THE EFFECT OF WETTING AGENTS ON THE GROWTH OF TUBERCLE BACILLI

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(Received for publication, April 1, 1948)

It is possible to obtain finely dispersed growth of tubercle bacilli by adding to the media in which they are cultivated certain non-ionic wetting agents. The polyoxyethylene esters of oleic acid (Atlas G-2144), lauric acid (Atlas G-2124), sorbitan monooleate (Tween 80), sorbitan monostearate (Tween 60), sorbitan monopalmitate (Tween 40), and sorbitan monolaurate (Tween 20), have proven especially effective in this respect. In addition to their wetting effect on the cell, certain of the water-dispersible esters just mentioned are capable of enhancing the growth of many strains of tubercle bacilli, probably by supplying them with long chain fatty acids in a non-toxic form available for metabolic utilization (3). Unfortunately, the ester linkage in these wetting agents is susceptible to enzymatic hydrolysis by lipases, a fact which prohibits their use in media containing animal tissues or fluids rich in these enzymes. It is therefore desirable to find other types of wetting agents capable of promoting dispersed growth of tubercle bacilli and stable in the presence of animal tissues. The present report describes the properties of an arylalkyl polyether of phenol (Triton A20) which in some respects fulfills these requirements.

EXPERIMENTAL

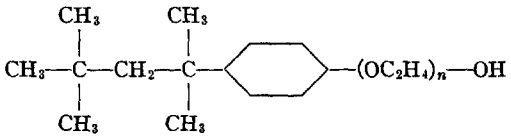
Cultures.—For the sake of brevity only experiments carried out with the human strain of tubercle bacillus H37 and with the avian strain Camden 3 will be described. Results with other strains have confirmed the findings to be presented here. Two variants of the culture H37 were used; both had been obtained originally from the Trudeau Laboratory through the generosity of Dr. W. Steenken, Jr. The virulent variant, H37Rv, was reisolated from the lung lesions of experimentally infected mice. The avirulent variant, H37Ra, was reisolated from a colony exhibiting characteristic morphology on oleic acid-albumin agar. The cultures were maintained by weekly transfers in Tween 80-albumin liquid medium and possessed the morphological characteristics of the virulent and avirulent forms, respectively (7). The avian strain Camden 3 was recently isolated in our laboratory from the liver of a tuberculous chicken and was also maintained in Tween-albumin agar. The cultures used for inoculation had been incubated for 8 to 10 days at 37°C. and contained approximately 0.35 mg. bacilli (dry weight) per cc. of medium.

Media.—The basal medium has been described in preceding reports (6); bovine serum albumin was added aseptically (in a final concentration of 0.5 per cent) after autoclaving of the medium, with or without 1.5 per cent agar. The wetting agents were added prior to autoclaving.

The sphingomyelin used was sample 1127 of the collection of the late Dr. P. A. Levene (4). It was added to the medium in the form of a 1 per cent solution in distilled water, sterilized by three consecutive heatings at 80°C.

The nature and properties of the polyethylene esters of the Tween type have been described in earlier publications (3, 5).

Triton A20 is described by the manufacturer as an arylalkyl polyether of phenol. The related compound, Triton N100, has the following general formula.



The chemical structure of Triton A20 is slightly different from that of Triton N100 and has not been published. Although both these products are related non-ionic wetting agents, Triton A20 is much less toxic than Triton N100 for tubercle bacilli and for experimental animals. For this reason, it has been selected for further study.

Triton A20 is miscible in all proportions with water and can be handled like Tween 80 in the preparation of liquid and agar media; as in the case of Tween 80, its aqueous solutions are cloudy above 80–90°C. but become limpid again at lower temperatures. Triton A20 is distributed by the manufacturer as a 20 per cent solution in water. The concentrations reported in the present publication are corrected in terms of the original material.

The media were distributed in 4 cc. amounts in Pyrex test tubes, 25 mm. in diameter. As described elsewhere, aluminum caps were used instead of cotton plugs during autoclaving and incubation (5).

Comparative Effects of Triton A20 and Tween 80 on the Growth of Tubercle Bacilli.—All cultures of tubercle bacilli so far tested are capable of giving macroscopical evidence of growth within 10 to 15 days in the basal medium to which has been added bovine serum albumin, even when the inoculum contains only a few living cells; growth in this plain albumin medium always consists of large clumps of bacilli. Tween 80 in concentration of 0.02 to 0.2 per cent increases the amount of growth and changes its character from granular to diffuse. The effect of Triton A20 was studied in the following experiment.

