THE INHIBITORY EFFECT OF LIPASE ON BACTERIAL GROWTH IN MEDIA CONTAINING FATTY ACID ESTERS

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Received for publication September 2, 1947

It has been reported from this laboratory that serum albumin is essential for the initiation of growth by small inocula of tubercle bacilli in a liquid medium containing "tween 80"[2] which promotes submerged growth of these organisms (Dubos, 1945; Dubos and Davis, 1946). The albumin acts as a protective rather than a nutritive growth factor, binding traces of unesterified fatty acid which inhibit growth of small inocula (Davis and Dubos, 1947).

The commercial bovine serum albumin, however, also makes the medium somewhat unstable, which is particularly important because tubercle bacilli grow so slowly. Inocula containing only small numbers of bacilli (ca. 10 to 20) require a period of cultivation of 2 weeks to attain visible growth, yet after sterile incubation of the tween-albumin medium at 37 C for 2 weeks, it was unable to support growth of even large inocula. It appeared possible that in the race between bacterial multiplication and spontaneous deterioration of the medium even smaller inocula might be prevented from surviving. This prediction could be confirmed after the deterioration was traced to hydrolysis of tween 80 by lipase in the commercial albumin, which released free fatty acid in bacteriostatic quantities exceeding the binding capacity of the albumin. Elimination of the lipase permitted cultivation of even smaller inocula, such as 2 or 3 bacteria.

Lipolysis was also found to account for the bacteriostatic effect of several materials of biological origin (sera, bacterial culture filtrates), observed only in media containing tween 80. This effect calls attention to precautions necessary in using media containing tween.

The results of the experiments involving lipase, which have received a preliminary report (Davis and Dubos, 1946), are presented in this paper. Although the bacteriological work was confined to human tubercle bacilli, the principles evolved are applicable to the cultivation, in the presence of esters, of any bacteria that are sensitive to free fatty acid.

EXPERIMENTAL METHODS AND RESULTS

The methods of cultivation have been reported elsewhere (Dubos and Davis, 1946). The semisynthetic liquid medium contained a salt mixture buffered at pH 7.0, enzymatic casein hydrolyzate, yeast autolyzate, glucose, 0.05 per cent tween 80, and 0.1 per cent bovine serum albumin (fraction V, Armour), unless

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[2] Trade name of a standardized commercial polyoxyethylene derivative of sorbitan monooleate.

otherwise specified. It was distributed in amounts of 5 ml in wide (25-mm) metal-capped test tubes. The inoculum was a standard laboratory strain of virulent human tubercle bacilli (H37Rv) that had been grown for many passages in the tween-albumin medium. The inocula are designated in the tables in terms of volume of a 7- to 10-day-old culture in the same medium, containing approximately 1 mg moist weight of organisms per ml; in one experiment (table 6) accurately measured inocula were used. Growth is recorded in the tables in terms of a visual estimate ranging from 0 (no visible growth) to 4 (full growth, approximately 2 mg moist weight per ml).

In experiments involving prolonged storage of media at 37 C, evaporation was prevented by parafilm covering the cotton plugs.

Chemical estimations of lipase activity were based on a method of extracting and titrating fatty acid that was developed for this purpose (Davis, 1947*a*). A somewhat similar method of measuring lipase activity was developed simultaneously by Archibald (1946).

Deterioration of Tween-Albumin Medium

It was repeatedly observed that sterile incubation for 14 to 21 days at 37 C destroyed the capacity of the medium to support the growth of even large inocula of tubercle bacilli. Since the minimal growth requirements of tubercle bacilli are very simple, the deterioration of the medium during the incubation seemed likely to have been caused by formation of a bacteriostatic product rather than by destruction of a growth factor. The medium could be stored for much longer periods in the refrigerator without deterioration.

