THE EFFECT OF "TWEEN 80," BOVINE ALBUMIN, GLYCEROL, AND GLUCOSE ON THE GROWTH OF MYCOBACTERIUM TUBERCULOSIS VAR. HOMINIS (H37Rv)\textsuperscript{1}

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In a series of publications (Dubos, 1945; Dubos and Davis, 1946; Davis and Dubos, 1946\textsuperscript{a}; Davis and Dubos, 1946\textsuperscript{b}; Dubos, 1946; Dubos, Davis, Middlebrook, and Pierce, 1946; Dubos, 1947; Davis and Dubos, 1947; Dubos and Middlebrook, 1947) the addition of certain wetting agents to synthetic culture media used for the growth of virulent tubercle bacilli has been advocated. The synthetic nonionic detergent "tween 80" was found to be the most suitable. The advantages claimed were that this substance exerted a stimulating effect on the growth of tubercle bacilli and produced a diffuse homogeneous growth similar to that of many non-acid-fast bacteria instead of the granular or flaky growth usually encountered. Commercial preparations of tween 80, however, contained a sufficient amount of unesterified oleic acid to exert a growth-inhibitory effect on small inocula of tubercle bacilli. This could be removed chemically from the commercial material or counteracted by the addition of purified serum albumin to the medium. These investigators also reported that glucose and glycerol increased the total amount of growth of tubercle bacilli but not the initial rate of multiplication, and that glycerol exerted an inhibitory effect on the growth of small inocula in the presence of tween 80 that was not apparent in the absence of the oleic acid ester.

Quantitative determination of the growth rate of \textit{Mycobacterium tuberculosis} var. \textit{hominis} has been difficult because of the lack of reliable methods. Youmans (1946), however, has shown that nitrogen determinations can be used for accurately determining the amount of growth of tubercle bacilli, and the effect that alteration of the composition of the medium and the presence of growth inhibitory substances has on the rate of growth. In this manner the effect of various substances on the rate of growth may be evaluated on a quantitative basis. This technique is necessitated by the fact that, without a dispersing agent in the medium, tubercle bacilli form flaky clumps, which eliminates the application of turbidimetric, plating, or direct counting methods for the estimation of the amount of growth.

\textbf{METHODS}

\textit{Basal media.} Two chemically defined media were employed. The first was the modified Proskauer and Beck synthetic medium (modified P & B synthetic medium), which has previously been employed by Youmans (1946). The

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\textsuperscript{1} Aided by a grant from Parke, Davis and Company, Detroit 32, Michigan.
second medium was that recommended by Dubos (1947), in which we employed asparagine as the nitrogen source. Only water redistilled from glass was employed. The pH of both media was adjusted to 7.0 using a Macbeth electrometer, and the media were sterilized in the autoclave at 10 pounds for 20 minutes.

_Tween 80_ (polyoxyethylene sorbitan monooleate), a synthetic nonionic detergent, was obtained from the Atlas Products Company of Wilmington, Delaware. Three fresh lots (ARL no. 3020, no. 3292, and no. 7939) of tween 80 were employed, and were stored in the icebox at 5 C. The purified oleic acid-free tween 80 was prepared from one of these (Lot no. 3020) as required, according to the method recommended by Davis (1947).2 With this lot 0.60 per cent acid, calculated as oleic acid, was extracted from the unpurified tween 80 and the yield of purified tween 80 was approximately 70 per cent. These results compare very favorably with those obtained by Davis (1947) in the purification of tween 80. Tween 80 was employed in the media in a concentration of 0.05 per cent. All solutions of tween 80 were freshly prepared just before being used.

**Albumin.** Bovine albumin powder (fraction V) prepared by Armour Laboratories, Chicago, Illinois, was employed. A 5.0 per cent solution in physiological saline was inactivated by heating the albumin solution for 30 minutes at 55 C to remove any substances that might liberate free oleic acid by enzymatic action after prolonged incubation with the water-soluble ester tween 80 (Dubos and Davis, 1946). Sterile solutions were then obtained by filtration through porcelain candles (Selas no. 2) or Berkefeld filters (normal porosity) and added to the autoclaved medium with aseptic precautions in amounts to give a final concentration of 0.2 per cent.

**Glassware.** Pyrex test tubes, 200 by 25 mm, were cleaned by standing them overnight in concentrated sulfuric acid or detergent cleaning solution.3 They were rinsed then 7 to 8 times with running tap water, 3 times with distilled water, and twice with redistilled water. When dry they were capped with loose-fitting aluminum caps and sterilized in the autoclave at 20 pounds pressure for 20 minutes. Following sterilization, 10.0 ml of sterile medium were introduced aseptically into each tube with an accurately calibrated volumetric pipette.

