NUTRITION OF MYCOBACTERIUM PHLEI

I. REQUIREMENTS FOR RAPID GROWTH

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Many of the mycobacteria grow on simple, chemically defined media (Kuhne, 1894; Proskauer and Beck, 1894; Sauton, 1912; Lockemann, 1919; Long and Seibert, 1926). However, these chemically defined media were designed either to support the growth of a large mass of tubercle bacilli or for the production of a tuberculin preparation free of nonspecific antigens. Long (1921–1922) and Merrill (1930, 1931) studied the ability of the different acid-fast bacteria to utilize various compounds as the sole source of carbon and nitrogen. Although differences in specific results are recorded by these authors, their general observations are in agreement. The saprophytes are able to utilize a number of compounds as the source of carbon when tested with ammonium ion as the source of nitrogen. In contrast the pathogens were more restricted; for example, the H-37 strain of the tubercle bacillus utilized only glucose and glycerol of the compounds tested. Similarly when tested with glycerol as the sole source of carbon, the saprophytes had a greater ability than the pathogens to utilize a variety of amino acids as the source of nitrogen. Ammonium ion would serve as the nitrogen source for all members of the group tested. That these chemically defined media do not furnish the optimum conditions for growth is recognized in practice by the use of the complex egg-containing media for the isolation of the tubercle bacillus from pathological material. A number of biological materials have been found which enhance the growth of the tubercle bacillus, i.e., potato extracts, orange juice and lemon juice (Uyei, 1930), phospholipid from egg yolk (Boissevain and Schultz, 1938), serum albumin (Boissevain, 1940; Powelson and McCarter, 1944). Dubos (1945) and Dubos and Davis (1946) have described media permitting more rapid growth of Mycobacterium tuberculosis. These media contain Tween 80 (an ester of oleic acid), albumin, enzymatic digest of casein, and yeast autolysate. Oleic acid has been substituted for Tween 80 in some media (Dubos and Middlebrook, 1947). Most previous work has been concerned with the growth requirements of the tubercle bacillus. Technical problems, however, make growth studies with this organism cumbersome and present difficulty in obtaining satisfactory quantitative data. Therefore, the basic information concerning the growth requirements of the acid-fast bacteria seems insecure when compared

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2 The studies reported in this paper were taken from a thesis submitted by the author to the Faculty of the Graduate School of Arts and Sciences of the Harvard University, Division of Medical Sciences, in partial fulfillment of the degree of Doctor of Philosophy.
to that concerning other organisms such as the diphtheria bacillus and the lactic acid bacilli.

In this study the growth requirements of *M. phlei* have been studied. The requirements for rapid growth have been studied, not those for minimal growth. Mueller (1940) has discussed the importance of differentiating between the requirements for normal growth and detectable growth. Practically speaking the objective has been to produce a chemically defined medium which would support growth at a rate equivalent to that obtained in a good complex medium. The strain of *M. phlei* studied grows rapidly when compared to the tubercle bacillus and since it is nonpathogenic is easily handled in the laboratory.

If the acid-fast bacteria represent a natural group of organisms derived from a common ancestor through variation and selection (Knight, 1936; Lwoff, 1943), it would seem possible that their nutritional requirements for optimal growth would have much in common. From this point of view it was hoped that basic information concerning the growth requirements of the pathogenic acid-fast bacteria might be gained.

The present paper describes experiments characterizing a complex medium supporting rapid growth of *M. phlei*. This is composed of an acid hydrolyzate of casein, glucose, the usual minerals, and an aqueous infusion of beef heart. The last named component enhances the growth rate of this microorganism. Observations concerning the growth enhancing effect of several nonionizing esters of long chain fatty acids are also presented.

**MATERIAL AND METHODS**

Experimental media employed contained the following salts per 10 ml: K$_2$HPO$_4$, 50 mg; MgSO$_4$·7H$_2$O, 5 mg; FeSO$_4$·7H$_2$O, 0.05 mg; CuSO$_4$·5H$_2$O, 0.01 mg; ZnSO$_4$, 0.008 mg. The MgSO$_4$·7H$_2$O and the trace metals were sterilized separately as a concentrated solution. Phenol red was present in all culture media (0.005 per cent) and the initial pH was adjusted to 7.2. To this salt mixture were added the carbon and nitrogen sources under consideration. Glucose was sterilized separately as a 40 per cent solution and was added to the final medium aseptically. Casamino acids (Difco) is a hydrochloric acid hydrolyzate of casein from which the excess chloride has been removed. Beef heart infusion was prepared by infusing two pounds of ground beef heart in one liter of tap water, boiling for 10 minutes, and filtering.