To detect the unstable component a group of partial media were prepared, each lacking in one of the major components. These were stored under sterile conditions for 21 days at 37 C. Portions of sterile concentrated solutions of each of the components were simultaneously stored in the refrigerator. At the end of the period of preliminary incubation the absent component was aseptically added to each incomplete batch of medium, so that the final composition of the various batches was identical except for changes that had taken place during storage. These media were then distributed in 5-ml volumes, inocula of various sizes were added, and the tubes were further incubated.

The results, which are presented in table 1, show that two components of the medium were involved rather than one; deterioration took place only if serum albumin and tween 80 were incubated simultaneously. Since other experiments had shown that tween 80 contains somewhat bacteriostatic concentrations of free oleic acid (Davis, 1947*a*) and, further, hydrolyzes slowly in aqueous solution (Davis, 1947*b*), and since serum is known to contain lipase, it appeared very likely that the commercial albumin was contaminated by traces of lipase that accelerated the hydrolysis of the tween, so that after a sufficient period of incubation the binding capacity of the albumin for fatty acid was exceeded.

This interpretation was strengthened by the observations (table 2) that further addition of albumin, at the end of the preliminary incubation, overcame the toxicity of deteriorated tween-albumin medium and that commercial crystalline

TABLE 1

Deterioration of incomplete media during storage

NATURE OF MEDIUM DURING STORAGE	TEMPERATURE OF STORAGE (C)	INOCULUM (MG MOIST WEIGHT)				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
		Growth*				
Complete	37	4	½	0	0	0
Glucose absent	37	4	1	0	0	0
Casein hydrolyzate absent	37	4	0	0	0	0
Mineral mixture absent	37	4	1	0	0	0
Albumin absent	37	4	3½	2½	½	±
Tween 80 absent	37	4	3	1½	½	¼
Complete	5	4	3½	2½	1½	¼
Complete, freshly prepared		4	3	2½	1	½

Incomplete media were stored for 21 days at 37 C. The deficiencies were then restored and the complete media inoculated in volumes of 5 ml. Complete media stored meanwhile in the refrigerator, or freshly prepared, were inoculated simultaneously.

* Growth recorded at 12 days.

TABLE 2

Restoration of medium deteriorated during storage: stability of medium containing crystalline albumin

MODIFICATION OF MEDIUM	TEMPERATURE OF STORAGE (C)	INOCULUM (MG MOIST WEIGHT)				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
		Growth				
None	5	4	4	3	2½	0
Crystalline albumin	5	4	3½	3	2½	2
Tween 60	5	4	4	2½	¼	0
None	37	4	0	0	0	0
Tween 60	37	0	0	0	0	0
Crystalline albumin	37	4	3½	3	2	2
Additional fract. V	37	4	3½	3	2½	1½

Complete media, containing 0.05 per cent tween 80 and 0.1 per cent fraction V, as well as media modified as indicated (tween 60, a stearic ester, substituted for tween 80, an oleic ester; crystalline albumin substituted for fraction V), were stored at 37 C and 5 C for 18 days. To one lot of unmodified medium additional fraction V (0.1 per cent) was added at the end of the period of storage. Volumes of 5 ml were inoculated and growth was recorded after 14 days of incubation.

The medium containing tween 60 and albumin which had been stored at 37 C showed a slight precipitate in all tubes following the second period of incubation, as well as complete absence of growth. At this stage of investigation this was the observation which led to the recognition of hydrolysis of the tweens by albumin, for oleic acid is more soluble than stearic acid, and hence there had been no visible evidence of hydrolysis of tween 80.

serum albumin, which is purer than fraction V, did not cause deterioration during storage.

