

EFFECT OF TWEEN 80 ON THE GROWTH OF TUBERCLE BACILLI IN AERATED CULTURES¹

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ABSTRACT

LYON, RICHARD H. (Veterans Administration Hospital, Minneapolis, Minn.), HERMAN C. LICHSTEIN, AND WENDELL H. HALL. Effect of Tween 80 on the growth of tubercle bacilli in aerated cultures. *J. Bacteriol.* **86**:280-284. 1963.—The effect of Tween 80 (polyoxyethylene sorbitan monooleate), glucose, and glycerol on aerated and stationary growth of *Mycobacterium tuberculosis* strain H₃₇Ra was examined in Dubos liquid medium. Previous studies established that aeration (rotation) of liquid cultures of *M. tuberculosis* strains H₃₇Ra and H₃₇Rv caused an inhibition of growth in a medium containing glucose as a source of carbohydrate. The present studies show that Tween 80 exerts a toxic effect on the growth of tubercle bacilli in aerated cultures when glucose is present in the medium as the sole source of carbohydrate, but not when glycerol is included. The role of hydrolytic products of Tween 80, *viz.* oleic acid and the polyoxyethylene derivative of sorbitol, is discussed. The hypothesis is submitted that glycerol protects against the growth suppression by aeration because it reduces the concentration of free fatty acids in the medium to subinhibitory levels.

The rate of growth of certain aerobic bacteria can be increased by aeration. In a previous publication (Lyon, Lichstein, and Hall, 1961), it was established that aeration of liquid cultures of *Mycobacterium tuberculosis* var. *hominis* strains H₃₇Rv and H₃₇Ra caused an inhibition of growth in a medium containing glucose as the

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source of carbohydrate. On the other hand, aeration of liquid cultures of certain saprophytic mycobacteria resulted in a stimulation of growth with either glucose or glycerol as the source of carbohydrate. This report deals with the effect of Tween 80 (polyoxyethylene sorbitan monooleate) on the growth of aerated cultures of *M. tuberculosis* strain H₃₇Ra. It is concluded that Tween 80 is in some manner responsible for growth inhibition in aerated liquid cultures lacking glycerol.

MATERIALS AND METHODS

M. tuberculosis strain H₃₇Ra was employed. The experimental liquid medium was that developed by Dubos and Middlebrook (1947). The tubercle bacilli were grown in 200 ml of medium dispensed in 500-ml Erlenmeyer flasks. The flasks were inoculated with 2 ml (equivalent to approximately 0.2 mg dry weight of bacilli) of a 7-day culture. Stationary cultures were rotated briefly by hand three times daily. The rotated (aerated) cultures were incubated in a rotary shaker at 140 rev/min. Incubation temperature was 37 C. Growth was measured as turbidity in a Coleman Junior spectrophotometer (model 6A) at a wavelength of 600 m μ . Samples (10 ml) were recovered at intervals, mixed with a Teflon grinder to insure a uniform suspension of bacilli, and transferred to cuvettes (19 × 150 mm) for reading. The cultures were observed over a 21-day period.

RESULTS AND DISCUSSION

Previous work (Lyon et al., 1961) showed that growth of strain H₃₇Ra was inhibited in aerated cultures that contained glucose as a source of carbohydrate. This effect was overcome by increasing the inoculum, replacing glucose with glycerol, or adding glycerol to glucose in the medium. The growth inhibition was not

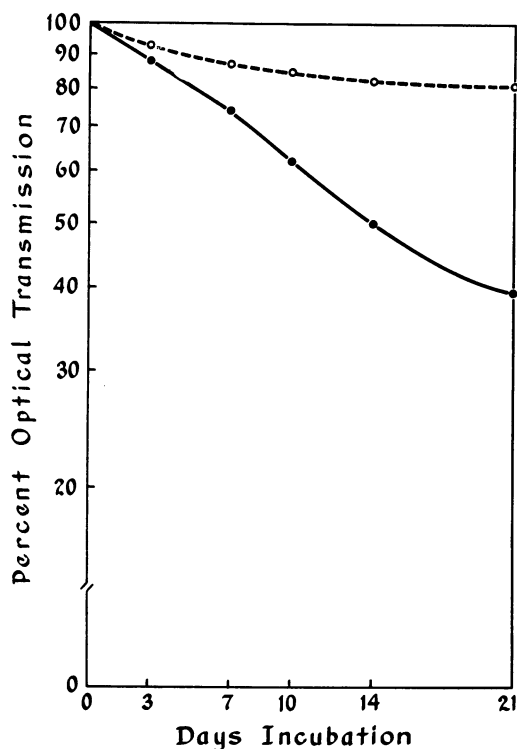


FIG. 1. Effect of aeration on the growth of *Mycobacterium tuberculosis* strain *H₃₇Ra* in Dubos liquid medium devoid of carbohydrate. ●, Stationary incubation; ○, rotary incubation.

observed in stationary cultures. Subsequent studies with liquid media devoid of glucose or glycerol revealed inhibition of growth in the rotated cultures (Fig. 1). Thus, it appeared that the suppressive effect of rotation was not necessarily dependent upon the presence of carbohydrate.

