FACTORS AFFECTING THE GROWTH OF TUBERCLE BACILLI IN LIQUID MEDIA

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Virulent tubercle bacilli are able to yield an abundant growth in simple synthetic media by synthesizing their structural and metabolic constituents from a few mineral salts and organic compounds; they are therefore, among the least exacting of pathogenic microorganisms in their growth requirements. On the other hand, it is often difficult to initiate the growth of small inocula of these organisms *in vitro;* furthermore all authentic strains of human, bovine, and to a lesser extent avian tubercle bacilli multiply extremely slowly, even in media enriched with the growth-promoting substances present in serum, egg yolk, potato extract, milk, etc. These characteristics render difficult the application to tubercle bacilli of quantitative bacteriological methods based on enumeration of living cells by plating or dilution techniques; they delay and at times prevent bacteriological diagnosis of tuberculosis; they hinder investigations concerned with pathogenesis, immunity, and chemotherapy.

In addition to these difficulties there are others, less obvious but equally important, which result from the heterogeneity of fully grown cultures of tubercle bacilli. On the one hand, cells present in these cultures vary greatly in age and therefore in physiological state. Heterogeneity of the cell population is further increased when mycobacteria are allowed to grow in the form of pellicles, heaped masses, or large clumps; the environmental conditions prevailing in the center of these masses differ greatly from those prevailing at the periphery and are probably reflected in structural and metabolic differences among the individual components of the bacterial population. It has been shown that a 2 week old culture of human tubercle bacilli contains a very large proportion of dead cells (Wilson and Schwabacher, 1935–36) and it is likely that many of these have undergone varying degrees of autolysis. This heterogeneity undoubtedly complicates the analysis of the factors affecting the rate of bacterial growth and the establishment of standard experimental infections. Even more probably it obscures the results of immunochemical analysis by leading to the study of artefacts resulting from autolysis of the cells and preventing the detection and isolation of important cellular components and metabolic products of the normal, physiologically active tubercle bacilli.

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The investigations to be described in this and subsequent papers are concerned with some of the factors which affect the initiation, rate, and mode of growth of tubercle bacilli. Since their ultimate objective is to develop more adequate methods for the bacteriological and immunological study of tuberculosis, it may be useful to state some of the specific requirements which a satisfactory medium should fulfill to this end.

1. Growth should be rapid.

2. For diagnostic work and for quantitative bacterial counts the medium should permit growth of very small inocula (preferably of a single cell).

3. Diffuse growth is desirable where physiological or chemical homogeneity of the culture is important.

4. There should be no variation of the culture in the course of growth (smooth ⇄ rough, virulent ⇄ avirulent, etc.).

5. Large yields of bacteria may be required for certain purposes such as the isolation and identification of cellular components or metabolic products.

It is not necessary that all these requirements be satisfied in one single type of medium. Thus, rapid and accurate diagnostic tests or bacterial counts can be obtained without abundant growth, and conversely, ability to initiate the growth of very small inocula is not essential where the aim is to produce large yields of bacilli or well dispersed growth.

The present paper will be limited to a consideration of the effect of certain substances on the initiation of growth of small inocula, and on the state of dispersion of the cultures developing in liquid media. The theoretical basis and implications of the findings will be more fully considered in subsequent publications.

Materials and Methods

Cultures of Mycobacteria[1].—The following strains were used.

Human—H37 RV: Jamaica 22: cultures recently isolated from tuberculous sputa.

Bovine—Ravenel.

Avian—12 strains from different sources; many recently isolated from diseased birds.

Saprophytic—Several chromogenic and non-pigmented strains.

Stock cultures were maintained on coagulated egg yolk-potato extract slants (Wooley and Petrik, 1931). New material for inoculation of experimental media was obtained from approximately 2 week old slants by homogenizing the cell suspension with glass beads, allowing the larger particles to settle, and diluting the supernatant bacterial suspension to an opacity corresponding approximately to 0.1 mg. (dry weight) per ml. Cultures growing diffusely in experimental liquid media were used for inoculation in many cases.

Glassware.—Most experiments were carried out in pyrex test tubes, 25 mm. diameter, containing 5 to 7 ml. of medium. If tubes of a smaller diameter are used, the volume of fluid

[1] These strains were obtained from many different sources. We wish to thank in particular the following individuals who contributed pure cultures to our collection: Dr. J. Freund, Dr. F. R. Beaudette, Dr. W. H. Feldman, Dr. W. A. Hagan, Dr. W. McDermott, Dr. W. Steenken, Dr. D. Yegian.

must be reduced; the amount of growth developing in the culture medium decreases as the depth of the fluid layer increases probably on account of the exacting oxygen requirements of the tubercle bacillus (Cohn, 1944).