Presence of Lipase in Commercial Serum Albumin

Direct proof of this explanation of the instability of the tween-albumin medium required demonstration of lipase in the serum albumin. All efforts to demonstrate lipase by the usual method of measuring changes in titrable acidity were unsuccessful even following incubation of tween solutions with albumin for as much as several days; the buffering power of the albumin concealed any lipolysis that might have taken place. Similar negative experiments were obtained with a classical lipase substrate, tributyrin. It was therefore necessary to develop a

TABLE 3

Measurement of lipase in albumin

ADDITION TO SUBSTRATE	TITER N/50 NaOH AT TIME:			
	0	24 hr	48 hr	72 hr
Control—none	0.13	0.14	0.13	0.16
Albumin (fract. V, unheated)	0.17	0.26	0.38	0.55
Albumin + 0.03% NaF	0.17	0.20	0.21	0.215
Albumin heated at 56 C in 1% NaCl, pH 5.6	0.15	0.14	0.14	0.135

The substrate consisted of 0.5 per cent tween 80 in 0.02 M phosphate buffer at pH 7.0, or in the mineral mixture (pH 7.0) used in the bacteriological medium; similar results were obtained in either medium. A series of tubes containing 10 ml of substrate and albumin (1 ml 5 per cent) and fluoride as indicated, were incubated at 37 C, with the addition of a crystal of thymol to inhibit bacterial growth. At 24-hour intervals a tube from each group was extracted and titrated for ether-soluble acid. The blank values are high because the tween was not freed of fatty acid before incubation.

more sensitive method, involving extraction of the free fatty acid from the substrate-albumin mixture[3] (Davis, 1947*a*).

Table 3 shows that the commercial serum albumin hydrolyzes tween 80. It further shows, in anticipation of the following section, that the lipolytic activity is destroyed by heating at 56 C for 30 minutes, indicating an enzymatic activity. The amount of lipase is so small that incubation for several days was necessary for its measurement.[4]

[3] Tween 80 was used as the substrate for measuring lipase since it was the ester of chief biological interest. A closely related ester was found to be hydrolyzed by albumin somewhat more rapidly and hence to serve as a more sensitive substrate. This product is designated by the Atlas Powder Company as G-2144; it consists of the polyoxyethylene derivative of oleic acid and differs from tween 80 in the absence of sorbitan.

[4] It is possible that the lipase activity might have been more simply measured in a Warburg respirometer, but it may be calculated from the data of table 3 that the addition of as large an amount of albumin as 0.2 ml of 5 per cent solution to a respirometer vessel would have resulted in the evolution of only 4 mm^3 of CO_2 per hour, assuming that the buffering capacity of the protein would not have interfered with the gaseous equilibration.

Elimination of Lipase from Albumin

It was found possible to eliminate the lipase activity by any of three methods: (a) heating the albumin sufficiently to destroy the lipase, (b) using purer albumin that is not contaminated by lipase, or (c) using fluoride as a lipase inhibitor.

Heated albumin. The capacity of serum to bind fatty acid is dependent upon the essentially native configuration of the protein molecule, being destroyed by enzymatic digestion or by heating to 100 C even under certain conditions that avoid coagulation of the denatured protein (Davis and Dubos, 1947). Fortunately, however, the lipase proved to be more thermolabile than the fatty-acid-binding capacity of the albumin. The lipase effect was completely destroyed by heating 5 per cent albumin solution at 56 C for 30 minutes. In order to avoid coagulation of the albumin at this temperature, it is necessary either to neutralize the albumin solution (which has an initial pH of approximately 5.6) or to add 1 or 2 per cent NaCl. The higher concentration of NaCl is safer, as 1 per cent is not sufficient to prevent coagulation if the temperature rises slightly above 56 C. Since the heated albumin solution tends to clog the filters by which it is sterilized, it is advisable to filter before heating.[5]

Since the coagulability of albumin varies markedly with salt concentration and pH, it was necessary to determine whether the conditions which reduced the danger of coagulation of the albumin might not also reduce the destruction of lipase. Table 4 shows that albumin heated at 56 C under any of the conditions described above causes no deterioration of the medium following incubation for 20 days, in contrast to unheated albumin. The possibility of slight differences in residual lipolytic activity of these albumins, of course, is not ruled out by this bacteriological evidence.