The effect of Tween 80, as well as glucose and glycerol, on aerated and stationary growth of strain *H₃₇Ra* in Dubos liquid medium was examined next. These studies showed that Tween 80 was responsible for the adverse effects of rotary incubation on growth of this organism (Fig. 2). Growth in the medium containing glycerol improved under rotary conditions, in comparison with that observed during stationary incubation, regardless of the presence or absence of Tween 80. However, in the medium containing glucose, the inhibitory effect of rotation was overcome only when Tween 80 was deleted from the medium. Thus, Tween 80 exerted a toxic

effect on the growth of tubercle bacilli in aerated cultures when glucose was present in the medium (Fig. 2) or if no carbohydrate was added to the medium (Fig. 1), but not when glycerol was the source of carbohydrate (Fig. 2).

Dubos and Davis (1946) encouraged dispersed growth of tubercle bacilli in liquid media by adding Tween 80 in order to depress surface tension. Dubos and Davis (1947) and Dubos and Middlebrook (1947) reported that the favorable effect of Tween 80 was reduced in the absence of serum albumin (the function of which is to bind free fatty acids) and by a low bacterial inoculum. These investigators indicated that oleic acid hydrolyzed from the Tween 80 molecule caused the alteration. Dubos (1946) had previously studied the effect of long-chain fatty acids on bacterial growth and had observed that oleic acid in concentrations as low as 10^{-6} M inhibited growth of tubercle bacilli in synthetic media. It is important to note that Dubos and Middlebrook (1947) used glucose as a source of carbohydrate in their media.

Tween 80 is an oleate ester of sorbitol and its anhydrides, condensed with polymers of ethylene dioxide. Hydrolysis of the ester linkage releases oleic acid. Davis (1947a) reported that the titer of free fatty acid in commercial preparations of Tween 80 corresponded to 3% of the total fatty acid present, or 0.6% by weight if the fatty acid is calculated as oleic acid. Moreover, this concentration of free oleic acid was sufficient to

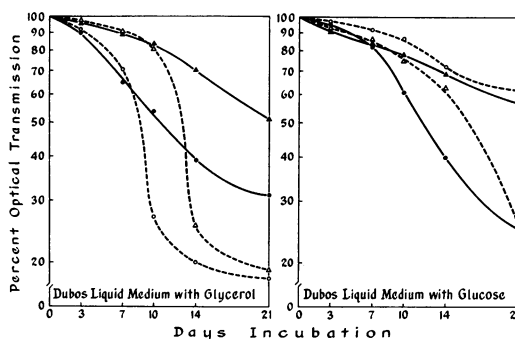


FIG. 2. Effect of Tween 80, glucose, and glycerol on aerated and stationary growth of *Mycobacterium tuberculosis* strain *H₃₇Ra* in Dubos liquid medium. ●, Stationary incubation with Tween 80; ○, rotary incubation with Tween 80; △, rotary incubation without Tween 80; ▲, stationary incubation without Tween 80.

account for the inhibitory effect of Tween 80 on the growth of small inocula of tubercle bacilli. In a subsequent paper, Davis (1947b) described a method of preparing fatty acid-free Tween 80 from the commercial product. In the present studies, the use of purified Tween 80 in Dubos liquid medium did not alter the growth patterns of strain H₃₇Ra described above.

Esterase and lipase activity of mycobacteria have been well established. Minami and Yamane (1954) showed that esterases of the tubercle bacillus split Tween 80 into oleic acid and the polyoxyethylene derivative of sorbitol; further, they showed that each component is subsequently metabolized via the Krebs tricarboxylic acid cycle. Studies were therefore undertaken to determine the effect of these hydrolytic products on the growth of strain H₃₇Ra, utilizing the cultural conditions described. The effect of sorbitol on the growth of strain H₃₇Ra is currently under investigation and will be reported at a later date. Oleic acid (10 µg/ml, purified; obtained from Fisher Scientific Co., Pittsburgh, Pa.) added to Dubos liquid medium was found to be inhibitory under rotary conditions in glucose-containing