The presence of films of impurities (*e.g.* soaps, or other fatty materials and surface-active substances) which often contaminate bacteriological glassware is known to exert a deleterious effect on the growth of the tubercle bacillus: oily substances liberated from cotton plugs during sterilization by dry heat are particularly objectionable from this point of view (Drea, 1942; Youmans, 1944). Ideally, therefore, the glassware used for the growth of small inocula should be chemically clean and the use of cotton plugs should be avoided. Because of practical necessities, the following procedure has been followed in the experiments to be reported in this paper:—

The glassware was autoclaved, washed with hot solutions of green soap, and thoroughly rinsed in several changes of hot tap water. When cotton plugs were used, they were sterilized by autoclaving (15 pounds pressure for 20 minutes) and not by dry heat.

It is often advantageous and practical to replace cotton plugs by plastic, or aluminum caps (Drea, 1942). When this is done it is advisable to use loose fitting caps provided with ridges which permit free passage of air over the edge of the tubes and which prevent the establishment of semianaerobic conditions in the culture medium. No contamination has been observed to follow this practice.

Basal Medium.—The favorable effect of certain water-soluble lipids and of serum albumin on the growth of the tubercle bacillus (to be described later in this paper) can be readily demonstrated by adding these substances to any of the standard synthetic media (Dorset, Kirchner, Long, Sauton). We have found the following modification of Kirchner's medium to be easy to prepare, well buffered (0.025 M phosphate), stable, and favorable to the growth of small inocula.

Asparagine or hydrolysate of casein	1.0 gm.
$Na_2HPO_4 \cdot 12H_2O$	6.3 gm.
KH_2PO_4	1.0 gm.
Na_3 citrate $\cdot 2H_2O$	1.5 gm.
Dissolve, then add	
$MgSO_4 \cdot 7H_2O$	0.6 gm.
Distilled water to 1000.0 ml.	

The pH of the medium does not appear to be critical, and no inhibition of growth has been observed between pH 6.4 and 7.5.

As noted, asparagine can be replaced by acid or preferably enzymatic hydrolysates of casein which supply a less expensive and probably preferable source of nitrogen.[1] The addition of asparagine or glutamine, and of glucose or glycerol to the casein hydrolysate medium increases the total amount of growth but does not appear to facilitate its initiation.

Although only little information is available concerning the effect of water-soluble vitamins on the growth of the tubercle bacillus, it has been our practice to supply these materials in the form of yeast autolysate,[2] in a final concentration of approximately 0.02 per cent (added aseptically to the sterile medium). There is, however, no convincing evidence that this material adds to the efficiency of the medium.

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[1] Most experiments have been carried out with enzymatic hydrolysates of casein commercially available under the name of N-Z-Case and kindly supplied to us by the Sheffield Farms Co., 524 W. 57th St., New York. Acid hydrolysates of casein appear somewhat less satisfactory. The yeast autolysate used was also a commercial preparation available under the name of Vegex.

It should be emphasized that we have not attempted any systematic investigation of the composition of the basal medium. The only criterion used in the present experiments has been the ability of the medium to permit visible growth of very small inocula rather than the production of large yield of bacterial cells or of their products. On the basis of present information, it appears likely that the basal medium can be still further simplified while remaining adequate, or even becoming more favorable, for the initiation of growth of small inocula of tubercle bacilli.

The Effect of Water-Soluble Lipids on the Growth of Tubercle Bacilli

It has long been known that egg yolk constitutes one of the most favorable media for the growth of mycobacteria. Rapid and submerged growth of these organisms can be obtained in a medium prepared by diluting sterile egg yolk (unheated) in 10 volumes of distilled water (Besredka, 1921). During recent years evidence has been obtained that at least part of the growth-enhancing property of egg yolk resides in the phospholipid fraction which can be extracted from the original material with ether and precipitated with acetone (Boissevain and Schultz, 1938). Indeed, several reports in the literature suggest that various lipids can enhance the growth of the tubercle bacillus (Andrejew, 1943; Lominski, 1930).