**Determinations of growth of tubercle bacilli.** The method employed for the determination of the amount of growth of tubercle bacilli in the various media by micro-Kjeldahl nitrogen determinations followed the procedure used by Youmans (1946).

The virulent human type strain, H37Rv, of _M. tuberculosis_ was employed in all experiments. The amount of inoculum used ranged from 0.042 to 0.070 mg bacterial nitrogen per 10.0 ml of medium, but the inoculum was constant in all experiments in which comparisons were being made.

1 Purified tween 80 and data concerning chemical analysis were supplied by Leonard Doub of the Research Laboratories of Parke, Davis and Company, Detroit, Michigan.

RESULTS

The effect of unpurified tween 80 on the growth of H37Rv in modified Proskauer and Beck synthetic medium. Graph 1 shows a typical experiment in which 0.05 per cent unpurified tween 80 was added to the medium. It is apparent that the tween 80 exerted an inhibitory action on the growth of the tubercle bacilli. The actual decrease in the rate of growth in the presence of tween 80 as determined from the straight-line portions of the curves was 37.1 per cent. Table 1 shows the results of 7 similar experiments in which the percentage of inhibition of growth rate produced by 0.05 per cent tween 80 varied from 28.3 to 49.8.

### TABLE 1

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Concentration of Tween 80 in Per Cent</th>
<th>Inoculum</th>
<th>Time in Hours</th>
<th>Per Cent of Inhibition of Growth Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.00</td>
<td>0.059</td>
<td>0.119</td>
<td>0.280</td>
</tr>
<tr>
<td>B</td>
<td>0.00</td>
<td>0.070</td>
<td>0.117</td>
<td>0.124</td>
</tr>
<tr>
<td>C</td>
<td>0.00</td>
<td>0.042</td>
<td>0.102</td>
<td>0.176</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.053</td>
<td>0.106</td>
<td>0.150</td>
</tr>
<tr>
<td>E</td>
<td>0.00</td>
<td>0.049</td>
<td>0.126</td>
<td>0.202</td>
</tr>
<tr>
<td>F</td>
<td>0.00</td>
<td>0.049</td>
<td>0.072</td>
<td>0.108</td>
</tr>
<tr>
<td>G</td>
<td>0.00</td>
<td>0.049</td>
<td>0.109</td>
<td>0.161</td>
</tr>
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</table>

0.01 mg bacterial nitrogen is equivalent to approximately 0.23 mg of dried tubercle bacilli.

The effect of purified tween 80 on the growth of H37Rv in modified Proskauer and Beck synthetic medium. Graph 1 also shows the effect of purified tween 80 on the growth of the H37Rv strain. This purified material inhibited growth 23.3 per cent. Also shown in the graph is the effect on the growth of tubercle bacilli of the unpurified tween 80 from which the purified material was prepared. This produced considerably more inhibition of growth than the purified material. Two similar experiments with other preparations of purified tween 80 produced 20.7 and 24.9 per cent inhibition of growth, respectively.

The effect of purified and unpurified tween 80 on the growth of H37Rv in Dubos basal medium. Similar experiments were conducted using Dubos basal medium,
which contained glucose instead of glycerol, and in every case the same inhibitory effect of both purified and unpurified tween 80 was observed. The degree of inhibition by these agents was usually slightly less than with the Proskauer and Beck modified synthetic medium. However, accurate determinations of the degree of inhibition were difficult to measure since the rate of growth in this medium fell off so much more rapidly than in the modified Proskauer and Beck modified synthetic medium that it was difficult to find straight-line portions of the curve to use for this purpose. Graph 2 shows a typical experiment in which the unpurified tween 80 inhibited growth approximately 30 per cent, and the purified tween inhibited growth approximately 13 per cent, using the rate of growth during the first 5 days as the basis for the comparison.

Effect of glycerol\(^4\) (2.0 per cent) and glucose (0.2 per cent) and purified tween 80

\(^4\) Baker and Adamson reagent grade glycerin.
(0.05 per cent) on the growth of M. tuberculosis (H37Rv) in modified Proskauer and Beck and Dubos basal synthetic medium. Graph 3 shows the results obtained with modified Proskauer and Beck medium containing (1) 2.0 per cent glycerol; (2) 2.0 per cent glycerol plus 0.05 per cent tween 80; (3) 0.05 per cent tween 80; (4) neither glycerol nor tween 80. It is evident that omission of the glycerol resulted in marked reduction in the rate of growth of the tubercle bacilli (37.6 per cent). The addition of 0.05 per cent tween 80 to this medium containing no glycerol resulted in a stimulation of growth but not to the same degree as the addition of glycerol. The addition of both glycerol and tween 80 to the basal medium resulted in a rate of growth for a period of 7 days that was the same