Table 5 presents evidence that the deteriorated medium which had been incubated with unheated albumin contained more free fatty acid than that which had been stored in the refrigerator or incubated with heated albumin. Since albumin can protect tubercle bacilli against only 1 to 2 per cent of its weight of oleic acid (Davis and Dubos, 1947), the amount of fatty acid freed in the deteriorated medium (0.002 per cent) is sufficient to saturate the protective capacity of the albumin (0.1 per cent). Since some free oleic acid is present in the medium in addition to that specifically hydrolyzed at 37 C, and is further released during incubation, the failure of the medium to support growth is quantitatively accounted for by its content of oleic acid.

Crystalline serum albumin. Crystalline bovine serum albumin, which is purer than fraction V, is also commercially available (Armour). This material contains no significant amounts of lipase; the medium containing crystalline albumin

[5] Salt was observed to have a dual effect on the thermal stability of albumin. Whereas NaCl protected the albumin at pH 5.6 from coagulation when heated to 56 C, the opposite effect was observed at pH 7.0, at which a solution of albumin without added NaCl could be boiled without coagulation, but a solution with added NaCl (0.5 per cent or more) coagulated when boiled. The boiled, uncoagulated albumin, however, was denatured; not only had it lost its power to bind oleic acid (Davis and Dubos, 1947), but it precipitated on addition of acetic acid.

rather than fraction V showed no deterioration after incubation for 18 days, as was shown in table 2.

Fluoride. Fluoride ion is a well-known inhibitor of lipase (Loevenhart and Peirce, 1907). It is also a bacteriostatic agent, but fortunately the lipase in serum albumin was effectively inhibited by a concentration of NaF (0.01 per cent) that did not interfere with growth of tubercle bacilli in this medium. The absence of bacteriostasis may be related to the high concentration of Mg in this medium (0.1 per cent $MgSO_4 \cdot 7H_2O$), since the inhibitory effect of fluoride on

TABLE 4

Destruction of lipase in heated albumin (bacteriological evidence)

TYPE OF ALBUMIN	TEMPERATURE OF STORAGE	INOCULUM (MG MOIST WEIGHT)			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
		Growth*			
Unheated, 2% NaCl (clear)	37	±	0	0	0
	5	3	2	1	½
Heated, 2% NaCl (sl. opalescent)	37	3	2	1	½
	5	3	2	1	½
Heated, 1% NaCl (sl. opalescent)	37	3	2	1	½
	5	3	2	1	½
Heated, neutral, 2% NaCl (clear)	37	3	2	1	½
	5	3	2	1	½
Heated, neutral, 0 NaCl (clear)	37	3	2	1	½
	5	3	2	1	½

Sterile bovine serum albumin (fraction V) in a concentration of 5 per cent in various solutions was heated at 56 C for 30 minutes. Lots of medium containing 0.05 per cent tween 80 and 0.1 per cent of these various albumins, as well as unheated albumin, were stored for 20 days at 37 C and at 5 C; they were then distributed in 5-ml volumes and inoculated with dilutions of tubercle bacilli.

* Growth recorded at 9 days.

some enzymes has been shown to be due to the formation of magnesium fluorophosphate complexes (Warburg and Christian, 1942).

It is shown in table 3 that the addition of 0.03 per cent NaF essentially completely inhibits the lipase of fraction V. Evidence of the effectiveness of a bacteriological medium containing fluoride is presented in table 6.

Effect of Elimination of Lipase on Bacterial Growth

Table 6 also shows that elimination of lipase activity by any one of the three methods described above permitted growth of tubercle bacilli following inoculation with one further 10-fold serial dilution (10^{-8} mg moist weight) than was possible with unheated fraction V. Other similar comparative experiments showed a difference of two serial dilutions.