As was mentioned in an earlier publication, we have confirmed and extended the findings of others concerning the growth-promoting properties of phospholipid fractions extracted from natural materials and have established that similar and even more striking results can be obtained by the use of synthetic water-soluble lipids (Dubos, 1945). Positive results have been obtained with the following phospholipid fractions obtained from natural sources: an ether soluble-acetone insoluble fraction prepared from egg yolk; an oil-free phosphatide fraction from soy bean; phosphatidyl serine and other cephalin fractions separated from brain tissue and from erythrocytes.[3]

There are important objections to the use of these phospholipid fractions. (*a*) Their preparation is laborious; (*b*) although they exert some stimulating effect on the submerged growth of bacilli of human origin (H37 RV and Jamaica 22) they have only slight effect on the growth of avian bacilli (4 strains tested); (*c*) the bacilli grown in the presence of these phosphatide fractions multiply in the form of large clumps which are very resistant to further dispersion; (*d*) the phosphatide fractions rapidly become growth-inhibitory on standing (perhaps as a result of autooxidation and formation of organic peroxides, or of hydrolysis to free fatty acids, or both).

Fortunately, certain synthetic water-soluble lipids have also been found to exert a favorable effect on the growth of tubercle bacilli (Dubos, 1945). These lipids consist of esters of long chain fatty acids which are used for certain indus-

[3] Phosphatidyl serine and brain and red cell phosphatides were generously supplied by Dr. Jordi Folch-Pi. The soy bean phosphatide was obtained through the courtesy of Dr. A. Scharf of Associated Concentrates, Inc., Atlanta, Georgia.

trial purposes because of their surface activity (detergency, wetting properties, etc.). Most of our experiments have been carried out with products commercially available under the following names.[4]

G-2124—A polyoxyethylene ester of lauric acid.
G-2144—" " " " oleic "
Tween 20—A polyoxyethylene derivative of sorbitan[5] monolaurate.
Tween 40—" " " " " monopalmitate.
Tween 60—" " " " " monostearate.
Tween 80—" " " " " monooleate.
A polyoxyethylene derivative of sorbitan monoricinoleate.

It will be noticed that all these compounds are characterized by the possession of both lipophilic properties (determined by the long aliphatic chain of lauric, palmitic, stearic, oleic, or ricinoleic acid) and hydrophilic properties (supplied by the multiple oxygen-containing groups of the polyhydric alcohols and of the ethylene oxide chains). We are informed by the manufacturers that each of these commercial products consists of a mixture of related compounds, rather than constituting a single chemical entity. This is due to two facts: (*a*) the fatty acids used in the manufacture are commercial products prepared from natural sources and are therefore contaminated with other related and unrelated substances, (*b*) due to the nature of the esterifying process, the quantitative relation between fatty acid, alcohol, and ethylene oxide residues in any given preparation is a statistical one and does not correspond to a single chemical entity. Because of these limitations, it is to be expected that the properties of the different synthetic preparations will vary from one sample to another, especially when measured in terms of a sensitive biological assay, such as the growth of the tubercle bacillus. Despite these experimental difficulties the results obtained with some sixty samples of water-soluble lipids can be summarized as follows:—

All samples of esters of lauric and palmitic acid (G-2124, Tween 20, Tween 40) have proved inhibitory to the growth of the tubercle bacillus when added to synthetic media in concentrations of 0.005 per cent or less.

Results with the esters of stearic acid (Tween 60) have varied greatly from sample to sample. Some preparations were found to be inhibitory in concentrations of 0.01 per cent or less. Others exerted a favorable effect when added to synthetic media in concentrations up to 0.2 to 0.5 per cent; very satisfactory results for example have been obtained with batch No. 1411 of Tween 60. No information is available to account for the variation between different batches.

All samples of esters of oleic acid (G-2144, Tween 80) have exerted a favorable effect on the growth of all strains of tubercle bacilli tested. The polyoxyethyl-

[4] These products are manufactured by the Atlas Powder Co., Wilmington, Delaware, which generously contributed materials and advice to this study.

[5] Sorbitan is an anhydride of the 6-carbon polyhydric alcohol sorbitol.

ene esters (G-2144) have a tendency to form precipitates when added to serum and other proteins, which limits their usefulness in the preparation of certain bacteriological media.

The two samples of esters of ricinoleic acid which were tested (G-6486T and G-6506F.J.) supported growth of the tubercle bacillus but did not appear to be as satisfactory as the oleic acid esters.