Tween 80 and Triton A20 were added to the basal medium in final concentrations ranging from 0.003 to 0.3 per cent as indicated in Table I. Each test medium was inoculated with 0.003 or 0.0003 cc. of a 10 day old culture in Tween albumin medium of H37Rv or H37Ra diluted in 0.3 cc. of 5 per cent bovine serum albumin. This corresponded to an inoculum of approximately 3×10^{-4} and 3×10^{-5} mg. of bacilli (dry weight) per cc. of test medium. Macroscopical evidence of growth, checked in certain cases by microscopic examination, was read after 14 days' incubation at 37°C., and is recorded in Table I.

The results presented in Table I reveal that a definite dispersing effect on the growth of virulent and avirulent variants H37Rv and H37Ra can be detected with concentrations of Tween 80 or of Triton A20 as low as 0.01 per cent. However, the dispersing effect of Tween 80 is more complete than that of the latter wetting agent. Even with the highest concentrations of Triton A20 used, the cultures of H37Rv exhibited on careful macroscopic examination a finely granular growth which was shown by microscopic study to consist of long strands of bacilli.

The two wetting agents differ also in their effect on the yield of bacilli. Addition of Tween 80 to the medium brings about a definite enhancement of growth, probably caused by the oleic acid in the Tween molecule.[1] Increase in growth does not occur with Triton A20 which apparently is not utilized by tubercle bacilli.

Finally it is obvious that whereas Tween 80 enhances to a similar degree the growth of both the H37Rv and H37Ra variants, Triton A20 behaves very differently toward the two cultures as it inhibits the growth of the latter but

TABLE I

The Effect of Tween 80 and Triton A20 on the Growth of Tubercle Bacilli

Wetting agent added to the medium	Final concentration	Inoculum (mg. dry bacilli per cc. of medium) 3×10^{-4}		3×10^{-5}	
		H37Rv	H37Ra	H37Rv	H37Ra
	per cent				
Tween 80	0.03	8*f.d.‡	8 f.d.	6 f.d.	6 f.d.
" "	0.01	8 f.d.	8 f.d.	6 f.d.	6 f.d.
" "	0.003	6 g.	6 g.	5 g.	4 g.
Triton A20	0.3	4 d.	0	3 d.	0
" "	0.1	5 d.	2 d	4 d.	0
" "	0.03	5 d.	5 f.d.	4 d.	2 d
" "	0.01	5 d.	5 f.d.	4 d.	3 d
" "	0.003	5 g.	5 g.	3 g.	3 g.
H_2O		5 g.	5 g.	3 g	3 g.

* The amount of growth is recorded in terms of an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

‡ f.d. indicates that growth was finely dispersed, exhibiting no large clumps on microscopic examination. d. indicates that the growth was dispersed but consisted of clumps readily seen with a hand lens. g. indicates granular growth consisting of large clumps or flakes.

not that of the former. It should be emphasized at this time that the toxic effect on H37Ra reported in Table I has also been observed with a number of other avirulent variants of mammalian strains of tubercle bacilli whereas none of the variants appears to be unfavorably affected by Triton A20 in concentrations of 0.05 per cent or less.

[1] It has been shown elsewhere that under certain conditions, Tween 80 can exhibit toxicity owing to its contamination with free fatty acid; the toxicity can be overcome by addition of adequate amount of serum albumin to the medium (1–3). Furthermore, we have repeatedly observed that samples of Tween which had proved entirely non-toxic when first used, develop some 2 months after the container has been opened a type of toxicity which seems to be independent of the presence of fatty acids. It is essential therefore to use only selected samples of Tween if conclusions are to be drawn concerning the effect of polyoxyethylene derivatives of sorbitan monooleate on the growth of tubercle bacilli.

The Growth-Dispersing Effect of Tween 80 and Triton A20 in Serum Media.— It has been shown in preceding publications that incubation of Tween 80 with fraction V of plasma (serum albumin) results in the liberation of free fatty acid as a result of enzymatic hydrolysis of the ester by the lipase contaminating the albumin preparation (1, 2). In experiments previously reported, lipase activity had been minimized by heating the albumin for 30 minutes at 56°C. prior to addition to the medium. On the other hand, Tween 80 is rapidly hydrolyzed in the presence of tissues or tissue fluids and there is no convenient technique for abolishing the marked lipolytic activity of these materials. As there is no

TABLE II

The Influence of Blood Serum on the Growth-Dispersing Effect of Wetting Agents

Wetting agent added to the medium		Serum		Growth 14 days after inoculation with H37Rv (3×10^{-4} mg. dry weight per cc. medium)
	Final concentration	Mouse	Ox	
	per cent	*cc.*	*cc.*	
Tween 80	0.05	0	0	8*f.d.*
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
G 2144	"	0	0	8 f.d.
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
Triton A20	"	0	0	5 d.
" "	"	0.5	0	8 d.
" "	"	0	0.5	8 d.
H_2O		0	0	5 g.
"		0.5	0	8 g.
"		0	0.5	8 g.