To find how few bacteria constitute the minimum effective inoculum, an experiment was carried out with inoculation by means of dilutions of droplets

TABLE 5

Destruction of lipase in heated albumin (chemical evidence)

TYPE OF ALBUMIN	TEMPERATURE OF STORAGE (C)	TITER N/50	DIFFERENCE
Unheated	37	0.175	0.08
	5	0.095	
Heated	37	0.080	0.015
	5	0.065	

The media described in table 4 containing 0.1 per cent albumin, heated and unheated in 2 per cent NaCl, were stored for 20 days at 37 C and 5 C. Aliquots of 20 ml were then analyzed for free fatty acid.

The values obtained following storage at 5 C are only partly due to oleic acid, since albumin has a high blank value because of contamination by other ether-soluble acids (Davis, 1947*a*). The slight hydrolysis obtained with heated albumin at 37 C compared with 5 C is probably spontaneous (Davis, 1947*b*), although the presence of a trace of residual lipase activity cannot be excluded. The increased titer obtained following incubation with unheated albumin at 37 C (0.08 ml greater than at 5 C) is due to hydrolysis of tween and corresponds to 0.002 per cent oleic acid in the medium, i.e., one-fifth of the tween present.

TABLE 6

Growth of minimal inocula

ALBUMIN (0.1%)	INOCULUM (MG MOIST WEIGHT)			
	10^{-6}	10^{-7}	10^{-8}	10^{-9}
	Growth*			
Unheated (fraction V)	4	3½	0	0
Unheated + 0.01% NaF	4	3½	3	0
Heated (56 C, neutral)	4	3½	3	0
Crystalline unheated	4	3	2	0

Human tubercle bacilli (H37Rv) were grown for 7 days in the tween-albumin medium, with the addition of a small amount of phlei filtrate to promote dispersed growth (cf. section 7). The density of the growth in a portion of the culture was measured volumetrically after prolonged centrifugation to constant volume (1½ hours at 2,400 rpm) in a tube especially designed for this purpose, consisting of a capillary tube (0.9 mm in diameter) fused onto a wide (25-mm) test tube. The culture was adjusted to a concentration of 1 mm^3 of organisms per ml (i.e., 1 mg moist weight, or 0.2 mg dry weight, per ml) and serial 10-fold dilutions were made with especial care. Inocula were planted in tubes of media containing 0.05 per cent tween 80 and albumin and fluoride as indicated. Visible growth first appeared at 14 to 16 days; the results are recorded at 20 days.

* Growth recorded at 20 days.

containing a small number of tubercle bacilli counted under the microscope. The medium containing heated albumin developed visible growth regularly following inoculation with 2 or 3 bacilli with an incubation period approximately

the same as that of inocula of 10^{-8} mg. The medium containing unheated albumin required larger inocula than did 2 or 3 bacilli (table 7). Technical limitations prevented testing of known inocula of 1 bacterial cell, which would undoubtedly have initiated growth as well as 2 cells in the stable lipase-free medium.

Effect of Horse Serum on Bacterial Growth

The recognition of the lipase effect permitted an understanding of some hitherto puzzling observations, namely, the inhibitory effect of horse serum and of

TABLE 7

Inoculation of counted bacteria

NUMBER OF BACTERIA INOCULATED	ALBUMIN (0.1%)	
	Heated	Unheated
20	+	+
10	+	+
4 (16/4)	+	+
4 (16/4)	+	−
3 (30/10)	+	−
3 (30/10)	+	−
3 (30/10)	+	−
3 (30/10)	+	−
2 (20/10)	+	−
2 (20/10)	+	−
2 (20/10)	+	−
2 (2/1)	+	−

A culture of human tubercle bacilli grown as in table 6 was lightly centrifuged to remove clumps of bacteria. The supernatant was diluted in water so that the number of bacteria in a tiny hanging drop could be counted microscopically under the high dry objective. Comparable drops of the diluent usually contained one or two particles which might have been confused with bacilli. Since it was not found possible to eliminate consistently such particles, it was not possible to isolate 1 or 2 bacilli accurately. Droplets containing larger numbers of bacilli were therefore transferred to given volumes of glucose solution, of which aliquots were then inoculated alternately into tubes containing medium with heated and unheated albumin. Growth became visible at 14 to 16 days and heavier at 20 days. Sterile water was added to replace that which had evaporated. Further observation until 25 days showed no growth in the tubes that were negative at 16 days.