Of the different preparations tested, then, those marketed under the name of Tween 80 (polyoxyalkylene derivative of sorbitan monooleate) have been found to be the most satisfactory and the most reliable in their behavior. Tween 80 is a viscous yellow fluid which is soluble in all concentrations in water, and in the presence of the other constituents of the basal culture medium. Aqueous solutions become opalescent at high temperatures but again become perfectly clear upon cooling. Although aqueous solutions of Tween 80 are resistant to autoclaving there is definite evidence that they acquire growth inhibitory properties on standing for several weeks at room temperature: this deterioration appears to be catalyzed by certain biological materials, such as serum for example. Hydrolysis of the substance with release of free fatty acid, or oxidation and production of toxic peroxides, or both, may account for this unfavorable alteration: information concerning these possibilities will be presented in subsequent publications. In any case it appears advisable, until more is known of the factors affecting the stability of Tween 80, to use freshly prepared solutions of the material in making culture media and in particular not to store media containing both Tween and biological materials.

The effect of water-soluble esters on the growth of tubercle bacilli in synthetic media is illustrated in the following experiment.

The basal medium described earlier in this paper was distributed in 5 ml. amounts in pyrex tubes (25 mm. diameter). The esters were diluted in distilled water and 1 ml. of these dilutions added per tube of medium to give final concentrations of the esters ranging from 0.01 to 0.3 per cent. Four types of esters were used: the polyoxyethylene esters of lauric and of oleic acid (G-2124, G-2144) and polyoxyethylene derivatives of sorbitan monolaurate and monooleate. The media were autoclaved at 15 pounds pressure for 20 minutes. They were inoculated with 0.001 mg. of tubercle bacilli (either avian or human strains) obtained from 2 week old cultures on egg yolk slants and resuspended in 0.5 ml. of a solution containing 2 per cent glucose and 0.2 per cent yeast autolysate. The cultures were incubated for 10 days at 37°C. and occurrence of growth was determined by macroscopic and microscopic examination (Table I).

The results presented in Table I reveal that the two esters of lauric acid (Tween 20, G-2124) completely inhibited the growth of the human strain and also exhibited definite toxicity for the avian strain. On the contrary the two esters of oleic acid (Tween 80, G-2144) greatly enhanced the growth of the avian culture, the opacity of the culture increasing with increasing concentration of the esters in the medium (up to 0.1 per cent). In the case of the human strain, addition of the oleic acid esters to the control medium changed the character of the growth from one consisting of large compact granules to one in

which isolated cells and microscopic loose clumps prevailed; the bacilli constituting this more dispersed growth were typical in morphology and acid fastness. Since it is not possible to compare by turbidity the amount of growth present

TABLE I

Effect of Water-Soluble Esters on the Growth of Tubercle Bacilli in Synthetic Liquid Media

Ester added to basal medium		Final concentration	Growth (10 days) following inoculation with 0.001 mg. (dry weight)	
			Avian strain (Kirchberg)	Human strain (H37 RV)
		per cent		
Control		0	+ d	+ g
Polyoxyethylene ester of	Lauric acid (G-2124)	0.01	++ d	—
		0.03	++ d	—
		0.1	—	—
		0.3	—	—
	Oleic acid (G-2144)	0.01	++ d	+ d
		0.03	++++ d	+ d
		0.1	++++++ d	+ d
		0.3	++++++ d	+ d
Polyoxyethylene derivative of	Sorbitan monolaurate (Tween 20)	0.01	++ d	—
		0.03	—	—
		0.1	—	—
		0.3	—	—
	Sorbitan monooleate (Tween 80)	0.01	++ d	+ d
		0.03	++++ d	+ d
		0.1	++++++ d	+ d
		0.3	++++++ d	+ d

g, granular type of growth; microscopic examination reveals tight clumps of acid-fast bacilli.

d, diffuse type of growth; in the case of the avian culture microscopic examination revealed population of individual cells; in the case of human bacilli the population consisted of microscopic clumps of loosely packed cells which stained acid-fast.

Growth of avian bacilli—where it occurred—was diffuse. The amount of growth could be estimated from the density of the bacterial suspension (+, ++, ++++++).

The human bacilli grew in the form of large clumps in the control medium (no ester added); growth was much more dispersed in the presence of the oleic esters. Because of the difficulty of comparing the density of these two types of bacterial suspensions no estimation is given of the amount of growth produced by the cultures of the human strain.

in a granular type of growth with that in a more diffuse bacterial suspension, and for lack of other adequate quantitative data, no statement can be made at this time concerning the effect of the oleic esters on the amount of growth obtained in the cultures of human bacilli.