A culture of *M. phlei* was obtained from the stock collection of this laboratory; its original source is not known. This strain grows readily on egg medium, tryptic agar, and tryptic digest broth. Compared to non-acid-fast bacteria its growth is slow since it requires 72 to 96 hours to produce on tryptic digest agar colonies equal in size to those produced by the colon bacillus in 18 to 24 hours. The inocula were prepared from an overnight culture grown in tryptic digest broth (a tryptic digest of beef heart equivalent to 10 per cent meat). This was centrifuged, the original medium decanted, and the cells resuspended in distilled water to give a turbidity about equal to a MacFarlane nephelometer standard no. 1 or no. 2. Of this suspension 0.1 ml was used to inoculate each 10 ml of
medium. The amount of growth was estimated by determining the bacterial nitrogen on suitable aliquots of the culture medium, usually 5 or 10 ml. This method was first used for the diphtheria bacillus by Mueller (1935) and more recently by Youmans (1946) for the acid-fast bacteria. The analyses were made essentially as described by Mueller. The cultures were grown in Erlenmeyer flasks at 37 C and were constantly agitated on a Camp type shaker during incubation to ensure adequate aeration of the medium.

RESULTS

*M. phlei* grows well in a medium containing glucose, casamino acids, and beef heart infusion in addition to the basal salt mixture. The effect of each component on the growth rate and total crop of bacteria was studied to select its optimum concentration. The procedure was to vary the initial concentration of each component while holding the other two constant.

<table>
<thead>
<tr>
<th>TIME IN HOURS</th>
<th>PER CENT BEEF HEART INFUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>48</td>
<td>—</td>
</tr>
<tr>
<td>72</td>
<td>0.32</td>
</tr>
<tr>
<td>96</td>
<td>1.47</td>
</tr>
<tr>
<td>120</td>
<td>4.12</td>
</tr>
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</table>

The values in the table are milligrams of bacterial nitrogen per 10 ml of medium. The cultures had an initial volume of 50 ml contained in 250 ml Erlenmeyer flasks. Aliquots of 5 ml were used for the nitrogen analyses.

The initial concentrations of glucose and casamino acids over the range studied did not affect the growth rate but did affect the total amount of bacterial nitrogen produced. Thus in the presence of 50 per cent beef heart infusion and an adequate supply of casamino acids, the glucose concentration limited the total growth. For example, after 240 hours of incubation the glucose free medium contained 2.3 mg of bacterial nitrogen per 10 ml as compared to 5.1, 9.0, 12.4, and 15.2 mg per 10 ml for media containing 1.0, 2.0, 3.0, and 4.0 per cent glucose, respectively. Earlier in the incubation period (72 hours) the glucose containing flasks had produced equal amounts of bacterial nitrogen (3.55 ± 0.15 mg per 10 ml). When casamino acids limited the total growth, calculations indicated that approximately 90 per cent of the nitrogen supplied in the casein hydrolyzate was utilized by *M. phlei*. In subsequent experiments 2 per cent glucose and 1 per cent casamino acids were selected as suitable concentrations to employ.

The initial concentration of beef heart infusion had a marked effect on the growth rate of *M. phlei* (table 1). The amount of beef heart infusion does not
affect the total crop under these conditions. In this medium without beef heart infusion growth in 48 hours was negligible. Upon the addition of beef heart infusion the growth rate was increased and a considerable amount of bacterial nitrogen was formed in 48 hours. At this time maximal growth had not been reached. The observation below resulted from attempts to duplicate the stimulatory action of heart infusion with chemically defined substances. Substances tested were added to the basal mineral solution containing 1 per cent casamino acids and 2 per cent glucose.

Tweens have been shown to enhance the growth of the tubercle bacillus (Dubos, 1947). The tweens are all polyoxyethylene derivatives of certain fatty esters of sorbitan (Atlas Surface Active Agents, 1948). They differ only in the particular fatty acid component and are named as follows: (a) Tween 80—oleic acid; (b) Tween 60—stearic acid; (c) Tween 40—palmitic acid; (d) Tween 20—lauric acid. These compounds all enhanced the growth rate of M. phlei when added to the basal casamino acids-glucose medium (table 2). Tween 80 had the

TABLE 2

The effect of compounds related to Tween 80 on the growth of Mycobacterium phlei

<table>
<thead>
<tr>
<th>Tween Tested</th>
<th>0.01</th>
<th>0.05</th>
<th>0.10</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (oleate)</td>
<td>0.1</td>
<td>3.9</td>
<td>4.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Tween 60 (stearate)</td>
<td>0.1</td>
<td>2.6</td>
<td>4.1</td>
<td>4.7</td>
</tr>
<tr>
<td>Tween 40 (palmitate)</td>
<td>0.1</td>
<td>2.1</td>
<td>2.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Tween 20 (laurate)</td>
<td>0.1</td>
<td>2.6</td>
<td>3.7</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The values in the table are mg of bacterial nitrogen per 10 ml of medium.