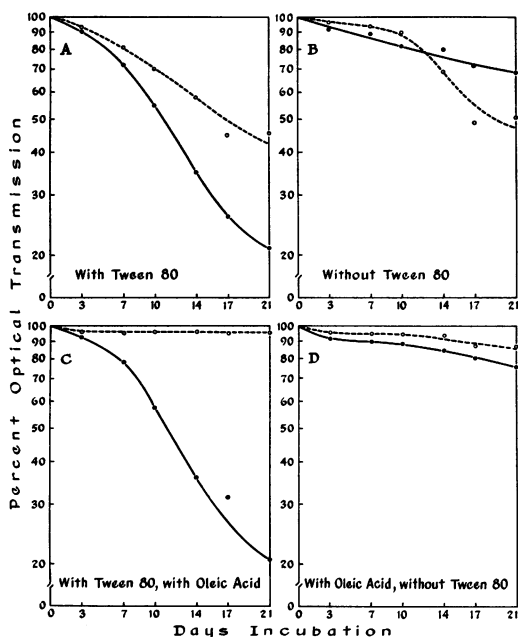


FIG. 3. Effect of Tween 80 and of oleic acid (10 µg/ml of medium) on the growth of *Mycobacterium tuberculosis* strain H₃₇Ra in Dubos liquid medium. ●, Stationary incubation; ○, rotary incubation. Glucose as the source of carbohydrate.

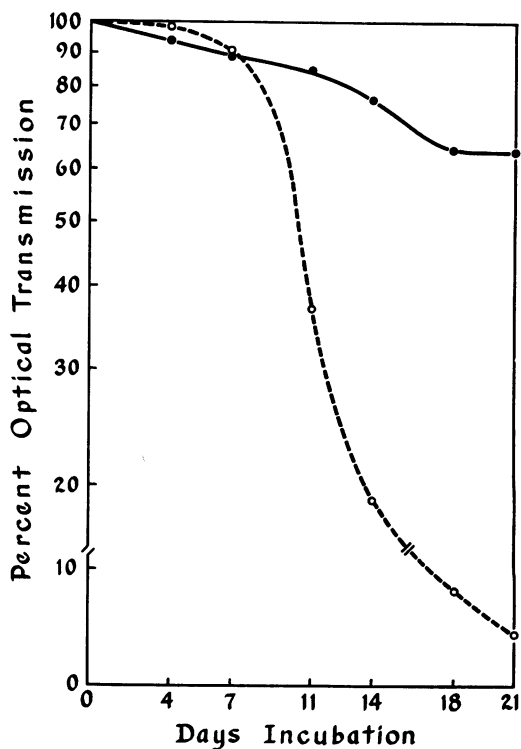


FIG. 4. Effect of oleic acid (10 µg/ml) on the growth of *Mycobacterium tuberculosis* strain H₃₇Ra in Dubos liquid medium (with glycerol, without Tween 80). ●, Stationary incubation; ○, rotary incubation.

media (Fig. 3C). The addition of oleic acid to media devoid of Tween 80 (Fig. 3D) also resulted in growth inhibition under rotary conditions. In contrast, oleic acid did not inhibit growth when added in concentrations as high as 50 µg/ml of medium when glycerol was substituted for glucose (Fig. 4). Tween 80 is beneficial for growth of this strain in liquid media under stationary conditions of incubation (Fig. 3A, B, C, and D). This may be attributed to the dispersed growth caused by the wetting agent and, according to Minami (1957), the protective effect of Tween 80 selectively adsorbed to the cell surface that prevents the adsorption and inhibitory action of oleate.

Our studies indicate that, in the concentrations used, oleic acid is toxic to the growth of strain H₃₇Ra in a glucose-containing medium under rotary conditions. The effect is not manifest when the medium contains glycerol, either as the sole source of carbohydrate or in combination

with glucose. The hypothesis is submitted that glycerol, in addition to its function as a carbohydrate, has a protective role (Fig. 5). It has been established previously that the lipid content of tubercle bacilli is greater when the organisms are grown in the presence of glycerol (Drea and Andrejew, 1953). Although definitive experimental proof is not available, a possible mode of action is the esterification of fatty acid with glycerol, probably at the cell surface, forming lipid materials. Such a mechanism could effectively reduce the concentration of free fatty acids in the medium to subinhibitory or even stimulatory levels.