The Effect of Glucose and Glycerol on the Growth of Tubercle Bacilli in Synthetic Media

It has been observed that certain samples of glycerol (C.P. grade) contain impurities which are toxic for the tubercle bacillus. While this toxicity may not be apparent when large inocula are used, it becomes manifest when the medium is inoculated with decreasing numbers of organisms. Even the best sample of glycerol available (Kahlbaum twice redistilled) has been found to exert a certain inhibitory effect on the growth of small inocula when added in concentration of 5 per cent to the basal medium. The inhibitory effect of this high concentration, however, may be of a different nature and due for example to physical factors, since toxicity is also observed when glucose or mannitol in final concentration of 5 per cent is substituted for glycerol.

It has been established in recent experiments that, in the presence of Tween, glycerol exerts an inhibitory effect on the growth of small inocula of human tubercle bacilli which is not detectable in the absence of the oleic ester; the incompatibility between the two substances will be more fully described in a subsequent report.

Although glucose can be used as source of energy by tubercle bacilli, it gives rise to toxic products when autoclaved with the other components of the medium. Caramelization products which are formed under these conditions inhibit the growth of small inocula even when the final concentration of the sugar does not exceed 0.2 to 0.5 per cent. If the glucose is to be used, therefore, it should be added aseptically to the medium in the form of a solution sterilized separately by autoclaving or filtration.

The effect of glucose and of samples of glycerol of different purity on the growth of tubercle bacilli in synthetic liquid media containing 0.05 per cent of oleic acid ester is illustrated in the following experiment.

The basal medium described above, to which was added 0.05 per cent of the water-soluble sorbitan monoleate, Tween 80, was distributed in 5 ml. amounts per test tube (25 mm. diameter). To these was added 1 ml. of solutions of glucose or glycerol in distilled water, to give final concentrations of 5 per cent and 0.5 per cent of these substances. Three samples of glycerol were used: C.P., Reagent (Merck), and redistilled (Kahlbaum). The glucose and glycerol solutions were added to the medium before autoclaving: in another set of tubes the glucose was added aseptically in the form of a sterile (filtered) solution instead of being autoclaved with the medium. The tubes were inoculated with 0.01 or 0.0001 mg. (dry weight) of suspensions of human or avian bacilli resuspended in 0.5 ml. of a 0.2 per cent solution of yeast autolysate. The results of growth were read after 10 days' incubation at 37°C. (Table II).

The results presented in Table II reveal that, in a final concentration of 5 per cent, glycerol and glucose delayed or inhibited completely, the growth of small inocula of tubercle bacilli. C.P. glycerol is more toxic than Reagent glycerol

which in turn is slightly more toxic than glycerol purified by redistillation. The medium autoclaved with 5 per cent or 0.5 per cent glucose developed a brown color and supported growth very poorly. It is worthy of notice that during the early phases of growth the addition of glycerol or glucose does not appreciably increase the growth developing in the presence of the oleic ester. A beneficial effect of the alcohol and sugar is, however, apparent after more prolonged incubation.

TABLE II

Effect of Glycerol and Glucose on the Growth of Tubercle Bacilli in Liquid Media Containing 0.05 Per Cent of Water-Soluble Oleic Ester (Tween 80)

Material added to the medium	Final concentration	Growth (10 days) following inoculation with		
		Avian bacilli 0.0001 mg.	Human bacilli (H37 RV)	
			0.01 mg.	0.0001 mg.
	per cent			
Control	0	+++++	+++	++
Glycerol C.P.	5	—	—	—
" "	0.5	+++++	++	—
Glycerol Reagent	5	+++	+	—
" "	0.5	++++++	+++	++
Glycerol redistilled	5	+++	++	+
" "	0.5	++++++	+++	+++
Glucose (filtered)	5	+++	++	+
" "	0.5	++++++	++++++	++++
Glucose (autoclaved with medium)	5	+	—	—
" " " "	0.5	++++++	++	—

In the case of the avian culture microscopic examination revealed a population of individual cells; in the case of the human bacilli the population consisted of microscopic clumps of loosely packed cells which stained acid-fast.

The amount of growth could be estimated from the density of the bacterial suspension (+, ++++, ++++++).