+ = visible growth at 16 to 25 days.

− = no visible growth at 25 days.

certain bacterial culture filtrates on growth in the tween medium. Early in the course of studying possible growth factors for the tubercle bacillus, it was observed that the substitution of bovine or human serum for bovine serum albumin promoted growth, but of a flocculent rather than of a more or less dispersed type; horse serum, however, completely inhibited growth. The effect of horse serum was surprising since it has been used in the past in certain media designed for the cultivation of tubercle bacilli. It was even more surprising that, though horse serum is inhibitory in concentrations ranging from 2 to 10 per cent, a

higher concentration (20 or 40 per cent) permits growth of large inocula (table 8). The nature of the phenomenon was suspected following the further observations that sufficient bovine albumin overcomes the toxic effect and that in the absence of tween horse serum is not inhibitory (table 8).

TABLE 8

Bacteriostasis by horse serum

SUBSTANCE ADDED TO 5 ML MEDIUM	VOLUME ADDED (ML)	INOCULUM (MG MOIST WEIGHT)		
		10^{-2}	10^{-4}	10^{-6}
		Growth		
Medium containing 0.05% tween 80				
Control	0	3½	½	0
Albumin 5%	0.1	4	4	2
Bovine serum	0.1	4	0	0
	0.5	4	4	2
Human serum	0.3	4	4	3
Horse serum	0.1	0	0	0
	0.3	0	0	0
	1.0	4	0	0
	2.0	4	0	0
Heated horse serum (56 C, 30 min)	0.1	½	0	0
	0.3	4	0	0
	1.0	4	3	0
	2.0	2	1	½
Horse serum + 5% albumin	0.1 + 0.1	0	0	0
Horse serum + 5% albumin	0.1 + 0.5	4	3	3
Medium containing 0 tween 80				
Control	0	4	1	0
Horse serum	0.1	4	2	0

These results were explained when the horse serum used in these experiments was found to contain approximately three times as much lipase as did the human serum. The toxicity shown by horse serum but not by human serum can thus be accounted for by the higher concentration of lipase in horse serum, together with its known lower concentration of albumin (Moore, 1945). The disappearance of the toxicity of horse serum at very high concentrations occurs when the binding capacity of the albumin added in the serum exceeds the amount of tween

80 available as substrate for the lipase.[6] The lipase in horse serum was apparently only partly destroyed by heating at 56 C for 30 minutes (table 8).

Bacteriostatic Effect of Bacterial Culture Filtrates

Filtrates of the timothy grass bacillus (*Mycobacterium phlei*) have been reported to furnish an essential growth factor which permits *in vitro* cultivation of another mycobacterium, Johne's bacillus (Woolley and McCarter, 1940). The effect of such filtrates on tubercle bacilli was therefore studied. *M. phlei* was grown for 3 to 7 days in the tween-albumin medium, and the cultures were filtered through sintered glass (Corning UF). The sterile filtrates, however, not only did not promote growth of tubercle bacilli but were bacteriostatic in high dilutions. Further studies revealed that the phlei filtrate was bacteriostatic only in the presence of tween 80 and that its bacteriostatic activity was destroyed by autoclaving. It therefore appeared that we were dealing with lipase, rather than with a directly acting antibiotic agent. This hypothesis was confirmed by chemical tests which showed that the phlei filtrate contained lipase in a much higher concentration than had been observed in any sera or in 5 per cent albumin.