The cultures had an initial volume of 20 ml and were contained in 125 ml Erlenmeyer flasks.

The cultures were incubated 48 hours at 37 C. Aliquots of 5 ml were used for the nitrogen analyses.

Nitrogen source is 1 per cent casamino acids and the carbon source is 2 per cent glucose.

TABLE 3

The stimulatory action of oleic acid on the growth of Mycobacterium phlei in the presence of Tween 80

<table>
<thead>
<tr>
<th>Oleic Acid, mg per 20 ml</th>
<th>Tween 60, 0.5 per cent</th>
<th>Tween 80, 0.5 per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5.8</td>
<td>6.5</td>
</tr>
<tr>
<td>0.05</td>
<td>5.8</td>
<td>6.2</td>
</tr>
<tr>
<td>0.5</td>
<td>6.8</td>
<td>6.0</td>
</tr>
<tr>
<td>2.5</td>
<td>6.6</td>
<td>5.8</td>
</tr>
</tbody>
</table>

The values in columns 2 and 3 are mg of bacterial nitrogen per 10 ml of culture.

The cultures had an initial volume of 20 ml and were contained in 125 ml Erlenmeyer flasks.

The cultures were incubated 48 hours at 37 C. Nitrogen source is 1 per cent casamino acids and the carbon source is 2 per cent glucose.
greatest effect on the growth rate, and at all concentrations a small but distinct differential existed between the effectiveness of this compound and that of any of the others.

The greater activity of Tween 80 in promoting growth appears to be related to its fatty acid component. The addition of oleic acid to Tween 60 (stearate) caused a growth promoting effect equivalent to that of Tween 80 (oleate); however, the addition of oleic acid in the same concentrations to Tween 80 proved to have a slightly inhibitory effect (table 3). The fact that the other tweens appeared to be more nearly equivalent to Tween 80 in their lower concentrations indicates that their activity is a function of the compounds themselves and not due to traces of oleic acid present as contamination.

TABLE 4

<table>
<thead>
<tr>
<th>PER CENT TWEEN 80</th>
<th>EGG YOLK PHOSPHATIDE, MG PER 10 ML</th>
<th>BACTERIAL NITROGEN, MG PER 10 ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>2.6</td>
</tr>
<tr>
<td>0</td>
<td>27</td>
<td>5.3</td>
</tr>
<tr>
<td>0</td>
<td>36</td>
<td>4.2</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>0.5</td>
<td>9</td>
<td>5.6</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1</td>
<td>9</td>
<td>4.7</td>
</tr>
</tbody>
</table>

The cultures had an initial volume of 20 ml and were contained in 125 ml Erlenmeyer flasks.

The cultures were incubated 48 hours at 37 C. Aliquots of 5 ml were used for the nitrogen analyses.

Nitrogen source is 1 per cent casamino acids and the carbon source is 2 per cent glucose.

Oleic acid in small amounts has been shown to enhance the growth of *M. tuberculosis*, but in larger amounts it is inhibitory. Serum albumin is added to media containing Tween 80 in order to bind the free oleic acid present and thus prevent its toxic effect (Davis and Dubos, 1947). The growth of *M. phlei* was affected by oleic acid in a similar manner. Free oleic acid added to the basal medium produced no effect and when added to a medium containing beef heart infusion was inhibitory. However, oleic acid added to the basal medium containing 0.5 per cent bovine serum albumin (Armour's Fraction V) stimulated the growth of *M. phlei*. A concentration of 1 mg per 10 ml of medium produced an increase in bacterial nitrogen of approximately 1 mg per 10 ml.

