hour period, the bacteria were kept in the logarithmic phase of growth through the prompt addition of a fresh liter of medium, the effect could be reproduced as often as desired.

The logarithms of the values for turbidity and live count are plotted against time in figure 2. It may be seen from this figure that the rapid regeneration rate of the organisms in still media during the first hour was continued after shaking began. During the logarithmic phase the number of live organisms doubled every 40 minutes, but after about 2½ hours of shaking the regeneration rate decreased sharply. The relative increase in mass during the shaking period was only 3-fold, whereas that in numbers was 30-fold (table 1, figure 1). It is apparent that there is an extensive mean loss in mass for the individual organism during this period of rapid growth. However, the yield of antigen, as judged

1 The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children’s Hospital, Cincinnati, Ohio.
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by the mouse test (Barnes and Dewey, to be published), was that expected from the increased numbers of organisms. Further study of this phenomenon is required to elucidate its place in bacterial growth.

Both smooth and rough strains of type III (Z), the "Karim Khan," and a Sonnei strain of Shigella were grown successfully. The virulent strain of type III (Z), when cultivated by the aeration method, showed a ratio of toxicity to protective power in mice that was not significantly different from that shown by this strain when grown by usual broth methods in small amounts (table 2).

**TABLE 1**

*Rapid growth run, no. 11, Flexner III (Z) dysentery bacilli*

<table>
<thead>
<tr>
<th>SOURCE OF SAMPLE G SIGNIFIES GROWTH RUN</th>
<th>LIVE COUNT</th>
<th>MASS</th>
<th>TURBIDITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>log units*</td>
<td>g/L</td>
<td>log units*</td>
</tr>
<tr>
<td>Prerun inoculum†</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G at -55/60 hr</td>
<td>1.6</td>
<td>0.33</td>
<td>0.52</td>
</tr>
<tr>
<td>G at 0 hr</td>
<td>4.8</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>G at 2 5/60 hr</td>
<td>44</td>
<td>9.17</td>
<td>1.96</td>
</tr>
<tr>
<td>G at 3 5/60 hr</td>
<td>102</td>
<td>21.2</td>
<td>2.33</td>
</tr>
<tr>
<td>G at 4 5/60 hr</td>
<td>129</td>
<td>26.9</td>
<td>2.43</td>
</tr>
<tr>
<td>G at 5 5/60 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G at 6 5/60 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Log values are increased by one to avoid negative values.
* Based on value of unity arbitrarily assigned at zero time.
† Arbitrary units of concentration expressed as 1,000 (2 - log T), where T = % transmission; 800 units represent 1 gram of dried organisms at higher concentration levels.
‡ This 17-hr-old culture was grown by ordinary methods but in the enriched medium described; 100 ml of this culture were used as an inoculum for each liter of medium in the shaking flasks. The two values recorded are about one-tenth of those reached by the growth curves at the end of the shaking period (see also figure 1).
§ This one value was taken from a graph constructed from the other, experimental, live count data in this column.

The apparatus may be set up also to operate continually day and night with fresh medium constantly flowing into the flasks and fully grown harvest being drawn off at the same rate. Under the continual ingress of medium and egress of harvest, the type Z organisms grew at a very rapid rate. At the end of 96 hours of continuous growth, the bacteria were multiplying as well as during the early phases of the period. Because of difficulties in maintaining a steady flow of fresh medium and harvest, the average yield was about 3 g of bacteria (dry weight) per liter every 3 hours. Thus for the continuous operation there was a 40- to 50-fold increase over that rate of growth which takes place under ordinary methods of culturing.

This extremely rapid rate of growth is a very sensitive indicator of culture conditions. For example, restriction of growth to a concentration one-tenth
that of the maximum occurred in one flask when the diameter of the air inlet tube was decreased to one-fourth of that leading to the other flask. Also it was found that whereas ordinary growth was easily achieved with the virulent type Z organisms, rapid regeneration was not accomplished until potassium salts were added to the medium. It seems likely that the effects of various substances and conditions on growth rate could be measured sensitively by the estimation

![Graph showing growth of Flexner III (Z) dysentery bacilli](image)

**FIG. 1. Simultaneous Changes in Mass, Turbidity, and Live Count During Growth of Flexner III (Z) Dysentery Bacilli**

of both rate of growth and maximal concentration under the conditions of this method.