An extremely sensitive method of detecting lipase, in smaller concentrations than can be detected by any available chemical method, is offered by incubation of a tween medium (without albumin) with a bacterial filtrate (or another source of lipase), followed by inoculation with tubercle bacilli. As little as 3 micrograms of oleic acid per ml, released following several days of incubation, can be detected. The specificity of the bacteriostasis as a test for lipase can be tested by control experiments in a medium without tween. By this means it was possible to demonstrate traces of lipase in the culture filtrate of an avian tubercle bacillus, but none in the filtrate of the human strain H37Rv.

Dispersing Effect of Phlei Filtrate

The growth of tubercle bacilli in the tween-albumin medium is only partly dispersed, most of the bacteria occurring in microscopic clumps (Middlebrook *et al.*, 1947). It would be desirable for several purposes to secure completely dispersed growth. The closest approach to this objective attained so far has been furnished by the addition to the medium of phlei filtrate in small amounts, which only slightly inhibit growth; the organisms grow in such a medium predominantly as single cells. It has not been determined whether this dispersing effect is dependent upon the lipase in the phlei filtrate.

The proper amount of phlei filtrate to provide dispersal without marked inhibition of growth must be empirically determined. Excellent results have been obtained by adding to the medium, containing 0.05 per cent tween 80 and 0.2 per cent albumin, 5 to 10 per cent of its volume of the filtrate of a 3-day

[6] The lipolytic mechanism here suggested to account for the gross antibacterial action of horse serum, manifested only in the presence of tween 80, is not considered to conflict with numerous earlier reports that various sera, including antisera to tubercle bacilli, were inhibitory to tubercle bacilli in media without tween.

culture of *M. phlei* grown in the same medium. The success of inocula consisting of very few cells from such a medium (tables 6 and 7) indicates clearly that though the phlei filtrate has a somewhat inhibitory effect on growth, the bacteria grown in this medium are perfectly viable.

DISCUSSION

It has been demonstrated that bovine serum albumin, although indispensable for the growth of small inocula of tubercle bacilli (especially in the "tween" medium), also exerts an inhibitory effect because of contamination of the commercial product (fraction V) by serum lipase; this enzyme releases free fatty acid in bacteriostatic concentrations from tween 80. After eliminating the lipase activity, growth can be initiated by smaller inocula (2 or 3 cells, 10^{-8} mg moist weight) than were previously effective in the tween-albumin medium. A similar effect can be obtained in spite of the lipase by the use of very high concentrations of albumin, which are capable of binding all the fatty acid available from hydrolysis of the tween.

Since two bacilli initiate growth regularly, it is reasonably certain that single viable cells inoculated into the tween-albumin medium without lipase would also grow; the smallest successful inocula demonstrated here were limited to an average of two cells simply because of technical limitations. The reduction of the minimum effective inoculum from about 10 to only 1 or 2 bacteria is obviously of practical importance and will permit more effective use of the medium, or of solid modifications of it, in diagnostic or quantitative work.

The ability to initiate growth with very few cells also furnishes interesting information concerning the inoculum as well as the receptivity of the medium, namely, that no significant proportion of the cells of a 7-day culture in the tween-albumin medium is sterile. This behavior contrasts with the high proportion of sterile cells found in surface growth (Wilson and Schwabacher, 1936–1937).

Three methods have been found for eliminating lipase activity from albumin—the use of heated fraction V, of crystalline albumin, or of unheated fraction V with added NaF. In general, heated fraction V has seemed more useful than crystalline albumin. Not only is it less expensive, but it produces somewhat heavier growth of tubercle bacilli. The promotion of heavier growth has been traced to a dialyzable, thermostable contaminant of the fraction V that is absent from the crystalline albumin (Dubos, 1947). The third method of eliminating lipase activity, inhibition by addition of fluoride, has been shown to be effective in liquid media, but has not been extensively studied.