The Effect of Serum Albumin on the Growth of Tubercle Bacilli

It is known that purified serum albumin facilitates the submerged growth of tubercle bacilli in synthetic liquid media (Boissevain, 1940; Powelson and McCarter, 1944). We have confirmed this finding with purified albumin prepared from human, bovine, and equine serum and found that these three preparations have essentially the same beneficial effect. Bovine albumin was therefore

selected for further experimentation since it is available as a commercial product.[6]

Bovine albumin can be obtained as a desiccated product (designated as serum fraction V) prepared from plasma by alcoholic precipitation, or as a more highly purified crystalline product. These two preparations are readily soluble in water. Their aqueous solutions are slightly acid (pH 5.6). They can be neutralized but in the amounts used they exert a negligible effect on the pH of a well buffered medium. The albumin solution cannot be sterilized by autoclaving since this treatment denatures the protein. Sterile solutions of 5 per cent albumin can be prepared by filtration through porcelain candles (Selas No. 2) or sintered glass filters (Corning U.F.). The sterile solution is added to the autoclaved medium in the desired amount with aseptic precautions.

Both crystalline serum albumin and fraction V have a remarkable enhancing effect on the growth of virulent tubercle bacilli. As will be shown in a subsequent publication the crystalline protein presents a certain advantage over fraction V since, upon prolonged incubation with the water-soluble ester Tween 80, the latter may give rise to small amounts of toxic substances which probably consist of free oleic acid liberated by enzymatic action. It is possible fortunately to minimize this unfavorable effect by heating the albumin solution (fraction V) for 30 minutes at 56°C., a treatment which appears sufficient to destroy most of the lipolytic activity of the preparation. The albumin can be made stable to this degree of heating by dissolving the protein in 2 per cent NaCl or neutralizing. Bovine albumin (fraction V) heated under these conditions appears satisfactory for the growth of small inocula of the tubercle bacillus. Its beneficial effect is illustrated in the following experiment.

To 5 ml. of the basal medium (in tubes 25 mm. in diameter), was added 0.2 ml. of 5 per cent bovine albumin, or 0.5 ml. of 0.5 per cent Tween 80 or both, the medium being made up to 5.7 cc. with distilled water in each case. The tubes were inoculated with 10^{-3}, 10^{-5}, or 10^{-7} mg. of tubercle bacilli (human or avian) obtained from a 2 week old culture on egg yolk slants and resuspended in 0.5 ml. of a solution containing 2 per cent glucose and 0.2 per cent yeast autolysate. Growth was recorded after 3, 8, and 15 days of incubation at 37°C. (Table III).

The results presented in Table III reveal that albumin alone facilitates the initiation of growth of small inocula (10^{-7} mg.) of tubercle bacilli in synthetic liquid media, but does not increase very markedly the total amount of growth produced (particularly in the case of avian bacilli). The water-soluble sorbitan

[6] The preliminary experiments with human and bovine albumin were carried out with preparations obtained through the courtesy of Dr. E. J. Cohn. The products of plasma fractionation employed in this work were developed by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The bovine albumin used in subsequent experiments was obtained from Armour Laboratories, Chicago, Illinois.

monooleate (Tween 80) alone does not permit growth of the smallest inocula of human bacilli. When albumin and water-soluble ester are both present in the medium, the smallest inocula (10^{-7} mg.) of avian and human bacilli give detectable growth within 8 to 11 days and fairly abundant and dispersed growth

TABLE III

Effect of Bovine Serum Albumin (Fraction V) and of Water-Soluble Ester of Oleic Acid (Tween 80) on the Growth of Tubercle Bacilli in Synthetic Liquid Medium

Basal medium		Inoculum	Growth after different periods of incubation					
Albumin	Tween 80		3 days	8 days	15 days	3 days	8 days	15 days
			Avian bacilli (Kirchberg)			Human bacilli (H37 RV)		
per cent	*per cent*	*mg.*						
0	0	10^{-3}	+ d	+ d	+ d	—	+ g	+ g
0	0	10^{-5}	—	+ d	+ d	—	—	—
0	0	10^{-7}	—	—	—	—	—	—
0	0.05	10^{-3}	+++ d	+++++ d	+++++++ d	—	+ d	+ d
0	0.05	10^{-5}	—	+++ d	+++++++ d	—	+ g	+ d
0	0.05	10^{-7}	—	—	+ d	—	—	—
0.2	0	10^{-3}	—	++ d	++ d	—	+ g	+ g
0.2	0	10^{-5}	—	+ d	++ d	—	+ g	+ g
0.2	0	10^{-7}	—	—	+ d	—	—	+ g
0.2	0.05	10^{-3}	+++ d	+++++ d	+++++++ d	+ g	+ d	+ d
0.2	0.05	10^{-5}	—	++++ d	+++++++ d	—	+ d	+ d
0.2	0.05	10^{-7}	—	++ d	+++++++ d	—	—	+ d

g, granular type of growth; microscopic examination reveals tight clumps of acid-fast bacilli.