The phospholipid of egg yolk is a naturally occurring substance which resembles the tweens in being a water-dispersable carrier of fatty acids. It has been reported to enhance the growth of the tubercle bacillus (Boissevain and Schultz, 1938). The ether-soluble, acetone-insoluble fraction of egg yolk was separated and reprecipitated. An alcoholic solution of the precipitate was prepared, and an aliquot was assayed for dry weight of phospholipid. Samples of this product
were tested in the basal medium for growth promoting activity. The action of the phospholipid was similar to that of Tween 80, giving about the same maximum response. When added to an optimal amount of Tween 80 it did not further stimulate the growth of *M. phlei*. When it was added to a suboptimal amount of Tween 80, it stimulated the growth rate, approaching the maximum (table 4).

**DISCUSSION**

Experiments concerning beef heart infusion, casamino acids, and glucose have been presented because they serve to characterize the requirements for rapid growth of *M. phlei*. In the presence of a suitable carbon source, glucose, and a suitable nitrogen source, casamino acids, the growth of this microorganism is slow. The addition of beef heart infusion to media containing these components permits rapid growth. Thus in a medium containing suitable carbon and nitrogen sources this bacterium grows at a slow rate which can be enhanced by the addition of suitable substances. Tween 80, a water soluble ester of oleic acids, stimulates the growth of *M. phlei*. Oleic acid alone, in the presence of bovine serum albumin, also enhances the growth rate. The phospholipid fraction separated from egg yolk was as active as Tween 80, these two being the most active substances studied. Tween 60 (stearate), Tween 40 (palmitate), and Tween 20 (laurate) also enhance the growth rate and are nearly comparable to Tween 80. These observations are similar to those of Dubos and Davis (1946) concerning the behavior of the tubercle bacillus. Oleic acid is known to be an essential growth factor for several organisms. It is required for growth from small inocula by the diphtheria bacillus (Cohen and Mueller, 1940; Cohen, Snyder, and Mueller, 1941), by *Clostridium tetani* and *C. welchii* (Feeney, Mueller, and Miller, 1943), and for *Erysipelothrix rhusiopathiae* (Hutner, 1942). The growth of several species of lactic acid bacteria is enhanced by oleic acid or Tween 80 (Guirard, Snell, and Williams, 1946). The action of these compounds on these microorganisms is complicated because these substances will partially or completely replace biotin (Williams and Fieger, 1946; Williams, Broquist, and Snell, 1947). The lactic acid bacteria are more specific in their requirement for Tween 80 than is *M. phlei*. The other tweens were active only in very high concentrations, this activity being attributed to contamination of the compounds with free oleic acid. Free oleic acid also inhibits the growth of the lactic acid bacteria; however, in the presence of Tween 40 this property is neutralized and the combination is as active as Tween 80. A similar observation is described in the present paper. Tween 60, although active in enhancing the growth of *M. phlei*, is less active than Tween 80. A combination of Tween 60 and oleic acid, however, is equivalent to Tween 80 in enhancing the growth rate.

The concentration of Tween 80 and related compounds employed to produce the maximum growth stimulation of *M. phlei* is high when compared to the concentrations employed in culture media for *M. tuberculosis* (Dubos and Davis, 1946). *M. phlei* appears to be considerably more resistant to the inhibitory action of oleic acid than does the tubercle bacillus since it grows in high concentrations of Tween 80 without the addition of bovine serum albumin. It would seem
possible that the high optimum concentration of Tween 80 is a result of the tolerance of the organism for oleic acid and the heavy growth being measured.

The growth promoting effects of the tweens cannot be ascribed to their fatty acid content alone. The same molecule functions as a protective growth factor as well. Davis and Dubos (1947) demonstrated that Tween 80 when freed from unesterified oleic acid would protect the tubercle bacillus against oleic acid added to the medium. In the case of acid-fast bacteria, tween also produces a more diffuse growth factor, a factor which would make oxygen and nutrients more available to the cells (Dubos and Davis, 1946). Very likely the stimulating action of these compounds on the growth rate of *M. phlei* is a resultant of these separate aspects.

**ACKNOWLEDGMENTS**

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**SUMMARY**

Experiments concerning the requirements for rapid growth of *Mycobacterium phlei* have been described. Casamino acids (Difco) will serve as a satisfactory nitrogen source, and glucose will serve as a carbon source. However, a medium composed of these two components and minerals only permits the organism to grow at a slow rate. The addition of an aqueous infusion of beef heart to such a medium enhances the growth rate. Growth is also enhanced by suitable concentrations of the nonionizing esters of long-chain fatty acids, the tweens, by the phospholipid fraction of egg yolk, and by oleic acid when tested in the presence of bovine serum albumin.

**REFERENCES**