* Symbols as in Table I.

enzyme of animal tissues known to be capable of attacking the ether linkages of the Triton A20 molecule, it was of interest to compare Triton A20 with two water-dispersible esters of oleic acid (G 2144 and Tween 80) with respect to their ability to disperse growth of tubercle bacilli in media with or without added serum.

The wetting agents were added to the basal medium in a final concentration of 0.05 per cent. Bovine or mouse serum sterilized by filtration through porcelain candles was added in amount of 0.3 cc. per 3 cc. of medium. The tubes containing the test media were inoculated with 0.003 cc. of 10 day old culture of H37Rv diluted in 0.3 cc. of 5 per cent bovine albumin. It is known from earlier studies that this amount of albumin is sufficient to overcome the toxicity of the free fatty acid which might be released by enzymatic lipolysis of Tween 80 or G 2144. Macroscopic evidence of growth confirmed by microscopic examination was read after 14 days' incubation at 37°C. (Table II).

The results presented in Table II confirm the fact that the dispersing effect of Triton A20 on the growth of tubercle bacilli in albumin medium without serum is less complete than that of the water-dispersible esters Tween 80 or G 2144. On the other hand it is seen that in the presence of whole serum these esters completely lose their dispersing activity whereas that of Triton A20 remains unimpaired. In view of the complexity of serum and of the many unknown interactions which certainly occur between its constituents, the tubercle bacilli, and the wetting agents, it is not possible to obtain convincing evidence concerning the factors which determine the dispersed or granular

TABLE III

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli

Added to the basal medium (final concentration)					Growth 14 days after inoculation with (mg. cc/cc. medium)						
Tween 80	Triton A20	Oleic acid	Sphingomyelin	Albumin	H37Rv			H37Ra			Avian
					3×10^{-4}	3×10^{-6}	3×10^{-8}	3×10^{-4}	3×10^{-6}	3×10^{-8}	3×10^{-6}
per cent	*per cent*	*per cent*	*per cent*	*per cent*							
0	0	0	0	0	3 g.*	1 g.	0	2 g.	1 g.	0	1 f.d.
0	0	0	0	0.5	6 g.	4 g.	2 g.	7 g.	5 g.	1 g.	1 f.d.
0.05	0	0	0	0	3 f.d.	0	0	3 f.d.	0	0	5 f.d.
"	0	0	0	0.5	8 f.d.	5 d.	3 f.d.	7 f.d.	4 f.d.	1 d.	8 f.d.
0	0.05	0	0	0	3 d.	2 d.	0	1 d.	0	0	1 f.d.
0	"	0.02	0	0	0	0	0	0	0	0	0
0	"	0	0	0.5	5 d.	4 d.	3 d.	3 d.	1 d.	0	1 f.d.
0	"	0.02	0	"	8 d.	6 d.	3 d.	2 d.	0	0	6 f.d.
0	"	0	0.05	0	8 d.	5 d.	2 g.	6 d.	2 g.	1 g.	5 f.d.
0	"	0	"	0.5	8 d.	6 d.	4 d.	7 d.	5 d.	4 d.	8 f.d.
0	0	0	"	0	4 g.	3 g.	2 g.	4 g.	3 g.	2 g.	5 f.d.
0	0	0	"	0.5	7 g.	4 g.	3 g.	7 g.	4 g.	3 g.	7 f.d.

* Symbols as in Table I.

character of the bacterial growth. Nevertheless, the facts observed are consistent with the hypothesis that the water-dispersible esters Tween 80 and G 2144 are destroyed by the serum lipase and thereby lose their wetting properties, whereas Triton A20 remains unaffected under the same conditions.

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli.—Although Triton A20 can disperse the growth of tubercle bacilli it never increases significantly the yield of growth. However enhancement of growth in its presence can occur when long chain fatty acids, or sphingomyelin, are added to the medium.

Tween 80, Triton A20, oleic acid, sphingomyelin, and albumin were added to the basal medium as indicated in Table III. The media were inoculated with 0.003, 0.0003, or 0.0000003 cc. of a 10 day old culture diluted in 0.3 cc. distilled water; these inocula corre-

sponded to approximately 3×10^{-4}, 3×10^{-6}, 3×10^{-8} mg. bacilli (dry weight) per cc. of medium. The amount and character of the bacterial growth were recorded after 14 days' incubation at 37°C.

The results presented in Table III illustrate again the striking differences between the wetting agents Tween 80 and Triton A20 with reference to their effects on the growth of tubercle bacilli. They confirm the finding of the preceding experiment that, in contrast with Tween 80, Triton A20 added to the basal medium with or without albumin does not increase significantly the amount of growth, although it has a definite dispersing effect on it.