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Growth of avian bacilli—where it occurred—was diffuse. The amount of growth could be estimated from the density of the bacterial suspension (+, ++++, +++++++).

The human bacilli grew in the form of large clumps in the control medium (no ester added); growth was much more dispersed in the presence of the oleic esters. Because of the difficulty of comparing the density of these two types of bacterial suspensions no estimation is given of the amount of growth produced by the cultures of the human strain.

within 2 weeks. The cultures of the human strain H37 RV growing in media containing the water-soluble ester consist at first of small loose clumps of acid-fast bacilli and become increasingly diffuse in appearance with further incubation. The oleic ester increases markedly the amount of growth produced by the avian strain, giving rise from the beginning to a culture essentially diffuse in character.

The Viability of Cultures Growing in Oleic Ester–Albumin Medium

Cultures of mycobacteria growing diffusely in media containing 0.05 per cent Tween 80, with or without serum albumin, have been carried through many transfers in these media without losing their viability and without any obvious modifications of their growth characteristics. The viability of the cultures is illustrated in the following experiment.

The human strain H37 RV and the avian strain Kirchberg were inoculated into media (7 ml. per tube 25 mm. diameter) containing 0.05 per cent Tween 80 and 0.2 per cent serum albumin; the inoculum consisted of 0.001 ml. of these cultures (2 weeks old) in the same medium. After 2 weeks' incubation at 37°C. the two cultures were found to consist of a heavy growth, corresponding to approximately 0.2 mg. dry weight of bacilli per ml. of culture (as determined by centrifugation and desiccation with acetone-ether). The two cultures were again inoculated into new media containing 0.05 per cent Tween 80—with or without 0.2 per cent albumin (heated at 56°)—using inocula ranging from 10^{-1} to 10^{-8} ml. of culture in 0.5 ml. of a diluent containing 2 per cent glucose and 0.2 per cent yeast autolysate. Readings of growth made after 2 weeks' incubation at 37° revealed the following results.

Visible growth could be detected in all the tubes containing albumin, even with the smallest inoculum (10^{-8} ml.) of either the avian or the human strain. In the absence of albumin, growth developed with inocula of 10^{-1} to 10^{-7} ml. in the case of the avian strain and 10^{-1} to 10^{-5} ml. in the case of the human strain.

Cultures transferred to media not containing the oleic ester exhibit a characteristic behavior. The avian bacilli grow very scantily, whether albumin is present or absent; where growth occurs, however, it is essentially diffuse in character as revealed by microscopic examination. The human culture retains at first a fairly dispersed mode of growth; soon, however, a film forms at the surface of the medium, the growth "creeps up" the side of the glass wall, and all subsequent growth develops in the form of clumps of varying sizes. Although serum albumin facilitates the initiation of growth of these clumps it does not modify the granular character of the growth when they are transferred to new synthetic liquid media free of the water-soluble oleic ester.

DISCUSSION

The water-soluble esters of oleic acid used in the foregoing experiments appear to exert a multiple effect on the growth of the tubercle bacillus. They cause more rapid and abundant growth of the avian strains, and this enhancing effect is directly related to the concentration of the ester added to synthetic media. Experiments to be published elsewhere establish that the bacilli can also utilize other esters of oleic acid, as well as free oleic and stearic acid. The effect of the oleic esters on the growth of human strains is not so clearly defined although it can be stated that these substances increase the regularity and heaviness of growth in synthetic media.

The water-soluble esters cause mycobacteria to grow diffusely throughout the liquid medium rather than as a surface pellicle. That this result is due to the wetting properties of the Tween is suggested by a number of facts. Growth

developing in media containing non-wetting oleic esters (methyl oleate for example) or Na oleate, consists of large clumps which are extremely resistant to dispersion. Furthermore, when very small amounts of the water-soluble esters are added to the medium (0.01 per cent Tween 80 for example), growth is at first submerged; after a few days' incubation, however, a fine pellicle forms at the surface of the medium and further growth "creeps up" along the side of the glass wall, probably as a result of the exhaustion of the Tween by hydrolysis or utilization by the organism. Further addition of Tween to the medium again causes wetting of the cells and their dispersion throughout the medium.