The mechanism by which oleic acid exerts its toxic action on the growth of tubercle bacilli under aerobic conditions has not been resolved. The data herein presented make it known that the greatest toxic effect occurs during rotation

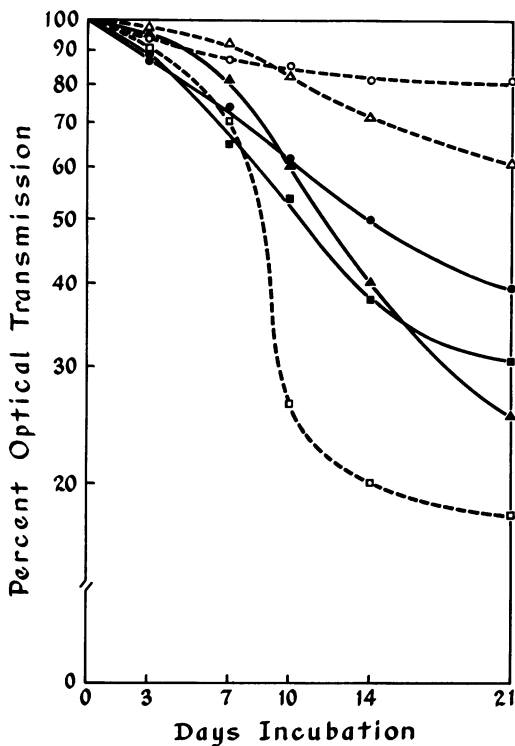


FIG. 5. Protective role of glycerol on the growth of *Mycobacterium tuberculosis* strain H₂₇Ra in Dubos liquid medium containing Tween 80. ●, Stationary incubation without carbohydrate; ○, rotary incubation without carbohydrate; ▲, stationary incubation with glucose; △, rotary incubation with glucose; ■, stationary incubation with glycerol; □, rotary incubation with glycerol.

of liquid cultures inoculated with small numbers of organisms. Rotation or aeration may promote the oxidation of unsaturated fatty acids, such as oleic acid, to peroxides which are toxic for this microorganism. Schaefer, Cohn, and Middlebrook (1955) reported a decrease in the rate of growth of several strains of tubercle bacilli in media containing an oleic acid-albumin complex. This effect was attributed to a stimulation of metabolic reactions produced by oleic acid which were not accompanied by an equally increased rate of CO₂ uptake by the bacilli. Growth of these strains was stimulated by adding biotin to the medium or increasing the CO₂ concentration. The possible relationship between the effects of oleic acid noted by these investigators and those described in the present paper is obscure.

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LITERATURE CITED

- DAVIS, B. D. 1947a. The estimation of small amounts of fatty acid in the presence of polyoxyethylene sorbitan partial fatty acid esters ("Tween") and of serum proteins. *Arch. Biochem.* **15**:351-358.
- DAVIS, B. D. 1947b. The preparation and stability of fatty acid-free polyoxyethylene sorbitan monooleate ("Tween"). *Arch. Biochem.* **15**:358-364.
- DREA, W. F., AND A. ANDREJEV. 1953. Nutrition and growth, p. 3-66. *In* The metabolism of the tubercle bacillus. Charles C Thomas, Publisher, Springfield, Ill.
- DUBOS, R. J. 1946. Effect of long chain fatty acids on bacterial growth. *Proc. Soc. Exptl. Biol. Med.* **63**:56-58.
- DUBOS, R. J., AND B. D. DAVIS. 1946. Factors affecting the growth of tubercle bacilli in liquid media. *J. Exptl. Med.* **83**:409-423.
- DUBOS, R. J., AND B. D. DAVIS. 1947. The binding of fatty acids by serum albumin—a protective growth factor in bacteriological media. *J. Exptl. Med.* **86**:215-228.
- DUBOS, R. J., AND G. MIDDLEBROOK. 1947. Media for tubercle bacilli. *Am. Rev. Tuberc.* **56**:334-345.
- LYON, R. H., H. C. LICHSTEIN, AND W. H. HALL. 1961. Factors affecting the growth of *Mycobacterium tuberculosis* strain H₂₇Ra in Dubos liquid medium containing Tween 80.

- bacterium tuberculosis* in aerobic and stationary cultures. Am. Rev. Respirat. Diseases **83**:255-260.
- MINAMI, K. 1957. Bactericidal action of oleic acid for tubercle bacilli. I. Quantitative and analytical survey of the action. J. Bacteriol. **73**:338-344.
- MINAMI, K., AND I. YAMANE. 1954. The metabolic process of Tween 80 by the tubercle bacillus. Symp. Enzyme Chem. (Tokyo) **10**:93-102.
- SCHAEFER, W. B., M. L. COHN, AND G. MIDDLEBROOK. The roles of biotin and carbon dioxide in the cultivation of *Mycobacterium tuberculosis*. J. Bacteriol. **69**:706-712.