TABLE 2
Effect of albumin (0.2 per cent) and tween 80 (0.05 per cent) on growth of tubercle bacilli (H37Rv) in modified P & B and Dubos media

<table>
<thead>
<tr>
<th>EXP. NO.</th>
<th>TYPE OF MEDIUM</th>
<th>ALBUMIN</th>
<th>PURIFIED TWEEN 80</th>
<th>UNPURIFIED TWEEN 80</th>
<th>MILLIGRAMS BACTERIAL NITROGEN</th>
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</thead>
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<tr>
<td></td>
<td>Modified</td>
<td></td>
<td></td>
<td></td>
<td>Inoculum Time in hours 72 120 168 216 264</td>
</tr>
<tr>
<td>H</td>
<td>P &amp; B</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>“” 0.2%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.046 0.132 0.210 0.288 0.372</td>
</tr>
<tr>
<td></td>
<td>“” 0.2% 0.05%</td>
<td>0.046</td>
<td>0.130 0.200 0.245 0.331</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>“”</td>
<td>—</td>
<td>—</td>
<td>0.046</td>
<td>0.109 0.168 0.256 0.345 0.491</td>
</tr>
<tr>
<td></td>
<td>“” 0.2% 0.05%</td>
<td>0.046</td>
<td>0.110 0.166 0.256 0.316 0.351</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Dubos</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>“” 0.2%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.046 0.108 0.169 0.213 0.288</td>
</tr>
<tr>
<td></td>
<td>“” 0.2% 0.05%</td>
<td>0.046</td>
<td>0.110 0.169 0.211 0.265 0.310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>“”</td>
<td>—</td>
<td>—</td>
<td>0.046</td>
<td>0.109 0.168 0.215 0.299 —</td>
</tr>
<tr>
<td></td>
<td>“” 0.2% 0.05%</td>
<td>0.046</td>
<td>0.110 0.169 0.211 0.265 0.310</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

as for the medium containing tween 80 alone, following which more growth was present in the medium containing both tween 80 and glycerol.

Graph 4 shows the results obtained using Dubos basal medium with and without glucose in the presence and absence of 0.05 per cent purified tween 80. The results obtained here are comparable to the results obtained with glycerol in the modified Proskauer and Beck synthetic medium.

The effect of albumin (0.2 per cent) in modified Proskauer and Beck and Dubos medium on the growth of tubercle bacilli (H37Rv). The addition of 0.2 per cent albumin to the basal media did not result in an increased rate of growth either in the early growth period or in the late phase. During the late growth period there was a tendency for an earlier decrease in rate of growth in the Proskauer and Beck medium containing albumin, but in all other respects the growth rate as well as the total amount of growth are essentially equal (graphs 5 and 6; table 2).
The effect of albumin (0.2 per cent) in the presence of tween 80 (0.05 per cent) on the growth of tubercle bacilli (H37Rv). The addition of tween 80 (0.05 per cent) in either the purified or unpurified state produced no significant differences in the rate of growth in Dubos medium or modified P and B medium in the presence of 0.2 per cent albumin for the first 5 days, but thereafter a decrease in growth was noted in both media which contained either purified or unpurified tween 80 (graphs 5 and 6; table 2).

DISCUSSION

It is apparent from the results of these experiments that unpurified tween 80 has a rather marked bacteriostatic effect on the large inocula of tubercle bacilli employed. This is in line with the observations of Dubos and Davis (1946) and Davis and Dubos (1946a) that this material is toxic to small inocula of tubercle bacilli. According to Dubos and Davis (1946a), Dubos, Davis, Middlebrook, and Pierce (1946), and Dubos and Middlebrook (1947), the toxicity of this material is due to the presence of unesterified oleic acid; and, when the oleic acid is chemically removed from the tween 80, the latter becomes entirely nontoxic for tubercle bacilli as determined by the growth of small inocula. Our results would indicate that tween 80 from which the free oleic acid has been removed still has a bacteriostatic action even on large inocula of tubercle bacilli. This, of course, might indicate that purification of the tween 80 had not been complete. The protective effect of bovine albumin noted in our experiments might also suggest that our purified tween 80 was not completely free of unesterified oleic acid. According to Davis (1947), purified tween 80 will slowly hydrolyze at 37 C to produce some free oleic acid, and it is possible that our findings could be accounted for on this basis. In view of the slow rate of hydrolysis of tween 80 and the prompt inhibition of growth observed in our experiments this explanation does not seem likely.
According to Dubos and Middlebrook (1947), lipases present in bacteria may accelerate the hydrolysis of tween 80. In view of the relatively large inocula employed in these experiments the possibility must be considered that the tubercle bacilli themselves might have hydrolyzed the tween 80 to form oleic acid with a consequent bacteriostatic effect. According to Dubos (1947) and Davis (1948), however, the toxicity of oleic acid can be neutralized by large inocula of tubercle bacilli. This would appear to exclude the size of the inoculum as a factor.