Failure to enhance growth is particularly well demonstrated in the case of the avian strain Camden 3, a fact in agreement with earlier observations that all avian strains tested give only very limited growth in the absence of long chain fatty acids (3). However, addition of oleic acid to the albumin medium containing A20 definitely enhances growth which remains dispersed despite the presence of the fatty acid. The growth-promoting effect of sphingomyelin first demonstrated in the preceding communication (4) is here confirmed. It is shown moreover that the beneficial effect of sphingomyelin, rendered even more evident in the presence of Triton A20 which increases the solubility of this phospholipid, is expressed not only in terms of greater density of the culture at the end of the incubation period, but also by the fact that it allows the growth of minute inocula even in the absence of serum albumin (3).

Comparison of the results obtained with the virulent (H37Rv) and avirulent (H37Ra) variants illustrates again the selective inhibitory effect of Triton A20 on the growth of the latter culture. It is clear however, that the inhibitory effect of the wetting agent can be corrected by the addition of sphingomyelin to the medium, even in protein-free media.

Comparative Effects of Tween 80 and Triton A20 on the Morphological Characteristics of Cultures H37Rv and H37Ra.—Unlike Tween 80, Triton A20 can inhibit the growth of the avirulent variants of mammalian tubercle bacilli in concentrations which exert no detectable toxic effects on the virulent forms (Tables I and III). This difference in antibacterial activity between the two types of wetting agents is paralleled by differences in their effect on the morphological aspects of the cultures growing in their presence. Only a brief statement of these findings will be presented here as the relation of morphological characteristics to virulence is to be treated more extensively in a subsequent communication.

It will be recalled that, in the absence of a wetting agent, the virulent organisms exhibit a marked tendency to adhere to one another in the direction of their long axis; this tendency results in the formation of strands of bacilli, which can be very long and at times extend over several microscopic fields, and which are several cells in thickness. In contrast to this serpentine pattern of growth, the avirulent forms exhibit either random growth or perhaps a rosette

arrangement of the cells (7). Attenuated strains, which possess, like the BCG culture in use in our laboratory, a slight degree of "invasiveness" also exhibit intermediate morphological characteristics. On the other hand, in media containing 0.1 per cent Tween 80, cultures of the virulent organisms and of the attenuated forms grow without producing the cords characteristic of growth in the plain albumin medium, and cannot be differentiated from the totally avirulent variants. A different morphological picture is obtained in media containing the same concentration of Triton A20 (0.1 per cent). Although growth of the virulent variants in Triton A20 liquid media appears fairly disperse, and their colonies in Triton A20-albumin agar are much less rugose than those developing on plain albumin agar, microscopic examination reveals that these cultures exhibit a definite serpentine pattern of growth (cord formation). It would thus appear that Triton A20 prevents the formation of the amorphous large clumps which correspond to the granular mode of growth of tubercle bacilli in general, but does not interfere with the tendency of the virulent cells to orient themselves in the typical "cords." These observations suggest that two independent factors contribute to the morphology of cultures of tubercle bacilli: one, common to both virulent and avirulent forms, is overcome by both Tween 80 and Triton A20, the other, peculiar to the virulent variants, is affected only by Tween 80. It is also possible, however, that these differences are not of a qualitative nature but may be due to the possession by the virulent variants of larger amounts of a certain hydrophobic substance, much less abundant in the avirulent forms, and which is more readily wetted by the water-dispersible esters than by Triton A20.

Whatever the ultimate significance of these findings for the understanding of the nature of the differences in cellular structure between the virulent and avirulent variants, it appears that the wetting agents Tween 80 and Triton A20 may lend themselves to the development of selective media useful for the separation of the two types of variants and for the analysis of bacterial variation and its relation to virulence.

SUMMARY

Tween 80 and Triton A20 are two water-dispersible, non-ionic, surface-active agents which favor dispersed growth of tubercle bacilli in aqueous media probably by wetting the bacterial surface.

Tween 80 is a polyoxyethylene ester of sorbitan monooleate and is liable to enzymatic hydrolysis by lipases. Triton A20 in an arylalkyl polyether of phenol which appears resistant to the known enzymes of animal tissues.

Tween 80 loses its ability to disperse cultures of tubercle bacilli in media containing serum; Triton A20 does not.

Tween 80 increases the yield of growth, probably by supplying oleic acid to the bacilli; Triton A20 does not.

In concentrations sufficient to cause dispersed browth, Tween 80 (purified by removal of unesterified fatty acid) and Triton A20 are completely innocuous for virulent tubercle bacilli. However, Triton A20 exhibits a marked toxic effect on the avirulent variants of mammalian strains; Tween 80 does not.

The two wetting agents also differ in their effects on the morphological aspects of the bacterial cultures. Whereas Triton A20 prevents the formation of large amorphous bacillary clumps, it is less effective in preventing the orientation of the virulent bacilli resulting in the formation of long bacillary strands. Tween 80 on the contrary prevents also the formation of these cords of bacilli and exerts therefore a more effective dispersing effect on cultures of virulent tubercle bacilli.

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