Long chain fatty acids are extremely toxic to the tubercle bacillus; as little as 0.1 to 1 γ of oleic acid per ml. of synthetic medium is sufficient to cause retardation or inhibition of growth. This toxic effect is neutralized by the addition of serum albumin to the system. Tween 80 also exhibits an inhibitory effect which can be detected at higher concentrations and which can also be neutralized by serum albumin.[7] In fact, albumin protects the tubercle bacilli—and other cells as well—from the toxic action not only of long chain fatty acids but also of many other substances, heavy metals, phenolic compounds, chlorine compounds, anionic and cationic detergents, etc. It appears possible therefore, that the beneficial effect of albumin on the growth of the tubercle bacillus—and of other microbial cells—may be due not to supplying some growth-promoting nutrient, but rather to its ability to protect the organism against an inimical environment. It is interesting in this respect that many other proteins (gelatin, gliadin, edestin, lactoglobulin, egg albumin, casein, serum globulin (fractions II, III, IV), pressed yeast juice, etc.), fail both to protect the tubercle bacillus against the toxic effect of fatty acids and to enhance the growth of small inocula in synthetic media.

Although the experiments reported in the present paper deal only with one human (H37 RV) and one avian (Kirchberg) strain, similar results have been obtained with two other human, one bovine, twelve avian, and six saprophytic strains of mycobacteria. It is worth emphasizing again that all avian strains tested are much more resistant than the human and bovine strains to the toxic effect of oleic acid and of high concentrations of Tween 80 and much less dependent upon the protective effect of albumin. The amount of growth produced by avian bacilli is much increased by the addition of Tween or oleic acid to synthetic media. It must be kept in mind, however, that whereas the human strains used were in the "rough" dissociation phase, the avian strains were all in the smooth phase. It is possible, therefore, that the cultural differences observed do not differentiate human and avian strains but correspond in reality to differences in the dissociation phase of the cultures used.

Tubercle bacilli growing rapidly and diffusely in media containing water-

[7] The slight toxic action of Tween 80 is due to the small amounts of free fatty acids which contaminate the commercial preparations; essentially non-toxic preparations can be obtained by a method of purification to be described elsewhere.

soluble esters of oleic acid exhibit characteristic acid-fast staining and are essentially free of beads and granules in the young cultures. Moreover, these bacilli when transferred back to the classical synthetic or egg yolk media again give rise to the typical mode of growth, showing no indication that their fundamental biological properties have been altered by cultivation in the presence of the water-soluble lipids.

Typical diffuse cultures of acid-fast bacilli have been obtained by inoculating positive human sputa (after treatment with sodium hydroxide), or tissues of experimental animals infected with human and avian bacilli, directly into the Tween-albumin medium. It appears, therefore, that the medium can support the growth not only of laboratory-adapted strains, but also of cultures directly obtained from pathological material. There is, however, no indication that in its present form, the medium can be utilized with advantage for the large scale cultivation of tubercle bacilli or for diagnostic work. It is likely, for example, that the rate of diffusion of oxygen throughout the medium will become one of the limiting factors when the rapid production of very dense bacterial suspensions is desired, thus rendering necessary some form of forced aeration of the culture. On the other hand, the extraordinary enhancing effect of oleic esters on the growth of avian tubercle bacilli suggests that the peculiar and slow mode of growth of pathogenic mycobacteria is not due only to intrinsic structural and metabolic characters of the organisms, but may depend in part upon gross deficiencies in the composition of the media used for their cultivation. It is probable therefore that the medium described in the present paper is still deficient in a number of unidentified substances which are essential for optimal growth of pathogenic mycobacteria.

SUMMARY

1. Certain water-soluble esters of long chain fatty acids (in particular of oleic acid) favor submerged and diffuse growth of mycobacteria throughout the depth of synthetic liquid media.

2. Esters of oleic acid increase considerably the amount of growth yielded by avian strains in synthetic media.

3. The addition of serum albumin to synthetic liquid media permits visible growth of minimal inocula of virulent human tubercle bacilli (10^{-8} mg.) within 11 to 15 days.

4. Cultures growing diffusely in media containing the water-soluble esters—with or without albumin—consist of cells of classical morphology and staining properties, which again exhibit the usual mode of growth when returned to the standard synthetic or egg yolk media.

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