It would seem, exclusive of the role played by any free oleic acid present in tween 80, that this substance by virtue of its surface-active properties might possibly be bacteriostatic per se. In any event, in the media employed and under the conditions of the experiments, there is no indication of the marked enhancement of growth reported by Dubos (1947). In this connection it is of interest to note that cultures of tubercle bacilli in media containing tween 80 usually appear visually, because of the diffuse type of growth, to have more organisms present; but in all cases, except when the glucose or glycerol were omitted from the media, in which actual quantitative measurements of the amount of tubercle bacilli present were made, a greater total mass of organisms was found in the media that were without tween 80 and that showed the clumped form of growth.

It is also apparent from these results that both glycerol and glucose markedly stimulate growth. Furthermore, purified tween 80 also stimulates growth when added to either of the media in the absence of glucose or glycerol, but not to nearly the same degree as either glucose or glycerol alone. Purified tween 80 when added to either of the media in the presence of glycerol or glucose inhibited growth as compared with either glucose or glycerol alone. This is in contrast to the findings of Dubos and Davis (1946) and Dubos, Davis, Middlebrook, and Pierce (1946) that glycerol inhibited the growth of small inocula in the presence of tween 80; actually the converse held in our experiments, since the tween 80 inhibited growth in the presence not only of glycerol but also of glucose, even though it stimulated growth slightly in the absence of these agents.

Although it is possible that glycerol might have an inhibitory effect on small numbers of tubercle bacilli and stimulate growth of larger numbers, it would also seem reasonable that another substance (tween 80) that was toxic for large inocula might also have a growth-retarding effect on small inocula. This is supported by work using small inocula (Youmans, 1948).

Under the conditions of these experiments, using large inocula, the addition of serum albumin (0.2 per cent) resulted in neither an increased initial rate of growth nor an increased total yield. According to Dubos and Davis (1946) and Davis and Dubos (1947), although the addition of crystalline serum albumin to the medium often permits the initiation of growth of minute inocula that would not grow in the absence of the protein, it does not increase appreciably the final density of the culture. However, less pure protein (fraction V) apparently contained heat-stable impurities that could be separated from the albumin and that increased markedly the amount of growth yielded by tubercle bacilli in
synthetic media. In our experiments no such enhancing effect resulted from the addition of fraction V serum albumin to the basal medium. In fact, during the late growth period there was a tendency toward a slight decrease in rate of growth in the Proskauer and Beck medium containing albumin, but the initial growth rates were not significantly different. These divergent results, of course, might be accounted for on the basis of differences in the samples of bovine albumin employed.

The addition of purified and unpurified tween 80 to either medium containing 0.2 per cent albumin resulted in no significant difference in the rate of growth for the first 5 days; thereafter, a decrease in the rate of growth was noted which was more marked in the modified Proskauer and Beck medium. Thus it appears that the albumin protected against the early inhibitory effect of tween 80 but not for a period greater than 5 days. The combined bovine serum albumin and tween 80 mixture definitely failed to increase either the rate of growth or total yield as compared to the results when using only Dubos basal medium or modified Proskauer and Beck synthetic media.

Though the role of these various agents as tested seems definite, it does not necessarily follow that under conditions in which different concentrations were used the same results would be noted. Because of the laboriousness of this quantitative method the present experiments were limited to the use of these substances in the concentrations recommended in the literature. The answer to questions relative to the effect of varying the concentrations must await further quantitative study. Furthermore, it cannot be concluded that all strains of virulent human type tubercle bacilli would behave under the conditions of the experiments in the same manner as the H37Rv strain.

CONCLUSIONS

Unpurified "tween 80" (0.05 per cent) markedly inhibited the growth rate of virulent tubercle bacilli (H37Rv) in modified Proskauer and Beck synthetic media.

Purified tween 80 (0.05 per cent) exerted a similar bacteriostatic effect but to a lesser degree.

The same inhibitory effect of this surface-active agent was noted using Dubos basal medium.

Glucose and glycerol markedly stimulated the rate of growth.

Although purified tween 80 inhibited growth in the presence of glucose and glycerol, it stimulated growth slightly in the absence of these agents.

Bovine serum albumin (fraction V; 0.2 per cent) did not stimulate growth of the tubercle bacillus.

Bovine serum albumin (0.2 per cent) protected tubercle bacilli against the inhibitory effect of purified and unpurified tween 80 (0.05 per cent) but only during the first 5 days of growth.

The modified P and B synthetic medium containing 2.0 per cent glycerol supported growth of tubercle bacilli at a maximum rate for a longer period than the Dubos medium containing 0.2 per cent glucose.
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