Two explanations have been advanced for the slowing and final cessation of growth at the end of the logarithmic phase. One, the older, ascribes the interruption of growth to a gradual accumulation of inhibitory substances derived from bacterial metabolism during the growth of the culture. The other is supported by evidence indicating that the limits of necessary conditions for growth are exceeded by the demands of the large number of organisms present at the end of the logarithmic growth phase (Topley and Wilson, 1936). If the enriched medium, used in the method described here, was not shaken but was
merely incubated for 18 to 20 hours, the concentration of organisms was about one-tenth that achieved in the ensuing 2½ hours of shaking (table 1). As there was essentially only one variable, it is apparent that shaking was responsible for the continuation of growth as well as for the increased growth rate. Elucida-

![Graph showing logarithmic changes in live count and turbidity during growth of Flexner III (Z) dysentery bacilli.](http://jb.asm.org/)

Fig. 2. Logarithmic Changes in Live Count and Turbidity During Growth of Flexner III (Z) Dysentery Bacilli

A number of possible explanations of the shaking effect are suggested. One is that through the increased surface area, greater accessibility to the oxygen supply is provided. Another is that shaking permits volatile toxins, such as excess of carbon dioxide, to be removed more rapidly, or others to be oxidized.
Another possibility is that the production of inhibitory substances proceeds more slowly in the presence of adequate supplies of oxygen. Various combinations of these effects also provide possible explanations for the observations.

Among the numerous unsolved problems concerning the nature of growth in normal and pathological cells is the fact that little is known concerning the potentialities for growth which are inherent in a system of normal cells when the only variables are the accessibility and rates of removal of metabolites. The rates of growth achieved here clearly show how tremendous these potentialities are. They suggest the possibility that rate of growth as well as its extent in biological systems can be altered greatly by merely changing the rate of supply and perhaps removal of metabolically active substances. It may not be necessary to assume that there is any change in cancer cells other than that producing a slight change in the rate at which nutrient substances and oxygen are made available to the cellular mechanism. For example, this might involve only a change in permeability of the cell membrane.

Rates of access and removal of metabolites with respect to bacterial cells may be a factor in the bacterial growth rate within the body of a host, and hence of importance in the virulence of invading organisms. The adaptation of bacteria to new environments may also be influenced by this same condition. Finally, this rapid adaptation of growth mechanisms which are ordinarily highly patterned is compatible with the findings of Schoenheimer (1942) and his school, namely, that synthetic and degradative cellular processes are going on in fundamental cellular components rapidly and continuously.

**EXPERIMENTAL PROCEDURES**

Procedure. One hundred ml of an 18- to 20-hour-old culture of *Shigella* organisms are added to each of two 6-liter Florence flasks containing a liter of culture medium apiece. The flasks are shaken mechanically at 37 C while sterile air is passed into the air space above the liquid. The rate of growth may be estimated by withdrawing, at intervals, 1 to 10 ml of medium through outlets near the bottom of the flasks and, after suitable dilution, measuring the turbidity.

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**TABLE 2**

Comparison of different methods of culture of Flexner III (Z) dysentery bacilli

A: Ordinary broth-grown organisms, centrifuged and washed with saline, killed by heat at 60 C for 30 minutes

B: Organisms from continuous, rapid-growth run, treated in same way as A

<table>
<thead>
<tr>
<th>TOXICITY</th>
<th>PROTECTION</th>
<th>TOXIC UNITS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dilution equal to 1 toxic unit)</td>
<td>(Dilution equal to 50% deaths)</td>
<td>(Required to give 50% protection)</td>
</tr>
<tr>
<td>A—1:97</td>
<td>A—1:19.7</td>
<td>A—4.9</td>
</tr>
<tr>
<td>B—1:52</td>
<td>B—1:13.9</td>
<td>B—3.7</td>
</tr>
</tbody>
</table>

* The vaccines were compared by toxicity-protection test in mice (Barnes and Dewey). The data were analyzed by Mrs. Estelle Brown with the Reed-Muensh method.
by transmitted light in an electric photometer. A filter is used which transmits a band of light having a mean wave length of 660 mμ. By this means one can follow the growth curve of the organisms. By plotting the unit concentration of organisms, 1,000 (2-logarithm of the transmission), against the passage of time, one can relate any later readings to the curve so obtained (figure 3), thus permitting removal of the culture at any phase of growth. If desired, a unit of concentration on the graph may be easily standardized in terms of milligrams of nitrogen or grams of dried organisms per liter.

Near the end of the logarithmic phase of growth, which occurs about 4 to 4 1/2 hours after the shaking of the first batch has begun, the harvest is tapped off and a liter of fresh medium is added to each flask, a suitable inoculum always being present in the foam and draining medium left in the flasks from the previous run. The organisms will now grow to the previous maximum concentration in 2 1/2 hours, having remained in the logarithmic phase of growth.