The deterioration of the tween medium on incubation with bacterial filtrates or other biological materials furnishes an exceedingly delicate test for lipase; in the absence of albumin, lipolysis, which yields as little as 3 micrograms of oleic acid per ml, can be detected by its inhibition of growth of tubercle bacilli. By this method no lipase could be detected in the filtrate of human tubercle bacilli. In view of the extraordinary sensitivity of this method, this observation suggests that the utilization of the oleic acid of tween 80 as a growth factor by the tubercle bacillus (Dubos, 1947) is not preceded by extracellular hydrolysis of the ester

in the medium. The mechanism by which esterification permits fatty acids to be nutritive may thus be somewhat different from the effect of serum albumin, which reversibly binds fatty acid (Davis and Dubos, 1947) and presumably releases free fatty acid into the medium in extremely low concentrations as the fatty acid is utilized by the bacteria. In the one case the organism would be expected to absorb the ester, in the other the free acid.

It was observed that horse serum is bacteriostatic in a medium containing tween 80 but not in a medium without it. The phenomenon can be accounted for by the relatively high concentration of lipase and low concentration of albumin in horse serum. This observation is of general significance insofar as it emphasizes the need for guarding against lipolytic effects when a medium containing tween is inoculated with materials which may contain lipase (e.g., in the cultivation of tubercle bacilli from blood or other tissues). In such circumstances it may be anticipated that the danger of bacteriostasis by free fatty acid can be avoided by the addition of fluoride or of sufficient albumin to bind all the fatty acid which may be released from the available tween. Where dispersed growth is not desired, it has been shown that the oleic acid can be supplied as a growth factor by including oleic acid in the medium rather than tween 80, together with sufficient albumin to bind it, thus eliminating all danger of lipolysis (Dubos, 1947).

A bacteriostatic agent in filtrates of *Mycobacterium phlei* proved to be simply lipase, which released free fatty acid from tween 80. This phenomenon calls to mind the analogous result reported a few years ago for notatin (penicillin B), which proved to be glucose oxidase; its bacteriostatic effect arose from the formation of hydrogen peroxide in the presence of glucose (Birkinshaw and Raistrick, 1943). It may be anticipated that the tween-albumin medium will be used in chemotherapeutic testing, since it has several advantages for this purpose: uniform inocula, uniform exposure of the bacteria to the medium, more rapid results, and ready quantitation of growth. But in testing the activity of antibacterial agents of biological origin in this medium it is important, as shown by the experience with phlei filtrate, to distinguish lipolytic from direct antibacterial effects.

SUMMARY

Commercial bovine serum albumin (fraction V), which permits growth of small inocula of tubercle bacilli in a liquid medium containing "tween 80" (a water-soluble ester of oleic acid), also makes the medium somewhat unstable. This effect is due to the presence in the albumin of a small amount of lipase, which in 2 weeks releases enough fatty acid from tween to exceed the binding capacity of the albumin; the unbound oleic acid then reaches a bacteriostatic concentration.

The lipase effect can be eliminated in several ways: (1) destruction of the lipase by heating a neutral solution of fraction V at 56 C, which does not destroy the capacity of the albumin to bind fatty acid; (2) use of commercial crystalline bovine serum albumin, which is not perceptibly contaminated by lipase; (3)

addition to the medium of 0.01 per cent NaF, which inhibits lipase but is not bacteriostatic to the tubercle bacillus. The effect can also be overcome by the use of a high concentration of albumin.

When the lipase is eliminated by any of these methods, it is possible to initiate growth with smaller inocula (10^{-8} mg moist weight, or 2 cells) than are effective in the presence of unheated fraction V or in the absence of albumin.

Lipolytic activity also accounts for the bacteriostatic effect in this medium of horse serum and of a culture filtrate of *Mycobacterium phlei*. This experience emphasizes the importance of guarding against lipolytic effects when materials of biological origin are introduced into a tween-containing medium for diagnostic work (e.g., blood culture) or chemotherapeutic studies (e.g., impure antibiotics).

The use of "tween 80" and tubercle bacilli provides an exceedingly sensitive bioassay for lipase, which showed no trace of lipase in filtrates of cultures of growing human tubercle bacilli.

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