GROWTH-ENHANCING FACTOR FOR HUMAN TUBERCLE BACILLI USING MINUTE INOCULA

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It has been difficult to cultivate human tubercle bacilli in agar medium unless bovine serum, or a fraction therefrom, was added aseptically, since the factor present in the serum is thermodabile (Dubos and Middlebrook, 1947). On the other hand, egg yolk or whole egg media may be used, but the difficulties in preparing the media render the methods uncertain and cumbersome. Hence, a thermostable agar medium which can be prepared by autoclave sterilization has been needed.

For three years the author studied the metabolism of “tween-80” by the tubercle bacilli (Minami et al., 1954; Minami and Yamane, 1955) and devised a medium in which tween-80 was the sole source of carbon (Yamane et al., 1954), but this medium did not permit growth from small inocula. The latter was accomplished by adding whole egg yolk to the tween-agar medium (Yamane et al., 1955). This growth factor could be extracted from the yolk by fat solvents and has been obtained in a crystalline form. It is heat stable, so that it can be added to tween-agar medium which can be sterilized by autoclaving, and its addition enables the bacilli to grow from small inocula. The present paper deals with the nature of this crystalline substance and the synthetic agar medium containing it.

METHODS

Mycobacterium tuberculosis var. hominis strain H37Rv, and a second strain recently isolated from the sputum of tuberculous patients, both cultivated by serial transplant on egg medium, were transferred to medium in which tween-80 was the sole source of carbon (Yamane et al., 1954) and incubated for 7 days at 37 C. These cultures were then diluted to 10^-2 mg dry weight per ml, using 0.05 per cent “triton” A-20 in saline as the diluent, and further 10-fold serial dilutions were prepared so that 0.1 ml represented 10^-3 to 10^-7 mg. These were used to inoculate, in duplicate, basal medium containing the fractions to be tested, medium containing egg yolk (Yamane, et al., 1955) and whole egg slanted agar. These were incubated for 14 days at 37 C and growth was observed.

The basal medium used was similar to that used previously and consisted of: KH₂PO₄, 3.0 g; Na₂HPO₄·12H₂O, 6.0 g; ferric ammonium citrate, 0.1 g; sodium glutamate, 5 g; asparagine, 0.5 g; sodium citrate, 0.1 g; β-alanine, 0.1 g; tween-80, 5.0 g; 2 per cent malachite green solution, 0.2 ml; purified (defatted) agar, 12 g; and distilled water, 1,000 ml. The agar was prepared by extracting its lipid impurity in boiling methanol, an indispensable part of the preparation of this medium. Sterilization was accomplished by autoclaving at 120 C for 15 min.

RESULTS

Extraction of the active crystalline principle. Egg yolk, 50 g, was treated with cold acetone to remove the acetone-soluble colored substances. The acetone-treated yolk was dried in vacuo to remove the acetone and extracted with 250 ml of ether. The ether extract of the yolk was concentrated and dried in vacuo to remove ether. The dried ether extract was dissolved in 250 ml of boiling ethanol. When this ethanol solution of the extract was kept overnight at 5 to 10 C, the white flocculated sediment appeared. This was the crude crystallized substance. This sediment was collected and redissolved in boiling ethanol, then kept overnight at 5 to 10 C, whence the crystallized sediment appeared. The collected crystals were washed with cold ethanol containing 0.5 per cent distilled water at 5 to 10 C. The extraction process is shown in figure 1. The crystalline principle was rosette-like when suspended in cold ethanol and examined under low power (figure 2). The melting point was 36 C but some preparations melted at 25 C while yet retaining growth-promoting activity. This crystal contained no nitrogen and phosphorus. The crystalline material was soluble in methanol,
Figure 1. Extraction of the active crystalline principle

The growth-promoting effect of the crystalline principle. The active principle is insoluble in water. It was added as the ether solution in various amounts to the basal medium and sterilized by autoclaving. Table 1 contains a record of the growth obtained after incubation at 37 C for 14 days.

It is evident that the principle increases growth at a concentration of 0.01 per cent or above. Although the basal medium permitted growth from 10^{-7} mg of inoculum at the 28th day, addition of the substance permitted growth...
by the 14th day. Similar results were obtained by means of the agar plate method. The activity of the principle thus appears to stand autoclaving. The medium contains rather large amounts of the buffering substances; therefore, it may be employed for the clinical detection of the bacilli without neutralizing the alkalized sputa of tuberculous patients.

**Figure 2.** Micrograph of the active crystalline principle.

**DISCUSSION**

Lominski (1933) reported that various lipid substances enhanced the growth of human tubercle bacilli. Later Boissevain and Schultz (1938) succeeded in extracting a lipid-like growth-promoting fraction from the egg yolk. This material was alcohol soluble and acetone-insoluble but was not pure, since in order to enhance growth it was necessary to add it in a concentration of about 0.3 per cent. This result was confirmed by the author. Lately Hirsch (1954) reported that Boissevain’s fraction was cholesterol or a related substance. But the author extracted the active principle in crystalline form by means of a method somewhat similar to Boissevain’s, and a concentration of 0.01 per cent in the medium was sufficient to enhance the growth from small inocula. Moreover, this substance hardly gave the Lieberman-Buchard’s reaction for sterols and thus seems to be a fatlike compound rather than a cholesterol-like compound.

The required concentration of the active principle in the medium is above 0.01 per cent. It is a rather large amount from the viewpoint of

<table>
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<th>Medium</th>
<th>Crystal (or Egg Component)</th>
<th>Amount of Inoculum (Mg)</th>
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<tr>
<td></td>
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<tr>
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<td>7</td>
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<tr>
<td>Tween agar*</td>
<td>Egg yolk</td>
<td>+++</td>
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<td>Ogawa’s medium</td>
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<td></td>
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The numbers in the cultural results show the average colony numbers of duplicate cultures and plus signs show growth of over 100 colonies per culture.

* Previously reported medium (Yamane et al., 1955).
the growth factor. Tubercle bacilli can grow in the synthetic medium containing no growth factor, when the inoculum is adequate; therefore, it can be said that tubercle bacilli require no growth factor. Thus, the present crystalline substance is a growth-promoting substance which probably does not function as the coenzyme factor but as some other physicochemical adjuvant like starch or charcoal (Uyei, 1930; Hirsch, 1954). When its chemical structure has been determined its true mode of action will be investigated.

ACKNOWLEDGMENT
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SUMMARY
A crystalline growth-enhancing factor for human tubercle bacilli was isolated from egg yolk. The substance is heat stable, soluble in ether and chloroform, and insoluble in water. It is required in concentrations above 0.01 per cent. Using this material, a thermostable synthetic agar medium could be devised for the detection of tubercle bacilli.

REFERENCES