Fig. 3. Growth Curve of Flexner III (Z) Dysentery Organisms Based on Measurements of Turbidity
After the growth curve of the organism in this apparatus has been characterized adequately, it is possible to operate on a continuous 24-hour a day basis. For the first liter in each flask the procedure is the same as described above. When the growth of organisms in this first liter approaches the end of the logarithmic phase of growth, fresh medium is started flowing continuously into the two shaking flasks, and at the same rate the fully grown culture material is allowed to flow out of the flasks at the bottom. This is collected in large bottles, to which a bactericidal agent is added from time to time.

**Media.** The medium used is much more concentrated than the usual medium and is composed of the following constituents per liter:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutri-peptone (Wilson)</td>
<td>20 g</td>
</tr>
<tr>
<td>Bacto beef extract (Difco)</td>
<td>10 g</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>18 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.7 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.0001 g</td>
</tr>
</tbody>
</table>

All of these constituents except the nicotinic acid and sugar are dissolved in the required amount of water, heated to 60 C, filtered hot through coarse filter paper, and then filtered in 2-liter lots through sterile Seitz filters. This medium remains clear. For large amounts, as the Seitz filtration is awkward, after filtration through paper the medium is autoclaved. Following this a moderate amount of precipitate settles out on standing; this can be removed by siphoning off the overlying medium. The nicotinic acid is dissolved in water (10 mg in 100 ml), sterilized 10 minutes in the autoclave, and added to the medium at the time of addition of the organisms. The glucose also is dissolved separately in some of the total water as a 5 per cent solution and sterilized separately.

**Apparatus.** The photograph (figure 4) shows the apparatus set up for continuous automatic use. However, if one wishes to grow only a few liters at a time, a large flask is not needed above the apparatus as a source of medium. The inlet tube for the medium (detail of area 6) in each shaking flask is then fitted up with a rubber stopper, and the outlets (12) are left plugged with cotton and unattached to rubber tubing (18 and 19). The medium is then introduced, a liter at a time, by siphons from small flasks.

The two central flasks (A and B) are the essential features. They are 6-liter Florence flasks with a small outlet tube fused into the side of each near the bottom. In the outlet tube is a glass stopcock (12). The stoppers in the flask necks are rubber, with three holes (see detail of area 6): one for air through which passes 7-mm glass tubing, one for introduction of the medium with 12.5-mm tubing, and one for an air vent (with 7-mm glass tubing) for which one or two ordinary distilling traps will suffice. The stoppers with these three glass fittings are wired firmly into the necks of the flasks, then covered with cotton and paper. Air is obtained directly from the compressed air line in the laboratory, is passed through 100 ml of concentrated sulfuric acid (22), then through an air trap (23), and finally through a sterilized drying tube (9) filled with cotton.
There must be no restrictions of any kind in the two air lines after the air leaves the T-tube (7).

The two Florence flasks are placed on a platform (13) and held in place by shallow metal receptacles (made from tin cans and attached to the platform) and by a plywood yoke (25) through which the necks of the flasks protrude. The yoke is held down at each end by a long bolt (11) and a wing nut (10). The flasks are well padded. The platform is made to move up and down on each side of an axis (24) in the direction of the arrows by an electric motor (17). A crank shaft is operated from the motor pulley (15) at 170 rpm, the platform and crank being joined to a wrist pin on the lower part of the platform by a pitman (14) from a crank pin. There is an excursion of the platform of 4 cm opposite the center of each flask. The center of the flasks is opposite a point
12 cm from the fulcrum (24) of the platform. The motion is just enough to keep the contents constantly and vigorously tossed about without causing them to strike violently against the walls.

For continuous operation the apparatus is set up as pictured so that media may be led in constantly from the upper bottle (C) through the siphon (3). The siphon may be started by blowing through a tube (1) and cotton filter (2). The rate of inflow is measured by means of drip counters (5) and controlled by clamps (4). The medium is run into each flask from the counters (5) through rubber tubes (16) at about one liter every two hours. The outlet tube (21) is raised or lowered to change the rate of egress of fluid. To permit better visualization, the outlet tube is pictured too low in the photograph and is usually placed about one-half inch below the level of fluid in the flasks when the platform is in a horizontal position. At this point the outlet behaves quite automatically and will control the level of liquid in the flasks within fairly wide limits of inflow of medium from above. One may control the operation through estimation of turbidity of the combined outflow from time to time. At times the turbidity of the medium from each flask may need to be measured separately.

ACKNOWLEDGMENT

We wish to thank Dr. Merlin Cooper for suggestions and Mr. J. Bell for constructing the shaking apparatus.

SUMMARY

A method is described by which Shigella paradysenteriae organisms may be grown continuously at the rate of 4 g (dry weight) per liter per 2½ hours with
simple laboratory apparatus. Certain relationships of the findings to problems of growth are noted.

REFERENCES

Barnes, F. W., and Dewey, M. To be published.
Hoberman, H., and Dubos, R. Unpublished observations.